PRESENT STUDY:

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

Chapter 2:

Introduction, Objectives and Materials and Methods

Chapter 2: Introduction, Objectives and Materials and Methods

2.1 Introduction

Microbial phytopathogens encounter plant cell wall as a major obstruction while invading and successfully colonizing the host. Typical plant cell wall is primarily composed of three polysaccharides namely cellulose (microfibrils), hemicellulose (xylan and xylan derivatives) and pectin, interconnected via ferulic acid bridges to form a rigid mesh-like structure (Harris and Hartley, 1977; Bunzel et al., 2001). This mesh-like network is required for providing strength and integrity to the plant cell wall. Phytopathogens, such as fungi and bacteria, deploy different ways to overcome this physical cell wall barrier, mainly to get entry into the host cell and colonise the tissue for nutrient acquisition. While bacterial pathogens prefer passive host entry via hydathodes or stomatal openings, fungal pathogens have evolved mechanisms to breach the primary barrier of plant hosts. The necrotrophic fungal pathogens use cell wall degrading enzymes (CWDE) to invade/colonise the plant tissue (Cosgrove, 2001); whereas the biotrophic or hemibiotrophic fungal pathogens, in addition to CWDE, depend on specialised host entry enabled by infection structures called as appressorium (Howard et al., 1991). While CWDEs are fairly studied with respect to virulence of phytopathogenic fungi, they are also implemented in industrial applications such as processing of plant cell walls for efficient production of biofuels. Ever-increasing collection of genome sequences reveals that CWDEs offer a wide diversity, both in terms of number and expansion of gene families, across phytopathogenic fungi (Kubicek et al., 2014). It is therefore important to study different CWDEs, which may lead to identification of novel virulence determinants in different phytopathogenic fungi.

Feruloyl esterases (Ferulic acid esterases, Fae; EC 3.1.1.73), a subclass of carboxylic acid esterases (EC 3.1.1.1), also belong to one such group of CWDEs. The Fae hydrolyses the ester bonds in the feruloyl-polysaccharide complex in the plant cell wall and thereby releases ferulic acid and polysaccharide (Faulds and Williamson, 1994; de Vries et al., 1997). Breakage of these ester bonds leads to loss of elasticity and plasticity and subsequent weakening of the plant cell wall. Feruloyl esterases have been classified into four classes - type A, B, C and D - depending upon sequence attributes and ability to act on wide range of substrates (Crepin et al., 2004). Crystal structure of feruloyl esterase from *Aspergillus niger* (AnFaeA) has revealed that it is a modular enzyme with a catalytic and a non-catalytic cellulose-binding domain (Hermoso et al., 2004). Involvement of fungal feruloyl esterases with differential activity during plant infection have been studied in different pathosystems. In *Fusarium graminearum*, feruloyl esterases, particularly FaeB1 and FaeD1, are found to be upregulated during infection or in response to aromatic compounds such as ferulic acid, caffeic acid and p-coumaric acid and also in the presence of carbon sources such as xylose, glucose and galactose (Balcerzak et al., 2012). However, FaeB1 and FaeD1 are not required for pathogenicity on wheat (Balcerzak et al., 2012). On the other hand, expression of Fae is not only upregulated during infection but also plays an essential role in pathogenesis in the apple tree canker pathogen *Valsa mali* (Xu et al., 2017).

Magnaporthe oryzae (synonym *Pyricularia oryzae*), a hemibiotrophic phytopathogenic filamentous fungus, causes blast disease in rice and other important cereal crops worldwide (Valent and Khang, 2010). Over several years, rice-blast disease has been widely used as a model pathosystem to study plant-pathogen interactions (Ebbole, 2007; Patkar et al., 2015). The infection cycle of *M. oryzae* starts with conidial germination in the presence of moisture, followed by perception of specific cues from the host surface, leading to

development of appressorium. The enormous turgor pressure generated inside the appressorium helps the fungus in penetrating the host cell. While the intracellular turgor pressure inside the appressorium contributes in generating the mechanical force, the localised loosening of the host cell wall underneath the appressorium is important and carried out by certain plant cell wall digesting enzymes secreted by the fungal pathogen. During subsequent host tissue invasion, the penetration peg differentiates into bulbous primary invasive hypha that elaborates within the first invaded cell. Once inside the first host cell, the fungal pathogen uses different strategies to evade plant immunity to colonise the tissue and for disease progression (Skamnioti and Gurr, 2008; Patkar et al., 2015).

Role of CWDEs in host penetration and thereby virulence has been studied to some extent in *M. oryzae*. Endo-xylanases and cellulases are significantly upregulated during plant infection and required for penetration and virulence in *M. oryzae* (Nguyen et al., 2011; Vu et al., 2012). Further, a secreted feruloyl esterase, encoded by MGG_01403.5, in *M. oryzae* is found to be expressed during post-penetration stage (72 hpi onwards) of rice infection, but does not play a significant role in pathogenesis of the fungus (Zheng et al., 2009). Interestingly, the *FAE* gene family in *M. oryzae* is relatively expanded when compared to that in non-pathogenic counterparts such as *Neurospora crassa* and *Aspergillus nidulans*, which have only one and three *FAE* genes, respectively (Dean et al., 2005). Hitherto, role of any other *FAEs*, particularly type B feruloyl esterases, has not been studied in *M. oryzae*. Here, a systematic study to decipher an important role of a particular Fae (Fae1), with respect to pathogenicity in *M. oryzae*, has been presented.

