2. MATERIALS AND METHODS

2.1 Fungal culture

M. oryzae B157, belonging to the international race IC9, was previously isolated in our laboratory from infected rice leaves (Kachroo *et al*., 1994). The fungus was grown and maintained on YEG medium (Glucose 1 g, yeast extract 0.2 g, H_2O to 100 ml) or oatmeal agar (Hi-Media, Mumbai, India). Fungal conidia were harvested by scraping the biomass grown on Oatmeal agar plates with a sterile surgical blade, resuspended in sterile water and purified by passing through sterile miracloth.

For study purpose the fungal biomass either from spores or from agar plugs was grown for 3 days in complete medium and then transferred to fresh medium and subjected to osmotic stress with 1 M NaCl, and 1.5 M Sorbitol, oxidative stress with 5-10 mM H_2O_2, 150 µM paraquat, antifungal stress with 1-100 mM fluodioxonil, vinclozonil, iprodione and resveratrol respectively either in broth or in agar plates. Minimal medium was used for some the plate assays.

2.2 *in silico* analysis of *MoHPT1*

Multiple sequence alignment of amino acid sequence was performed using the ClustalW (Thompson and and Gibson, 1994) program and the phylogenetic tree was constructed using the MEGA5 program (Tamura *et al*., 2011). Predictions of phosphorylation sites was done using NetPhosK (Blom *et al*., 1999) as well as GPS (Xue *et al*., 2008) tool.

2.3 Plasmid construction

- The full-length ORF of *MoHPT1* gene was amplified from *M. oryzae* B157 genomic DNA using gene specific primers MoHPT1F-5'ATGGTACCGTACGGTTGTTTGATGTCGG3' and MoHPT1R-5'ATGGATCCAGCCTGTCTTTCGTTTGAAG3'. The 1.5 kb amplified fragment was cloned in pBluescript KS+ at *BamH*I /*Kpn*I site and sequenced.
- The disruption vector was constructed for the *MoHPT1* gene by introducing hygromycin resistance gene (*hph*) cassette into the *SalI* site of the plasmid KS^{+}-*MoHPT1*. KS^{+}-*MoHPT1* was digested with *Sal*I restriction enzyme and subsequently ligated to the SalI digested *hph* cassette obtained from the pCAMGFP plasmid. The disruption construct made was checked by the release of 1.5 Kb fragment using *Sal*I. The clone was further confirmed by restriction digestion pattern obtained from *BamH*I-*Kpn*I digestion. The disruption vector was mobilised into dual selection vector pGKO2 using *BamH*I-*Kpn*I. The KS-*MoHPT1hph* and vector pGKO2 was digested with *BamH*I-*Kpn*I and then subsequently ligated. The mobilised cassette was checked with *BamH*I-*Kpn*I and confirmed using *Pvu*II.
- A Heme-aglutinin tagged construct was developed in a eukaryotic expression system so as to use it for co-immunoprecipitation experiments. The insert was released by *BamH*I and *Kpn*I from the pBKS-*MoHPT1* construct. The vector pRTDS having an enhanced CAM35S promoter and a 5'-HA tag was digested with *BamH*I and *Kpn*I. The compatible ends were ligated to get the gene cloned in the sense direction. The obtained clone was confirmed by restriction digestion with

*Kpn*I and *Xba*I. The expected bands of about 1.6 kb and 3.8 kb were obtained.

