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## CHAPTER 1: *Magnaporthe* Histidine Phosphotransferase is required for stress response signalling and survival

### 3.0 ABSTRACT

*M. oryzae* histidine phosphotransferase was found to be 84% identical to its *N. crassa* ortholog. The HPT in *M.oryzae* B157 was identified with two transcripts of the same gene. The longer transcript T0 was produced under the influence of light. Silencing of *MoHPT1* rendered the fungus less pathogenic than the wild type B157. The knock-down transformants of *MoHPT1* showed high sensitivity to osmotic stress, oxidative stress and cell wall stress. The phenylpyrrole antifungal aromatic compounds showed no effect on the wild type as well as the transformants. The expression analysis of the transformants showed a lower expression of *MoHPT1* and lower phosphorylation levels of *HOG1* in all the transformants. *MoHPT1* knock-down was also found to affect cell wall integrity signalling. *MoHPT1* knock-down affected sporulation and pathogenicity and also influenced laccase activity and expression. PAS domain containing Histidine kinases was induced in the presence of light was found to be affected in *MoHPT1* knock-down. The NCR and CCR plate assays showed that the gene has no positive regulatory effect on the nutrition repression pathways but instead showed a deficiency in the uptake of xylose as the sole carbon source. This study shows that *MoHPT1* has a pleiotropic role in stress signalling, development and pathogenicity.

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### 3.1 INTRODUCTION: Histidine phosphotransferase and it's role in stress signalling

The bacterial two component signal transduction (TCST) pathways regulate many signal transduction processes, including stress responses, motion, growth and development. Some bacterial species have over 300 two component proteins which co-regulate all these processes. Unlike the other signalling pathways, the component system universally displays the Histidine-Aspartic acid phosphorelay. A typical two component system (TCS) involves an external cue sensing transmembrane sensory kinase which is a histidine kinase and a response regulator. Upon activation, a sensor histidine kinase is autophosphorylated on a conserved histidine residue, which in turn phosphorylates a conserved aspartate residue in the receiver domain of a response regulator protein, which then signals for the necessary downstream expression processes. Although the number of phosphotransfer events in a pathway can vary, the phosphotransfer events in any given pathway ends in aspartyl phosphorylation followed by change in response regulator activity (Fassler & West, 2013).

Eukaryotic TCS is seen to be much more complicated than the prokaryotes. Phosphotransfer to or from a receiver domain Asp typically involves a histidine containing phosphotransfer (HPT) domain in eukaryotic TCST. TCST in eukaryotes involve more than one response regulator, both nuclear as well as cytoplasmic. Eukaryotic TSCT has evolved to form a multistep signal transduction rather than a simple signal transduction as that seen in prokaryotes. HPT forms an important component of this multistep signal transduction system and the protein shuttles between the nucleus and cytoplasm to activate the appropriate response regulator (RR) (Lu *et al.*,

2003). Such a TCST with an activated RR can regulate other downstream signalling pathways in response to a particular cue to bring about the proper response in favor of the survival and development of the fungus. The most studied example of a TCS regulated signalling in eukaryotes is that of *S. cerevisiae* where the stress activated MAPK *HOG1* is regulated by *SLN1* HK TCST pathway. The present study describes the TCS in *M. oryzae* B157, examining the native histidine phosphotransferase *MoHPT1* and its role as a member of the TCS in stress management, light sensitive response, cell wall integrity, oxidative stress management and pathogenicity. We show that *MoHPT1* in B157 is essential and therefore, used gene silencing to investigate the biology of *MoHPT1* at around 50 % of the gene expression. We expect that 50% lowering of the protein would mark a significant difference in the downstream signalling pathway.