2.2 Objectives

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

2.3 Materials and Methods

2.3.1 Fungal culture and growth conditions

M. oryzae wild-type (WT) B157 strain (MTCC accession no. 12236; Kachroo et al., 1994) belonging to the international race IC9 was used in this study. Fungus was grown and maintained on Prune Agar (PA) plates as described earlier (Soundararajan et al., 2004). Vegetative growth of the fungus on PA plates was allowed for 10 days at 28 °C, with initial 3 days incubation under dark conditions followed by 7 days incubation under constant illumination for conidiation. Vegetative growth was assessed by visual observation of the colony morphology and by measuring the colony diameter.

Conidia were harvested from a 10-day grown mycelial culture, as described previously (Patkar et al., 2010), followed by microscopic observation of the conidial morphology. Harvested conidial suspension was added into a hemocytometer and no. of conidia were counted in each of the four 1 mm corner squares. Conidial count ($\times 10^4$ conidia/mL) was calculated by taking average no. of conidia for all four 1 mm squares. Finally, conidial density was reported in terms of number of conidia per mL per unit area of the colony, by considering the area of the colony calculated before harvesting the conidia.

Assay for appressorial development was performed by spotting 20 µL conidial suspension (~10^4 conidia/mL) on an inductive (hydrophobic) cover glass (22 mm, no. 1; Microcil Ltd., India) for up to 24 hours at 25 °C under humid conditions, followed by assessment of appressorium formation by microscopic observation.

Composition of media used in this study:

Prune Agar (PA) medium (per L)

Yeast Extract	1.0 g
Lactose	2.5 g
Sucrose	2.5 g
Prune Juice	40 mL
Agar	20 g

Adjust the pH 6.5 with l0 M NaOH (before adding agar)

Complete medium for *M. oryzae* (per 100 mL)

Dextrose	1 g
Peptone	0.2 g
Yeast Extract	0.1 g
Cas Amino Acid (Tryptone)	0.1 g
$NaNO_3$	0.6 g
KCl	0.05 g
$MgSO_4$	0.05 g
KH_2PO_4	0.15 g
Final pH	6.5
Agar	2.0 g

Minimal medium (liquid) for *M. oryzae* (per 100 mL)

Dextrose	1 g
$NaNO_3$	0.6 g
KCl	0.05 g
$MgSO_4$	0.05 g
KH_2PO_4	0.15 g
Final pH	6.5

YEG (liquid) medium (per 100 mL)

Yeast Extract	0.2 g
Glucose	1.0 g

Basal Medium (BM) Agar (per L)

YNB w/o ammonium sulphate	1.7 g
Asparagine	2.0 g
NH_4NO_3	1.0 g
Glucose	10 g
Agar	20 g

Adjust pH to 6.0 with Na_2HPO_4 (before adding agar)

2.3.2 *In silico* analysis of fungal *FAE*s

Identification of putative feruloyl esterases in *M. oryzae* was done using NCBI protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) with known type B Fae sequences from *Aspergillus oryzae* (AoFaeB; PDB: 3WMT_B) and *Neurospora crassa* (NcFaeB; GenBank: AJ293029). Multiple sequence alignment of these putative Fae was carried out using ClustalW feature in MEGA tool to check the presence of GXSXG conserved motif (Dilokpimol et al., 2016). Presence of the characteristic α/β hydrolase domain was checked using the NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer et al., 2015). Further, percentage identity among all these *M. oryzae* Fae was checked by performing multiple sequence alignment of protein sequences using Clustal Omega (Sievers et al., 2011; Sievers and Higgins, 2018), followed by plotting the distance matrix heatmap using R tool.

Phylogenetic analysis of Fae in different host-specific isolates of *M. oryzae* (GY11, P131, Y34, PH14, US71, MZ5-1-6, CD156, BR32) was carried out using the annotated protein sequences, available in the NCBI and GEMO (http://genome.jouy.inra.fr/gemo/) databases, from these isolates. Protein sequences of putative Fae in *M. oryzae* 70-15 strain was used as a query to perform BlastP analysis with a custom database containing all the protein sequences from the aforementioned different isolates. Phylogenetic analyses were carried out with MEGA11 (Tamura et al., 2021), using the Maximum Likelihood method based on the JTT matrix-based model.

For phylogenetic analysis of feruloyl esterases across different fungal taxa, genome sequences of 26 representative fungal species were retrieved from the NCBI database. Proteomes were mined for the presence of the conserved domain Tannase (Pfam ID:

PF07519.13) or Esterase_PHB (Pfam ID: PF10503.13), using hmm search (hidden Markov model search) of HMMER suite ver. 3.3.2 (parameters -cut_tc; Eddy, 2011). Poorly aligned sequences were removed using TrimAl (Capella-Gutierrez et al., 2009; parameters -automated1). The Tannase domain containing protein sequences were curated manually and were aligned to construct the maximum likelihood phylogenetic tree, using IQ-TREE ver. 2.1.2 (parameters -m MFP -alrt 1000 -bb 1000 -nt AUTO; Minh et al., 2020). Best fit model (LG+R6) was chosen based on the Bayesian Information criterion (BIC), followed by assessment of phylogenetic tree for branch support with SH-like approximate likelihood ratio test (alrt) and ultrafast bootstrap (bb). Phylogenetic tree was visualized using iTOL (Letunic and Bork, 2021).