- Complementation experiments were carried out by expressing *MoHPT1* in *S. cerevisiae*. The ORF of *MoHPT1* was cloned into the 2µ yeast expression vector pYES2 *at Kpn*I and *BamH*I sites.
- Bacterial expression of *MoHPT1* for antigen production was carried out by cloning the gene in the pET30a vector and expressing it in *E.coli* BL21. The pET-*MoHPT1* construct was developed by cloning *MoHPT1* ORF from 1020th base pair to 1576th base pair from the KS-MoHPT1 at the *Nde*I/*Xho*I site so as to obtain a C-terminal His6X tag.
- The two isoforms of *MoHPT1* cDNA T1 and T0 were cloned in pBluescriptKS+ at *Eco*RV and sequenced using M13 primers.
- A 525 bp stretch from the ORF was amplified and used for an RNAi construct. This sequence was analysed by SI-FI software tool (http:// labtools.ipk-gatersleben.de/) for off target effects of siRNA in *Magnaporthe* transcriptome. There were no predicted off targets. The construct was then mobilised into pCAMBIA 1305 to obtain the binary construct pCAM-Silent *MoHPT1* (RNAi *MoHPT1*). The RNAi construct was prepared in the pSilent vector (Nakayashiki *et al*., 2005) to generate a hairpin loop when expressed in the fungus. The vector pSil-*MoHPT1* was constructed using 525 bp of *MoHPT1* ORF cloned in sense and antisense orientations on either side of the Cutinase intron at the *Bgl*II/*Kpn*1 sites and the *Hind*III site of the pSilent vector to build up the RNAi construct of the gene under *Aspergillus* derived constitutively expressed TrpC promoter and TrpC terminator. The *Xba*I cleaved fragment of the construct was then mobilised into pCAMBIA 1300 at the *Xba*I site to obtain the binary construct pSil-*MoHPT1*. The plasmid construct was confirmed by sequencing. It was used to

generate RNAi transformants using *Agrobacterium tumefaciens* mediated transformation (ATMT).

2.4 Complementation of Yeast *YPD1* heterozygous deletion mutant with *Magnaporthe MoHPT1*

The full length gene was cloned in the pYES2 vector carrying a URA3 selection marker for complementation experiments. Geneticin resistant BY4743 *YPD1/ypd1Δ* heterozygous deletion mutant was transformed with *MoHPT1*-pYES2 where *MoHPT1* was cloned under Gal1 promoter. Transformants were selected on Ura dropout plates containing geneticin to ensure transformation of the muatnt. The selected transformants were verified for the construct by *MoHPT1* specific PCR. For complementation assay, *MoHPT1* transformants were pre-grown in 4% Galactose medium, sporulated and treated with zymolase enzyme. Several dilutions were spread out on *ura* dropout medium with 4% galactose to select colonies expressing *MoHPT1*. The selected colonies were patched on geneticin 200 mg/l. Those selected on geneticin were assayed for mating type assay to verify haploid status of the complemented transformant.

2.5 Transformation of the fungus

The *A. tumefaciens* strain LBA4404/pSB1 was first subjected to transconjugation with pSil-*MoHPT1* carrying *E.coli* DH5α *via* triparental mating using Helper strain carrying plasmid pRK2013. The transconjugant *Agrobacterium* obtained was then used to carry out ATMT of *M. oryzae* as described (Mullins *et al*., 2001).

Briefly, *M. oryzae* spores were collected from 7-8 days old oatmeal agar plates and concentration adjusted to 10^6 spores/ml. *Agrobacterium* carrying the respective constructs was grown at 28 °C for 2 days in minimal medium (MM: Dextrose 10 g, KCl- 0.5 g, $MgSO_4$- 0.5 g, KH_2PO_4- 1.5 g, $NaNO_3$-6 g, pH-6.5). Subsequently, *A. tumefaciens* cells were diluted to OD_{600} = 0.15 in the induction medium (IM) and grown for another 6 h. Fungal spores were co-cultivated in the presence of 200µM Acetosyringone (Sigma Chemicals St. Louis, MO, USA) with *Agrobacterium* and *M. oryzae* transformants were selected on complete medium, CM supplemented with Hygromycin B (Sigma Chemical, St. Louis, MO, USA) to a final concentration of 200 µg/ml. Untransformed *M. oryzae* was used as a control. The transformants were maintained as monoconidial isolates to obtain pure cultures (Gupta and Chattoo, 2007).

2.6 Southern blot analysis

Fungal genomic DNA was extracted as described by Dellaporta *et al*. (1983). About 5 g of biomass grown for 3-4 days in complete medium was frozen in liquid nitrogen, ground to fine powder and resuspended in 15 ml DNA extraction buffer. Almost 1 ml 20% SDS was added to the mixture and incubated at 65 ^oC for 10 min after gentle mixing. To this mixture chilled 5 ml of 5 M potassium acetate was added gently mixed and incubated on ice for 20 min. To the supernatant collected after centrifugation for 20 min at 8000 rpm, 10 ml chilled isopropanol was added and incubated at -20 ^oC for 30 min. The mixture was centrifuged at 12000 rpm the supernatant was discarded and the precipitated DNA pellet was collected and air dried. The DNA pellet was further dissolved in 700 µl DNAse free milliQ water and treated

with RNAse (5 µl of 10 mg/ml stock). Choloroform :isoamylalcohol (400 µl) extraction of the DNA mixture was done and the supernatant was reprecipitated with 3 M sodium acetate pH 5.2 (75 µl) and isopropanol(500 µl). The precipitated DNA was was washed with 70% ethanol, air dried and redissolved in 100 µl DNAse free milliQ water.