## **3.2 RESULTS: Characterization of *MoHPT1* in *M. oryzae***

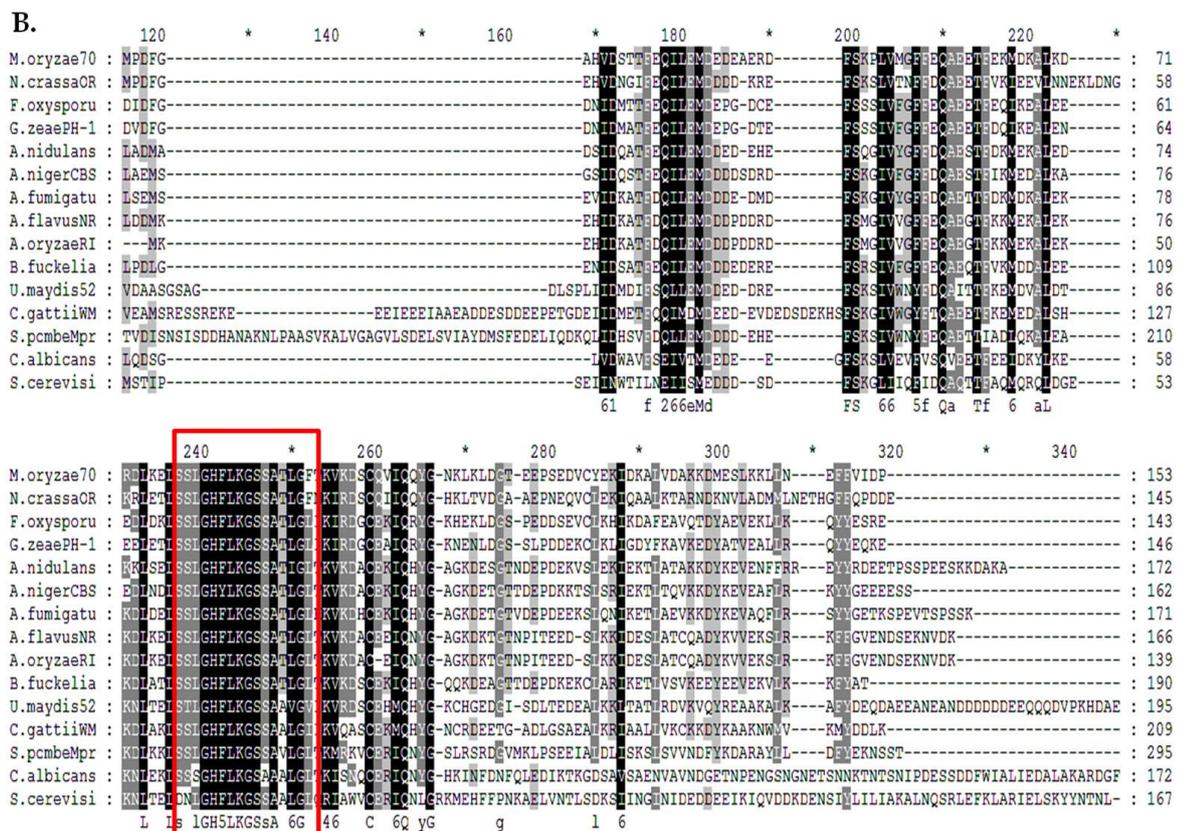
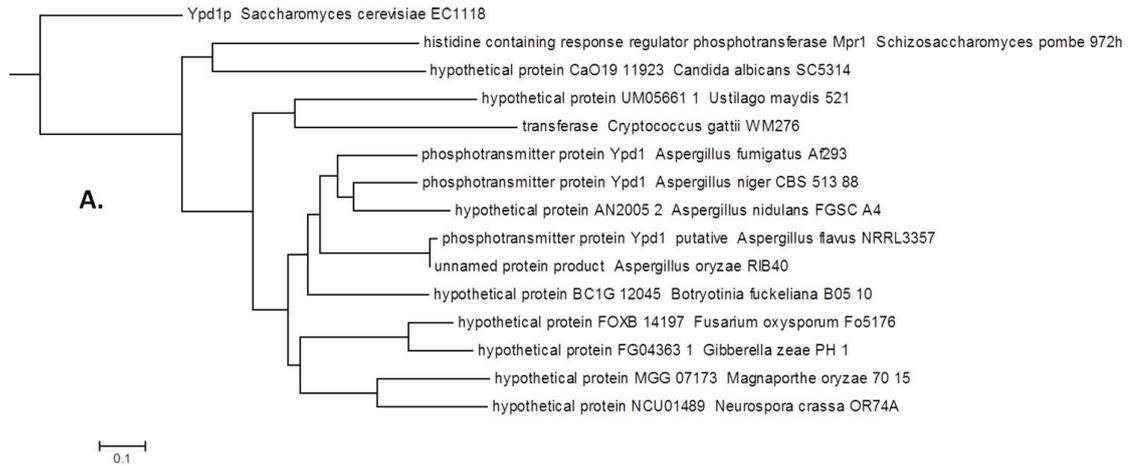
### **3.2.1 Identification of *MoHPT1* gene in *M. oryzae***