2.3.3 DNA isolation and manipulation

2.3.3.1 Plasmid isolation from *E. coli*

Plasmid isolation from *E. coli* was carried out by boiling-lysis method in a step-wise manner, as follows:

1. Inoculate a bacterial colony in 2–3 mL of LB broth in a test tube containing appropriate antibiotic, and allow it to grow overnight at 37 °C at 200 rpm.
2. Collect the overnight grown culture in 1.5 mL microfuge tubes and centrifuge for 30 sec at 13000 rpm (12470 × g) at RT.
3. Discard the supernatant and resuspend the pellet in left-over (~50-100 µL) supernatant by vortexing vigorously. To this resuspended culture pellet, add 300 µL of STET buffer (8% w/v sucrose, 10% v/v 0.5M EDTA pH 8, 5% v/v 1M Tris-Cl pH 8 and 0.1% v/v Triton X-100 (added after autoclaving)) and vortex it again.

4. Add ~10 µL of 50 mg/mL lysozyme (final ~1.66 mg/mL) solution and allow it to react for 3 min at RT.
5. Place the tubes in a boiling water bath for 45 sec.
6. Allow the tubes to cool down for 2 min, then centrifuge for 10 min at 13000 rpm.
7. Carefully remove the snot-like pellet using autoclaved tooth-picks.
8. Add 8 µL of 1 mg/mL RNase A and incubate in a dry bath at 65 °C for 20–30 min.
9. Add equal volume (~300 µL) of Chloroform-Isoamylalcohol (CI; 24:1) solution and mix well by inverting the tubes. Centrifuge at 13000 rpm for 10 min.
10. Carefully transfer the upper aqueous phase using a micropipette to a fresh tube by holding the tubes at 45° angle.
11. Add 1/10th volume (~ 30 µL) of 5% w/v CTAB (Cetyl Trimethyl Ammonium Bromide) solution and vortex vigorously.
12. Centrifuge at 13000 rpm for 10 min, and discard the supernatant.
13. Add 300 µL of 1.2 M NaCl solution and vortex vigorously to resuspend the pellet.
14. Precipitate the plasmid DNA by adding 300 µL of cold isopropanol and mix by inverting the tubes.
15. Centrifuge at 13000 rpm for 10 min and discard the supernatant.
16. Add 500 µL of cold 70% v/v ethanol and centrifuge at 13000 rpm for 10 min.
17. Decant the supernatant and allow the tubes to air-dry at RT (or 37 °C) in inverted position for 15–20 min.
18. To the pellet, containing plasmid DNA, add 20 µL of autoclaved Milli-Q® water and allow it dissolve at 65 °C in a dry bath for 10–15 min. Store at 4 °C.
19. Analyse the isolated plasmid DNA samples by 1% agarose gel electrophoresis.

2.3.3.2 Genomic DNA isolation from *M. oryzae*

Genomic DNA isolation from *M. oryzae* was performed using a following step-wise protocol (modified from Dellaporta et al., 1983).

1. Filter the fungal biomass grown in a suitable liquid medium, using Whatman® no. 1 filter-disc placed in a glass funnel.
2. Wash the biomass, at least thrice, with autoclaved distilled water and blot-dry using a tissue paper.
3. Take out the dried biomass in aluminium foil and proceed with the following steps. The dried biomass can also be stored at -20 °C for processing it at a later time.
4. Freeze the biomass in the presence of liquid nitrogen in a mortar and powder-grind using a pre-cooled pestle.

Further, genomic DNA isolation steps were essentially performed by the method reported earlier (Dellaporta et al., 1983).

5. Quickly transfer the powdered biomass (before it gets thawed) to 1.5 mL microfuge tubes containing 500 µL of DNA extraction buffer (10% v/v 1M Tris-Cl pH 8, 10% v/v 0.5M EDTA pH 8, 12.5% v/v 4M NaCl and 0.001% v/v β-mercaptoethanol) and vortex vigorously.
6. Add 50 µL of 20% w/v SDS solution, vortex and incubate at 65 °C in dry bath for 15 min.
7. Add 200 µL of 5M Potassium Acetate solution (5M potassium acetate – 60 mL, acetic acid – 11.5 mL and water – 28.5 mL) and mix well by inverting.
8. Place the tubes on ice for 30 min.

9. Centrifuge at 6000 rpm (2656 × g) for 20 min and then transfer the supernatant to a fresh tube.
10. Add 20 µL of 1 mg/mL RNase A and incubate in dry bath at 65 °C for 30 min.
11. Add equal volume of equilibrated phenol and mix by inverting the tubes.
12. Centrifuge at 13000 rpm for 5 min.
13. Carefully transfer (using pre-cut and autoclaved tips) the upper aqueous layer into a fresh microfuge tube.
14. Add equal volume of CI solution and mix well by inverting the tubes.
15. Centrifuge at 13000 rpm for 5 min.
16. Carefully transfer (using pre-cut and autoclaved tips) the upper aqueous layer into a fresh microfuge tube.
17. Add 30 µL of 3M sodium-acetate buffer, pH 5.2 and mix by inverting the tubes.
18. Add 300 µL of cold isopropanol and mix by inverting the tubes.
19. Keep the tubes at -20 °C for ~2 h.
20. Centrifuge at 13000 rpm for 10 min and then discard the supernatant.
21. Add 500 µL of cold 70% v/v ethanol to the pellet.
22. Centrifuge at 13000 rpm for 10 min and discard the supernatant.
23. Air-dry the pellet at RT (or 37 °C) for 15–20 min, by placing the tubes in inverted position (preferably in a laminar hood).
24. To the pellet, containing genomic DNA, add 30–40 µL of autoclaved Milli-Q® water and allow it dissolve at 65 °C in a dry bath for 10–15 min. Store at 4 °C.
25. Analyse the isolated genomic DNA samples by 1% agarose gel electrophoresis.