Southern blot analysis was carried out as previously described (Sambrook *et al*., 1989). About 10 µg genomic DNA was digested with appropriate restriction enzymes, precipitated and electrophoressed on 0.8% Agarose gels and blotted on to nylon membrane (Hybond N+, Amersham, GE Healthcare, Buckinghamshire, England). The blotted DNA was fixed to the nylon membrane by UV-crosslinking. The probe was labelled and hybridising bands were detected using Gene Images AlkPhos Direct Labeling and Detection System as per manufacturer's instructions (Amersham, Buckinghamshire, England).

2.7 Growth rate analysis of *M. oryzae* strains

The fungus was subjected to osmotic stress with 1 M NaCl and 1.5 M sorbitol, oxidative stress with 5-10 mM H_2O_2 and 150 µM paraquat for stress induced studies. Stress resistance of the knock-down transformants against antifungal agents phenylpyrrole and dicarboximide was examined by providing fludioxonil, vinclozonil and iprodione in YEG agar plates in a concentration range of 1-100 µM. The samples were also treated with 2 mM ABTS and other cell wall perturbing agents like SDS, Congo Red and Caffeine, either in broth or in agar plates for Laccase assay and cell

wall sensitivity assays respectively. Agar plugs of *M. oryzae* strains were spotted on fresh YEG supplemented with each of the above reagents and cultured at 28 °C for 3 days. The diameter of each colony was measured. All the experiments were performed in triplicate.

2.8 Melanin formation of *M. oryzae*

M. oryzae strains were pre-cultured for 3 days in 5 ml of YEG. Then, 200 µl of this pre-culture was seeded in 5 ml of YEGPCa (0.5% yeast extract, 2% glucose, 0.05% KH_2PO_4, 0.05% $NaH_2PO_4 \cdot 12H_2O$, 0.001% $CaCl_2$) (Yamaguchi *et al*., 1982) and cultured for 48 h and photographed. Carpropamid (20 ppm) was added after 24 h incubation.

2.9 Pathogenicity of *M. oryzae* strains on rice plants

In planta infections were carried out using conidial suspension of the rice blast fungus (1×10^5/ ml in 0.2% gelatin) and sprayed with an artist's airbrush onto the three leaf stage susceptible rice cultivar, CO-39, incubated in a wet box at 20 °C for 17 h and cultured in controlled environment chambers (light 14 h at 25 °C and dark 10 h at 20 °C) for 4–7 days for full symptoms to become apparent. The number of lesions on the third leaf of the infected plants was counted. Pathogenicity assays were repeated at least three times, using 12–14 leaves per assay. Detached leaf assay was carried out on 3 weeks old rice leaves by inoculating mycelia plugs previously grown on CM

medium and incubated on rice leaves placed on water agar containing kinetin (2 mg/l) plates in moist chamber. Infection lesions were analysed after 4–5 days.

2.10 Appressorium formation of *M. oryzae*

A 30 µl drop of the conidial suspension (1×10^4/ ml) was spotted on plastic cover slips or the hydrophobic side of Gelbond membranes (Hamer *et al*., 1988) and left in a humid environment at 25 °C. The frequency and morphology of appressorium formation were analysed after 16 h. Appressorium formation efficiency was measured at least 3 times, using more than 100 spores per assay.

2.11 Total RNA extraction and cDNA synthesis

Fungal biomass grown in liquid media was frozen in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies, California, USA). The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry. 5 µg of total RNA was used to synthesise the first strand cDNA using MoMuLV reverse transcriptase (NEB) and random hexamer in 20 µl reaction system.