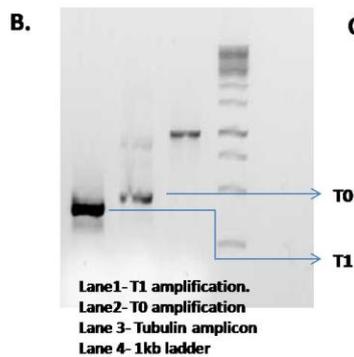
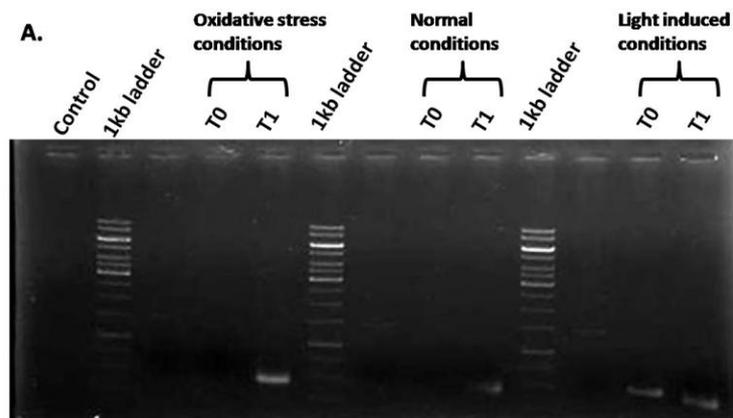
*M. oryzae* Histidine phosphotransferase (<http://www.broadinstitute.org/>) has about 44% identity to the yeast *YPD1*, with a coding sequence of 1558 bp containing two introns. The first intron spans from 44<sup>th</sup> base pair to 910<sup>th</sup> base pair of the ORF, whereas the second intron spans from 1095<sup>th</sup> base pair to 1216<sup>th</sup> base pair of the ORF. The predicted protein length of the gene was 161 amino acids. The sequence similarity at the protein level of *MoHPT1* to the histidine phosphotransferases from other fungi reflects their evolutionary relatedness, with the *N. crassa* *HPT* having the highest identity (84%) and *S. cerevisiae* the lowest (44%). Phylogenetic analysis of *M. oryzae* HPT protein

showed both MoHPT1 and NcHPT to be in the same clade (Fig. 7A). In addition, HPT1 sequence homology between these organisms was very high within the histidine phosphotransferase domain (Fig. 7B). Moreover, the essential histidine at the phosphorylation site at position 135 and other putative binding surface amino acids K138, G139, Q154, Q157, which could fold into a functional domain are conserved (Song *et al.*, 1999; Xu & West, 1999) (Fig. 7B).

### **3.2.2 Identification of two different transcripts of *MoHPT1* under different conditions**

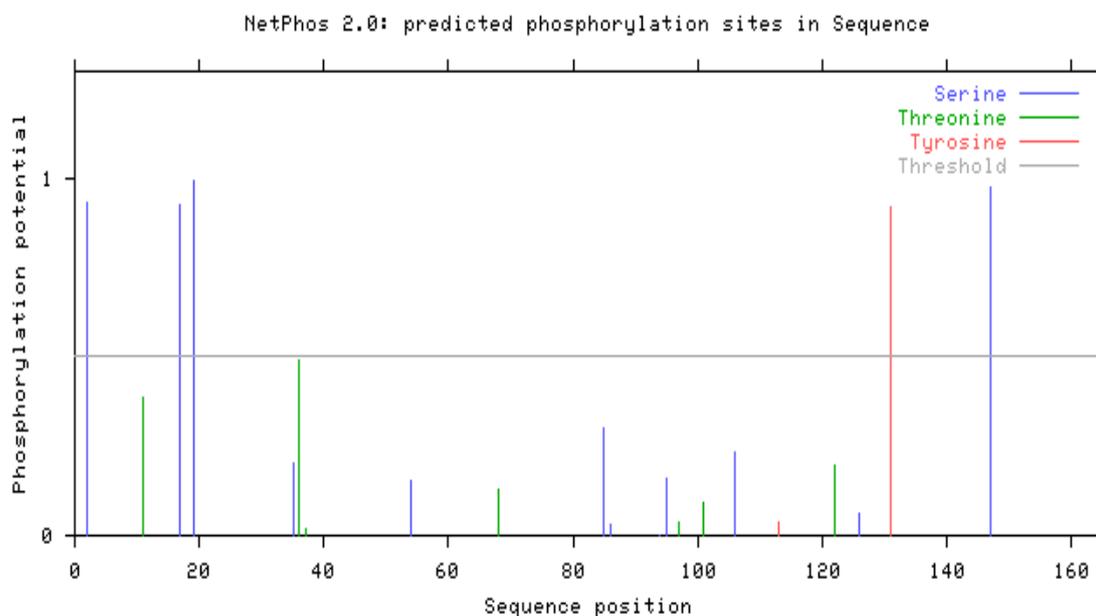
Two transcripts of *MoHPT1* were differentially expressed under normal and light induced conditions. The more abundant transcript, the smaller transcript T1 (408 bp), was seen to be expressed in all conditions, while the larger transcript T0 (462 bp) was comparatively highly expressed under light induced conditions. The two transcripts were amplified, cloned in pBluescript KS+, sequenced and analysed (Fig. 8A and 8B). The additional 54 bp stretch in T0' (as referred to in Broad database) coded for 18 amino acids which was further analysed by phosphorylation tools, NetphosK and GPS.3 (Fig. 9 and Appendix 3, Table 3). A potential Casein kinase phosphorylation site was identified in this additional N-terminal amino acid stretch.