2.3.3.3 PCR

PCR was carried out using Taq DNA polymerase (GoTaq®, Promega) or XT-5 DNA polymerase (GeNei™) and standard thermal cycler machines (Biorad-C1000 or ABI-Veriti). For PCR products amplified by Taq DNA polymerase, to be used for blunt-end cloning, were subjected to an end-filling reaction with T4 DNA polymerase (Fermentas) at 12 °C for 15 min as per the manufacturer's instructions. The PCR products, before further use, were purified either by sodium-acetate/ethanol precipitation (20 µL of 3M Na-acetate, pH 5.2 and 500 µL of cold absolute ethanol, followed by overnight incubation at -20 °C) or gel-extraction (QIAEX® kit, Qiagen) following manufacturer's instructions.

2.3.3.4 Restriction enzyme digestion

Restriction enzymes used throughout this study were obtained either from New England Biolabs (NEB, USA), GeNei (India) or Thermo Scientific (FastDigest; USA) and digestion of DNA was carried out in reaction buffers supplied with the corresponding enzymes and according to manufacturer instructions.

2.3.3.5 Ligation

Ligation reaction was carried out at 22 °C for 1–2 h using T4 DNA ligase (Genei or NEB) according to the manufacturer's instructions. Blunt-end ligation was generally carried out with 1:5-vector to insert molar ratio, while sticky-end ligation was performed at 1:3-vector to insert molar ratio. Ligated reaction mixture was then transferred to *E. coli* DH5α and the subsequent colonies were screened for the desired clones.

2.3.3.6 Southern blot hybridisation

Southern blot hybridisation of digested genomic DNA from fungal strains was carried out as follows:

1. Setup a digestion reaction with ~5–10 µg of genomic DNA with an appropriate restriction enzyme(s). Ensure complete digestion (~ 3 to 12 h depending upon the RE used) to avoid any signal due to undigested DNA.
2. Purify the digested genomic DNA sample by an overnight Na-acetate/ethanol precipitation, followed by resuspension in ≤ 30 µL of autoclaved Milli-Q® water.
3. Run the samples on a thick 1% agarose gel w/o ethidium bromide (EtBr) at 50 V (for ~4 h or till the dye front reaches the edge of the gel).
4. Stain the gel for 10 min with EtBr solution (20 µL EtBr/L) under gentle shaking condition and take a gel picture using a gel-documentation system (Bio-Rad Gel-Doc-XR system).
5. De-stain the gel with 2–3 repeated washes with distilled water.
6. Place the gel in a tray containing denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 min followed by neutralization buffer (1 M Tris-Cl pH-7.4, 1.5 M NaCl) for 45 min, under continuous gentle shaking condition.
7. Assemble the gel (inverted) and membrane together, as depicted in Figure 2.1 below, and leave it overnight for capillary transfer of DNA to the positively-charged membrane, in 10× SSC buffer (44.1 g of Na-citrate and 87.66 g of NaCl to final volume of 1 L with water, pH 7–8).

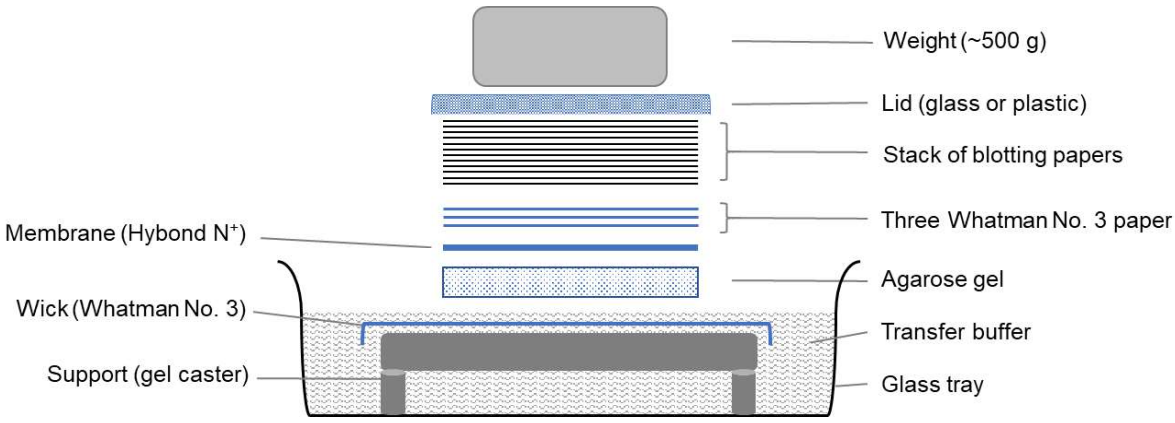

Figure 2.1. Schematic representation of the capillary DNA-transfer-setup for Southern blot hybridisation.

8. After capillary transfer, immobilize the DNA on the membrane by UV-crosslinking. Also, counter-stain the gel with EtBr to ensure complete/maximum transfer of DNA to the membrane.
9. Further steps such as preparation of labelled probe, hybridization and post-hybridization stringency washes and final signal generation and detection, was carried out using AlkPhos direct labelling and detection kit (GE Healthcare UK Ltd.), as per the manufacturer's instructions.