2.12 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time RT-PCR (qRT-PCR) was used to examine the expression pattern of *MoHPT1* under various conditions. Total RNA was isolated from 10^2

conidia/ml geminated in liquid complete medium and grown for 3 days. After 72 h of growth in complete medium each sample was exposed to stress conditions namely, 1 M NaCl, 1.5 M Sorbitol, 10 mM H_20_2 and light, for fifteen to thirty minutes before harvesting. qRT-PCR was performed by monitoring the increase in fluorescence of the SYBR Green dye on Light Cycler system (Roche Applied Science, Mannheim, Germany), or Taqman and SYBRgreen chemistry on Applied Biosystems, USA, according to the manufacturer's instructions. Each qRT-PCR quantification was carried out in triplicate using primers for individual genes (Table 1 and 2). The data were normalised against Tubulin gene. The Relative expression was calculated with $2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{\text{tubulin}})$ and fold changes were calculated by $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = (Ct_{\text{gene of interest}} - Ct_{\text{tubulin}})_{\text{test condition}} - (Ct_{\text{gene of interest}} - Ct_{\text{tubulin}})_{\text{control}}$. The primers used for the experiment are listed in the Appendix 3, Table 1.

2.13 Raising of antibodies against MoHPT1

A 435 bp segment of the *MoHPT1* cDNA which included the complete histidine phosphotransferase domain was cloned into pET30a for bacterial expression with a 6x His Tag. *E.coli* BL21 strain was transformed with the construct and was induced with 1 mM IPTG as per protocol and induction was checked at 2-6 h. Induced sample were run on SDS-PAGE with controls to confirm the induction of the desired protein. The protein was further purified using Ni-NTA resin (Thermo Scientific, MA, USA), concentrated and used for immunisation of New Zealand rabbits for generation of antibody. An initial immunisation dose of 300 µg was given, followed by two booster doses. The serum was collected after 21 days, purified using protein A column. The titre of the antibody was determined by ELISA and later used for western blot analysis.

2.14 Western blotting

Total protein was extracted from wild type *M. oryzae* mycelia grown in CM for 72 h, filtered and re-grown in CM with and without different stress treatments for 6 h. The protein samples were electrophoresced on 15% SDS-polyacrylamide gel, followed by electrotransfer to PVDF membrane (Hybond-P, GE Healthcare Life sciences). Antibody binding was visualised by the binding of a horseradish peroxidase-conjugated secondary antibody followed by detection with Super signal West pico chemiluminescence Substrate (Pierce, Rockford, USA). The stress activated phospho MAPK was detected by anti-phospho p38 MAPK antibody from Peirce Antibodies Thermo Fischer Scientific (MA, USA). Anti-Hog1 antibody from Santa Cruz Biotechnology, (Santa Cruz, CA, USA) was used to detect Hog1 expression.

2.15 Immunolocalisation and Microscopy

Calcofluor staining for chitin and DAPI (Sigma Chemicals, MO, USA) staining for nucleus was performed as per manufacturer's instructions. Antisera were raised in New Zealand white rabbits using bacterially expressed and purified MoHPT1 as the immunogen. Antisera were purified using Protein A column to provide IgG-enriched preparations. The titre of the preparation was checked by ELISA with purified MoHPT1 as the antigen. Western blot was done to confirm the activity of the anti-MoHPT1 preparation. The immunolocalisation of MoHPT1 was performed with the MoHPT1 IgG at different stages of fungal development. The samples (mycelium,

spores or appressoria) were fixed with 10% formaldehyde, 5% acetic acid and 85% ethanol for 30 min at room temperature and incubated in 0.1% toluidine blue for several hours to overnight. The fixed slides were further treated as previously described (Patkar and Chattoo, 2006). Primary antibody used was anti-MoHPT1. The slides were observed under an Olympus microscope BX51 and Zeiss LSM 700 confocal microscope.

2.16 Tail PCR

Tail PCR was performed as described by Mullins *et al.* (2001). The primary reaction consists of three cycles. Very low stringency cycle allows the amplification of the specific product. This cycle also amplifies many non-specific products. The third cycle that includes medium stringency of annealing temperature allows amplification of specific as well as nonspecific product. The secondary tail PCR consists of two cycles, high stringency and medium stringency cycle. The high stringency cycle was optimised to obtain specific product. Annealing at high stringency temperature was standardised to 62 ^{o}C. The tertiary reaction consists of only one cycle. The temperature standardised for the secondary TAIL PCR reaction was used for the tertiary reaction.
The reaction details are as given below:

Primary Reaction	**Secondary Reaction**	**Tertiary Reaction.**
94 ^{o}C- 30sec*	94 ^{o}C- 30sec*	94 ^{o}C- 30sec*
25 ^{o}C- 2min*	65 ^{o}C- 1min*	94 ^{o}C- 30sec****
72 ^{o}C- 2min*	72 ^{o}C- 2min*	44 ^{o}C- 1min****

94 ^{o}C- 30sec*	94 ^{o}C- 30sec***	72 ^{o}C- 2min****
65 ^{o}C- 1min*	65 ^{o}C- 1min***	72 ^{o}C- 5min*
72 ^{o}C- 2min*	72 ^{o}C- 2min***	
94 ^{o}C- 30sec***	94 ^{o}C- 30sec**	
44 ^{o}C- 1min***	44 ^{o}C- 1min**	
72 ^{o}C- 2min***	72 ^{o}C- 2min**	
94 ^{o}C- 30sec***	72 ^{o}C- 5min*	
65 ^{o}C- 1min***		
72 ^{o}C- 2min***		
72 ^{o}C- 5min*		

*one cycle, ** 12 cycles, *** 15 cycles , **** 25 cycles

2.17 Co-immunoprecipitation of MoHPT1

Biomass was harvested from fungal mycelia grown for 3 days in complete medium, homogenised with liquid nitrogen and resuspended in chilled immunoprecipitation buffer (Appendix 1) containing protease inhibitor cocktail and phosphatase inhibitor cocktail. The total cell lysate was centrifuged for 20 min at 14,000 xg at 4 °C, and the supernatant was filtered through 0.2 micron Acrodisc Syringe filter (Pall Corporation). The filtrate was precleared with equilibrated binding buffer (EB) and was combined with anti-MoHPT1/ anti-HA / anti-HOG1 or Control IgG (3–5 µL whole antiserum or 0.5–1 µg affinity-purified antibody/reaction) and incubated at 4 ^{o}C for 1 h on a rotating wheel. The antibody bound protein extract was incubated with 400 µl protein A agarose bed resin at 4 °C for 3 to 6 h with rotation.

The Supernatant was collected by centrifugation at 3000 xg for 15 sec and the resin resuspended in 1 ml EB followed by incubation with rotation for 20 min at 4 ^{o}C. This step was repeated 4 times. After the final supernatant removal, 20 µl sample buffer was added, boiled for 3 min and used for SDS-PAGE and western blot.

2.18 RNA extraction for RNA sequencing

Total RNA was extracted from mycelia or germinating conidia using the Qiagen RNeasy Plant Mini kit according to manufacturer's instructions. RNA was eluted in RNase-free water and checked for integrity and quantity on an Agilent 2100 Bioanalyzer according to manufacturer's instructions. RNA with integrity number of at least 6.5 was used for library preparations. RNA was prepared from at least two biological replicates and used for independent library preparations.

2.19 Library preparation for RNA-Seq

Sequencing libraries were prepared using mRNA-Seq Sample Preparation kit from Illumina from 9 mg of total RNA according to the manufacturer's instructions. Libraries were quantified and checked for quality on Agilent 2100 Bioanalyzer using a DNA 100 chip kit. Each library was diluted to 10 nM in Elution Buffer (Qiagen) and used for

sequencing using an Illumina Genome Analyser GX II platform.

2.20 Transcriptome Analysis and identification of differentially expressed genes

A 100 bp paired end sequencing was done on the Illumina HiSeq 1000. A total of 59 million reads for wildtype B157 and 70 million reads for *MoHPT1* knock-down transformant RA6 were generated. Based on the quality, sequence reads were trimmed (Q>30) using FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), to retain only high quality reads for further analysis. The pre-processed reads were aligned to the reference *M. oryzae* genome and gene model was downloaded from Ensembl database (Genome version MG8, strain 70-15). The alignment was performed using Tophat program (version 2.0.8) (Trapnell *et al.,* 2009) with default parameters. The aligned reads were used for estimating expression of the genes and transcripts using Cufflinks program (version 2.0.2) (Trapnell *et al.*, 2012). The differentially expressed genes (fold change > log_2) were classified into different groups on the basis of their functional domains. The validation of some of the differentially expressed genes was done using qRT-PCR with gene specific primers. The primers used for validation of transcriptome are listed in Appendix 3, Table 2**.** The validation qRT-PCR was repeated thrice.