**C.** MGG\_07173T1 | *Magnaporthe oryzae* 70-15 hypothetical protein, variant (408 nt)  
 ATGCCGATTTGGAGCCCATGTCGACAGCACGACGTTTCGAACAGATCCTGGAAATGGAC  
 GAAGATGAGGCCGAAAAGGGATTTTCAGCAAACCTTTAGTCATGGGATTCTTTGAACAAGCA  
 GAGGAGACTTTTGAAAAAATGGACAAGGCCCTGAAAGATCGTGACTTGAAAGAGCTTTCG  
 AGCCTTGGCCACTTCTCAAGGGTTCATCCGCCACTCTGGGCTTTACAAAGGTCAAGGAC  
 AGCTGCCAAGTGATTCAACAGTACGGAAACAAGCTGAAACTAGACGGCACTGAGGAGCCA  
 AGCGAGGATGTGTGCTATGAGAAGATCGATAAAGCACTGTGGATGCCAAGAAGGATATG  
 GAGAGTTTGAAGAACTTCTCAATGAGTTTTTCGTGATCGATCCTTAG

MGG\_07173T0 | *Magnaporthe oryzae* 70-15 hypothetical protein (462 nt)  
 ATGTA~~CT~~CTGTAAAGGACTCTGAAAAGTCCGATTCGGAAGAAAACGCCGACAAGATGCC  
 GATTTTGGAGCCCATGTCGACAGCACGACGTTTCGAACAGATCCTGGAAATGGACGAAGAT  
 GAGGCCGAAAAGGGATTTTCAGCAAACCTTTAGTCATGGGATTCTTTGAACAAGCAGAGGAG  
 ACTTTTGA AAAAATGGACAAGGCCCTGAAAGATCGTGACTTGAAAGAGCTTTCGAGCCTT  
 GGCCACTTCTCAAGGGTTCATCCGCCACTCTGGGCTTTACAAAGGTCAAGGACAGCTGC  
 CAAGTGATTCAACAGTACGGAAACAAGCTGAAACTAGACGGCACTGAGGAGCCAAGCGAG  
 GATGTGTGCTATGAGAAGATCGATAAAGCACTGTGGATGCCAAGAAGGATATGGAGAGT  
 TTGAAGAACTTCTCAATGAGTTTTTCGTGATCGATCCTTAG



#### NetphosK results

Site Kinase Score

**S-2 CKII 0.74**

S-2 CKI 0.53

**S-17 CKII 0.70**

**S-19 CKII 0.60**

S-35 CKII 0.55

T-36 PKC 0.59

T-37 CKI 0.56

S-54 PKA 0.68

T-68 CKII 0.52

T-68 CKI 0.59

**S-94 PKA 0.75**

S-95 PKA 0.69

T-97 PKC 0.75

T-101 PKC 0.63

T-122 CKII 0.53

S-126 CKII 0.69

**Y-131 SRC 0.54**

**S-147 PKC 0.79**

S-147 PKA 0.54

Highest Score: **0.79 PKC at position 147**

#### Net Phos 2- Serine predictions

Name Pos Context Score Pred

**Sequence 2 ---MSEEEE 0.935 \*S\***

**Sequence 17 VVEQSDSEE 0.929 \*S\***

**Sequence 19 EQSDSEENA 0.994 \*S\***

Sequence 35 AHVDSTTFE 0.205 .

Sequence 54 ERDFSKPLV 0.155 .

Sequence 85 LKELSSLGH 0.302 .

Sequence 86 KELSSLGHF 0.032 .

Sequence 94 FLKGSSATL 0.002 .