2.3.4 Protein sample preparation and estimation

Total protein isolation from secretory and intracellular fractions of *M. oryzae* culture grown in YEG liquid medium with or without rice-leaf extract, was carried out in a step-wise manner as follows:

Protein isolation from fungal biomass (intracellular fraction)

1. Collect the fungal biomass by passing the mycelial culture grown in a liquid medium through a Whatman® no. 1 filter-disc placed in a glass funnel.

2. Wash the biomass with distilled water for 2–3 times and then blot-dry using a tissue paper.
3. Freeze and powder-grind the fungal biomass in the presence of liquid nitrogen in a mortar & pestle.
4. Take the powdered biomass to 1.5 mL microfuge tubes and resuspend it in 1 mL of 1× PBS, pH 7.4.
5. Place the tubes on a tube-rotator (Tarsons) for 1 h at RT to allow the proteins to get solubilized in the buffer.
6. Centrifuge at 13000 rpm for 5 min, to remove the cell-debris, and collect the clear supernatant to a fresh tube.
7. Filter the supernatant by a 0.22 µm syringe filter (Millex®, Millipore), to remove any residual cell-debris and particulate matter.
8. Estimate the total protein concentration by Bradford reagent and standard curve method (B6916; Sigma-aldrich, USA) and then determine the Fae activity by a Fae-specific enzyme assay.

Protein sample preparation from culture supernatant (secretory fraction)

1. Collect the culture supernatant by filtering the mycelial culture grown in the aforesaid liquid medium.
2. Filter the supernatant by a 0.22 µm syringe filter (Millex®, Millipore), to remove any residual cell-debris and particulate matter.
3. Concentrate the clear supernatant upto ~30-fold i.e., ~35 mL to ~1.2 mL, using a 3 kDa MWCO Nanosep® (Pall) centrifugal filters, according to manufacturer's instructions.

4. Estimate the total protein concentration by Bradford reagent and standard curve method (B6916; Sigma-aldrich, USA) and then determine the Fae activity by a Fae-specific enzyme assay.

2.3.5 Bacterial and Yeast transformation

2.3.5.1 Bacterial transformation

Preparation of *E. coli* DH5α chemically-competent cells

Following steps for competent cells preparation were carried out aseptically under cold conditions.

1. Prepare an overnight grown *E. coli* DH5α culture in 3 mL of plain LB.
2. Add 1 mL of the above culture to 100 mL of plain LB prepared in a 1 L flask.
3. Incubate at 37 °C under shaking at 200 rpm for ~2–3 h till OD_{600nm} reaches 0.4.
4. Dispense the culture in 50 mL centrifuge tubes (pre-chilled) and centrifuge for 4 min at 4000 rpm at 4 °C.
5. Discard the supernatant and resuspend the pellet in 10 mL of chilled 0.1M $CaCl_2$ solution. Allow the pellet to homogenize by gently tapping the tubes.
6. Place the tubes on ice in a cold room for 45 min.
7. Centrifuge at 4000 rpm for 4 min at 4 °C.
8. Discard the supernatant and resuspend the pellet in 1 mL of chilled 0.1M $CaCl_2$ + 20% glycerol solution.
9. Make aliquots of 100 µL each under sterile condition in 1.5 mL microfuge tubes.
10. Store for long-term at -80 °C.

Transformation

1. Take one aliquot (100 µL) of *E. coli* DH5α competent cells. To it add the desired DNA sample (e.g., a vector DNA or a ligation-reaction mixture) and place this tube on ice for 30 min.
2. Give a heat-shock treatment in a water bath at 42 °C for exact 90 sec.
3. Immediately cool-down the tube on ice for 2 min.
4. Add 1 mL of LB to the tube and incubate for 45 min at 37 °C.
5. Spin briefly (30 sec), decant the supernatant and resuspend the cell-pellet in the residual ~100 µL of LB. This step is optional and recommended only when a ligation-reaction mixture is used to transform *E. coli*. For a parental vector-DNA-based transformation, take 100 µL of the unspun culture from step #4 above and proceed with step #6.
6. Spread the resuspended 100 µL culture, using a sterile spreader, onto LA-plates containing appropriate antibiotic.

Note: Transformation efficiency (CFU/µg of DNA) of the *E. coli* DH5α competent cells was calculated as:

$$\frac{\text{No. of colonies or transformants} \times \text{total volume (µL)}}{\text{Amount of DNA used (µg)} \times \text{volume plated (µL)}}$$

2.3.5.2 Yeast transformation

Following steps for yeast transformation were carried out under aseptic conditions.

1. Take a yeast (strain DBYα2445) culture, grown overnight in 5 mL-YPD medium containing 100 µg/mL ampicillin in a sugar-tube, for transformation.
2. Inoculate this 5 mL culture into a fresh 50 mL-YPD with ampicillin in a 100 mL flask and incubate at 28 °C under shaking at 200 rpm for ~4 h (OD_{600nm} = 0.85).
3. Centrifuge at 4000 rpm for 5 min at 25 °C.

4. Discard the supernatant and resuspend the pellet in 10 mL of sterile distilled water.
5. Centrifuge at 4000 rpm for 5 min at 25 °C.
6. Discard the supernatant and resuspend the pellet in 5 mL of sterile distilled water.
7. Distribute 1 mL each of this cell suspension into 5 microfuge tubes.
8. Centrifuge at 13000 rpm for 5 min at 25 °C.
9. Resuspend the pellet by adding the following components individually:

PEG 3500 (50% w/v)	240 µL
Lithium acetate (1 M)	36 µL
Salmon sperm - carrier DNA (10 mg/mL)	10 µL (boiled at ~100 °C for 5 m)
DNA sample (~1 µg/µL)	34 µL

10. Vortex well and incubate for 1 h at 42 °C in a water bath.
11. Gently spread (with only 3-4 strokes) the entire volume, using a sterile spreader, onto an appropriate selection medium.

2.3.6 *Magnaporthe oryzae* protoplast transformation

Following steps for protoplast transformation were performed under aseptic conditions.

1. Inoculate 30 µL of fungal spores (~10^4 – 10^5 conidia/mL) in 30 mL of liquid CM (in 100 mL flask) and allow it to grow at 28 °C, 200 rpm for 2–3 days (preferably 2 days).
2. Filter the biomass using Whatman® no. 1 disc and wash it thrice with sterile distilled water.
3. Resuspend the mycelia in 30 mL of 1M sorbitol taken in a 100 mL flask. Weigh and add ~30 mg of lysing enzymes (from *Trichoderma harzianum*; Sigma) to the mixture.
4. Incubate the mixture, for protoplasts formation, in shaking incubator at 28 °C at 80 – 100 rpm for 3–4 h (or preferably overnight).

5. Collect the protoplasts-containing filtrate in pre-chilled 50 mL Sorvall™ tubes by passing through a Miracloth® placed in a glass funnel.
6. Centrifuge the filtrate at 4000 rpm for 5 min at 4 °C and decant the supernatant.
7. Resuspend the pellet in 10 mL of 1 M sorbitol and follow above step no. 6.
8. Repeat the above step no. 7, for washing the pellet containing protoplasts.
9. Resuspend the protoplasts pellet in 10 mL STC buffer (1M sorbitol, 50mM Tris-Cl pH 8 and 50mM $CaCl_2$) and centrifuge at 4000 rpm for 5 min at 4 °C. Decant the supernatant.
10. Resuspend the pellet containing protoplasts in 1 mL STC buffer and transfer it to sterile 1.5 mL microfuge tubes (kept on ice). Count the protoplasts in haemocytometer and maintain density at $10^6 – 10^8$ cells/mL.
11. Mix 200–250 µL of the protoplast's suspension with 1–5 µg of the DNA sample; keep the volume of DNA sample as low as possible (maximum up to 30–35 µL).
12. Place the above tubes on ice for 15 min and then add 1 mL of PTC buffer (STC buffer + 40% w/v PEG 3350) to the mixture and incubate at RT for 30 min.
13. Transfer the above mixture into sterile sugar tubes and add 3 mL of CM, YEG or BM (depending on the selectable marker used) containing 1 M sorbitol.
14. Incubate the mixture at 28 °C under shaking at 80–100 rpm for 12–14 h.
15. Add 10 mL of molten regeneration medium (CM/YEG/BM + 0.4% agar) to the above mixture and mix properly and pour it onto at least four CM, YEG or BM-agar plates containing appropriate antibiotic(s).
16. Incubate the plates at 28 °C, until fungal colonies start appearing (~4–5 days). Pick the individual colonies, showing proper radial-mycelia growth, and inoculate on secondary selection plates (30- or 60-mm plates). Incubate the plates at 28 °C for ~4–5 days.

17. Screen the fungal transformants, growing on secondary selection plates, by PCR and/or Southern blot hybridization to check for the desired transformant(s).

2.3.7 Yeast Secretion Trap (YST) approach

Presence of functional signal peptide, if any, was confirmed using the Yeast Secretion Trap (YST) approach as described (Fig. 2.2; Lee et al., 2006).

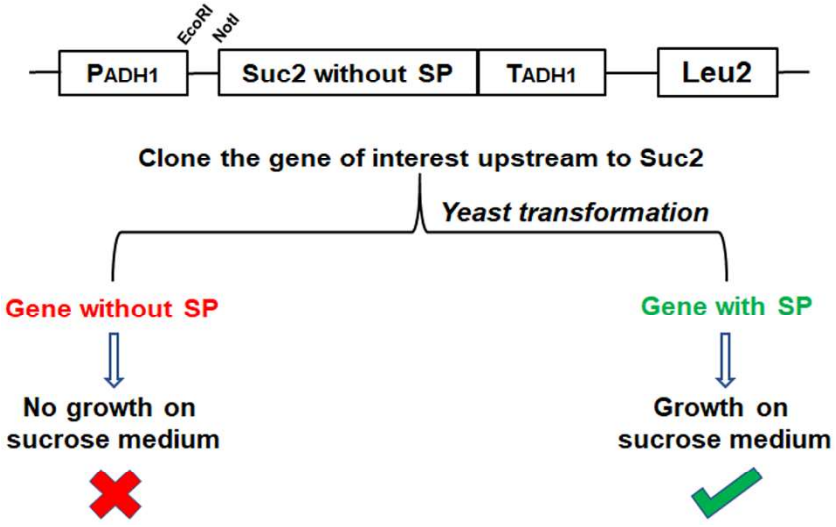

Figure 2.2. A schematic representation of the Yeast Secretion Trap strategy used to study presence of a signal peptide, if any, in Fae.

The ORFs of MGG_05529 and MGG_07294 were PCR-amplified from *M. oryzae* WT genomic DNA, using the following primer pairs (MGG_05529-F: 5'-ATGGACTCGTCAATCATTCACTGG-3' and MGG_05529-R: 5'-CCCCATTCCACTTTGACCTG-3'; MGG_07294-F: 5'-ATGCGTTTCTCCAGCATCTTC-3' and MGG_07294-R: 5'-CGCAATGAGACCAAAGAACC-3'). Individual PCR products were subjected to blunt-end cloning in pYST2 vector at *Not*I site after end-filling, followed by *E. coli* DH5α

transformation. Transformants obtained on Luria-agar plates containing 100 µg/mL ampicillin antibiotic were screened by RE digestion and those with desired restriction digestion pattern were confirmed by DNA sequencing. These recombinant plasmids from desired clones of *E. coli* DH5α were used for yeast (*Saccharomyces cerevisiae*) DBYα2445 (MATα, *suc2*Δ*–9*, *lys2–801*, *ura3–52*, *ade2–101*) transformation as described previously (Gietz and Woods, 2002). Selected transformants were screened and confirmed by colony PCR. Confirmed *S. cerevisiae* transformants were spotted on SD (YNB with $(NH_4)_2SO_4$, Lysine, Uracil and Adenine) + sucrose agar selection plates and incubated for 6 days at 28 °C.

2.3.8 Feruloyl esterase enzyme assay

Fae enzyme activity from intracellular and secretory protein fractions was determined by a biochemical spectrophotometric enzyme assay using Fae-specific substrate, 4-nitrophenyl ferulate (4-NPF; Institute of Chemistry, Slovak Academy of Sciences, Bratislava), as described previously (Mastihuba et al., 2002). Briefly, a 10.5 mM 4-NPF stock solution was prepared in DMSO followed by a 10-fold dilution with 0.1 M potassium phosphate buffer, pH 6.5 containing 2.5% v/v Triton X-100, to get a 1.05 mM 4-NPF working solution. To 1 mL of working 4-NPF solution, 100–200 µL of respective protein samples, as prepared earlier (section 2.3.4), was added and the reaction was incubated for 6 h at RT. Next, OD of the samples was measured at 410 nm and the amount of product (4-NP) released was estimated against the standard curve of 4-NP. Finally, specific activity (mU mg^{-1}) of Fae was calculated for intracellular and secretory protein fractions from both control and rice-extract-treated samples.

2.3.9 Quantitative real time reverse transcription PCR (qRT-PCR)

2.3.9.1 Total RNA isolation from *M. oryzae*

1. Filter the fungal biomass grown in a suitable liquid medium, using Whatman® no. 1 filter disc placed in a glass funnel.
2. Wash the biomass, at least thrice, with autoclaved distilled water and blot-dry using a tissue paper.
3. Take out the dried biomass in aluminium foil and proceed with the following steps. The dried biomass can also be stored for long-term at -80 °C.
4. Freeze the fungal biomass in the presence of liquid nitrogen in a mortar & and powder-grind using a pestle.

RNA isolation was performed using Trizol® reagent, essentially following manufacturer's instructions (https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf).

5. Quickly transfer the powdered biomass (~100 mg) to 1.5 mL microfuge tubes containing 1.0 mL of Trizol® reagent and mix by inverting the tubes.
6. Centrifuge for 5 min at 12000 × g at 4 °C and transfer the supernatant to a clean fresh tube.
7. Add 0.2 mL chloroform to it, mix well by inverting and incubate for 2–3 min at RT.
8. Centrifuge for 15 min at 12000 × g at 4 °C and transfer upper aqueous phase carefully to a fresh clean tube, while avoiding transfer of any interphase or lower organic phase.
9. Add 0.5 mL of cold isopropanol, mix by inverting the tubes and incubate for 10 min at RT.
10. Centrifuge for 10 min at 12000 rpm at 4 °C and then discard the supernatant.

11. Add 1 mL of 75% ethanol to the pellet, vortex briefly and then centrifuge for 5 min at 7500 × g at 4 °C.
12. Discard the supernatant and allow the pellet to air-dry (for ~30 min) at RT by placing the tubes in inverted position.
13. Resuspend the pellet in 20–50 µL of autoclaved Milli-Q® water and incubate the tubes in a dry bath at 65 °C for 5–10 min to dissolve the pellet.
14. Isolated RNA samples can be stored for long-term at -80 °C.
15. Estimate the total RNA concentration using Nanodrop® spectrophotometer.
16. Analyse the quality of the isolated RNA samples by 1% agarose gel electrophoresis.

2.3.9.2 First-strand cDNA synthesis

1. Place the frozen RNA samples on ice and allow it to get thawed. Then keep the tubes in a dry bath at 65 °C for 5 min.
2. Take 2 µg of total RNA for first-strand cDNA synthesis and to it add 2 µL of 20 µM oligo-$(dT)_{18}$ primers (Sigma, India).
3. Mix the above solution by tapping the tubes and give a short-spin.
4. Incubate the tubes for 15 min at 65 °C in a dry bath. Now, immediately cool the samples on ice for 2 – 5 min, and then give a short-spin.
5. Add 2 µL each of 10 mM dNTPs and 10× RT-buffer and make up the volume of the reaction mixture to 20 µL with autoclaved Milli-Q® water.
6. Finally, add 0.5 µL of 20-Units/µL M-MuLV reverse transcriptase (NEB, USA) enzyme for each cDNA synthesis reaction.
7. Give a short-spin and incubate the reaction mixture at 37 °C for 1.5–2 h.
8. Incubate the reaction mixture for 20 min at 65 °C to heat-inactivate the enzyme.

9. Check and confirm the efficiency of cDNA synthesis reaction by a conventional PCR using *M. oryzae* β-tubulin (Gene ID- MGG_00604) ORF-specific primers.

2.3.9.3 Quantitative real-time RT-PCR (qRT-PCR)

The first strand cDNA was subjected to qRT-PCR analysis using a Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. Optimized thermal cycling conditions for qRT-PCR were as follows: initial denaturation step at 95 °C for 15 min followed by 40 cycling reactions each at 95 °C (denaturation step) for 15 s and 66 °C (annealing and extension step) for 45 s. Transcript levels in each of the test conditions were calculated by $2^{-\Delta\Delta Ct}$ method (Livak method; Livak and Schmittgen, 2001) relative to that of the vegetative mycelia grown in liquid MM or CM, normalized against β-tubulin (MGG_00604) transcript levels as an endogenous (loading) control.

2.3.10 Plant infection assay

For infection assay, rice or barley plants were grown as follows: rice (cv. CO-39) or barley seeds were first, surface sterilized by treating with 30% v/v ethanol for 2.5 min at RT, followed by three-washes of distilled water. Surface-sterilized rice seeds, but not the barley seeds, were subjected to heat-treatment, wherein seeds were placed between the layers of moist tissue paper in a petri-plate and incubated at 52 °C for 1 h. Next, the rice or barley seeds were allowed to germinate under moist conditions for 3–4 days at RT, before transferring them to pots. Soil-mix for growing the plants was prepared by proportionately adding the following components: ~40% of garden/potting soil, ~40% of coco-peat, ~10%

of red soil and ~10% of vermiculite. Distilled water containing insecticide (Bayer; 1 mL/2 L water) was used to prepare the soil-mix, which was then added into the plastic pots for planting the germinated seeds. Now, plants were allowed to grow for ~3–4 weeks in a green-house maintained at 28 to 30 °C under humid conditions in a ~12 h light/dark cycle.

Whole-plant infection assay was carried out by spraying ~10^5 conidia/mL in 0.05% w/v gelatin or Tween 20 onto ~4-week-old rice or ~3-week-old barley plants, followed by incubation at 28 to 30 °C under humid conditions, initially for 24 h in the dark and then for 4 to 6 days under 14 h light and 10 h dark cycles. Development of disease symptoms was monitored regularly during the entire incubation period and recorded at an appropriate time (usually around 5 dpi). Similarly, detached-leaf infection assay was carried out, wherein, unlike whole-plants, excised leaves were surface-sterilized and then placed onto kinetin (2 mg/L) agar plates. Conidial suspension (~5x10^4 conidia/mL in 0.05% w/v gelatin or Tween 20) was then inoculated by placing 4–5 drops each of 20 µL per leaf-blade, followed by aforementioned photoperiod and incubation condition.

2.3.11 Host invasion and penetration assay

Host invasion by the fungal strains was checked on to the leaf sheaths obtained from 3–4-week-old host (rice, wheat or barley) plants, grown as mentioned above (section 2.3.10). Here, leaf sheaths were prepared by excising 2-3 cm long portions of the stem region and carefully removing the central slender tube using a fine-pointed forceps, leaving behind a hollow cylindrical sheath for the assay. Next, the leaf sheaths were inoculated with ~10 µL conidial suspension (~10^4 conidia/mL) followed by incubation at room temperature (<30 °C) under humid conditions for appropriate time points. At the end of incubation, remove

the midrib of the sheath tissue, mount it on a glass slide and cover it with a glass coverslip before observing under a microscope.

Host invasion was observed and quantified by scoring at least 100 appressoria each, using bright-field microscopy (Olympus BX51, Japan), for the development of invasive hypha in the plant cell underneath, at ~48 or 96 hpi. To check the effect of exogenous supply of glucose (2% w/v), ferulic acid (100 mM) or reduced glutathione (GSH, 20 mM) on host invasion by the blast fungus, the compound was added to the rice leaf sheaths, already inoculated with conidia, at 22 hpi, and the resultant invasive hyphal growth was observed at ~48 hpi.

Similarly, host penetration by the fungal strains was also checked by inoculating conidial suspension on to the leaf sheaths followed by microscopic observation for callose deposition by aniline blue staining. Inoculated rice sheath samples, at 36 hpi, were kept overnight in methanol for fixation and decolorization. Next, the sheaths were washed thrice with distilled water, followed by staining for 0.5–1 h with a freshly-prepared aniline blue solution (0.05% w/v aniline blue dye (water-soluble) dissolved in 0.067M K_2HPO_4, pH 9.0). The sheaths were then washed to remove the excess dye and observed for callose deposition at the site of host penetration, by fluorescence microscopy (Olympus BX51, Japan) using GFP filter.

2.3.12 Statistical analyses

Quantitative analyses for fungal Fae enzyme activity, transcript levels, vegetative growth, conidiation, appressorial development and host-invasion ability were carried out with three independent sets and the results are reported as mean ± standard deviation of mean (SDM). Statistical significance was determined by using two-tailed *t*-test; and *P*-values less than

0.05 (i.e., < 5%) were considered as statistically significant. $P < 0.05$ is denoted as * and likewise $P < 0.01$ as ** and $P < 0.001$ as ***.