Sequence 95 LKGSSATLG 0.161 .

Sequence 106 KVKDSCQVI 0.232 .

Sequence 126 TEEPSEDVC 0.061 .

**Sequence 147 KDMESLKKL 0.973 \*S\***

### 3.2.3 Cloning and construct preparation of the *MoHPT1* gene in different expression vectors

The following vector plasmids were developed for the characterisation of *MoHPT1* in *M. oryzae* (Fig. 10). The primers used are mentioned in Appendix 3, Table 1.

- The full length *MoHPT1* ORF was isolated from B157 strain using specific primers and sequenced. The 1.5 kb fragment of the *MoHPT1* ORF amplified was cloned into pBluescriptKS+ at *KpnI* and *BamHI* sites and sequenced. This basic construct was later used for the development of several other constructs.
- Complementation experiments were carried out by expressing *MoHPT1* in *S. cerevisiae*. The ORF of *MoHPT1* was cloned into the 2 $\mu$  yeast expression vector pYES2 at *KpnI* and *BamHI* sites under Gal1 promoter and *ura3* marker. The construct was used for complementation study in *S. cerevisiae*.
- Bacterial expression of *MoHPT1* was used to produce antigen for polyclonal antibody generation. A 435 bp segment of the *MoHPT1* cDNA was cloned into pET30a at *NdeI/XhoI* for bacterial expression with a 6xHis Tag.
- A hemagglutinin (HA) tagged *MoHPT1* was constructed by cloning *MoHPT1* in pRTDS vector with TEV (Tobacco Etch Virus) translational enhancer and CAMV 35S promoter at *BamHI/KpnI*. The HA-*MoHPT1* construct was mobilised into pCAMBIA 1300 binary vector at *XbaI*. This construct was used for HA tagged *MoHPT1* expression in *M. oryzae* for immunoprecipitation studies.
- *MoHPT1* has an internal *SalI* site. Hygromycin cassette obtained from pCAMGFP by *SalI* digestion was ligated into the *SalI* digested KS-*MoHPT1* to

## Constructs developed for the characterization of the gene

### Yeast expression construct of *MoHPT1* pYES



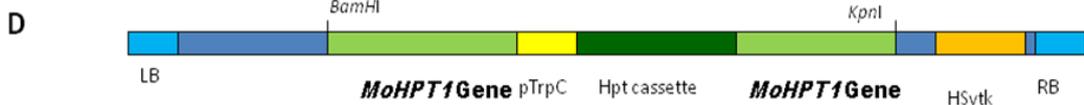
### Bacterial expression construct of *MoHPT1*



### *MoHPT1* with HA-tag for eukaryotic expression



### Disruption construct of *MoHPT1*



### RNAi construct of *MoHPT1*



### Antisense construct of *MoHPT1*



obtain the *MoHPT1* disruption construct. This KS-MoHPT1 Hyg was further mobilised into dual selection binary vector pGKO2 at *BamHI/KpnI* sites. The complete construct was confirmed by restriction digestion by *BamHI/KpnI* and *PvuII* enzymes.

A 521 bp stretch from the ORF (988<sup>th</sup> bp position to 1513<sup>rd</sup> bp position) was amplified and used for an RNAi construct. This particular sequence was analysed by SI-FI software tool ([http:// labtools.ipk-gatersleben.de/](http://labtools.ipk-gatersleben.de/)) for off target effects of siRNA in *Magnaporthe* transcriptome. There were no off targets predicted. This 521 bp was cloned in pBluescript-KS+ and then sub-cloned in pSilent vector. The RNAi construct was made in pSilent vector (Nakayashiki *et al.*, 2005) to generate a hairpin loop when expressed in the host. The construct was mobilised into pCAM1305 and verified by sequencing.

- Full length ORF of *MoHPT1* was also used to develop an antisense plasmid construct in pSilent vector under TrpC promoter and TrpC terminator at *BglIII/KpnI*. The construct was mobilised into pCAMBIA 1305.

All the binary constructs were used to generate *Agrobacterium* LBA4404 transconjugants and the positive transconjugants of HA-*MoHPT1*, RNAi and disruption constructs were used for ATMT of wild type *M. oryzae*.

### 3.2.4 Antibody Raising and Western blot of MoHPT1

*E. coli* BL21 strain was transformed with the pET30a-*MoHPT1* construct. The MoHPT1 protein was induced with 1mM IPTG at 28 °C incubated for 6 h. Induced sample was run on SDS-PAGE with controls (Fig. 11A). The protein was purified using

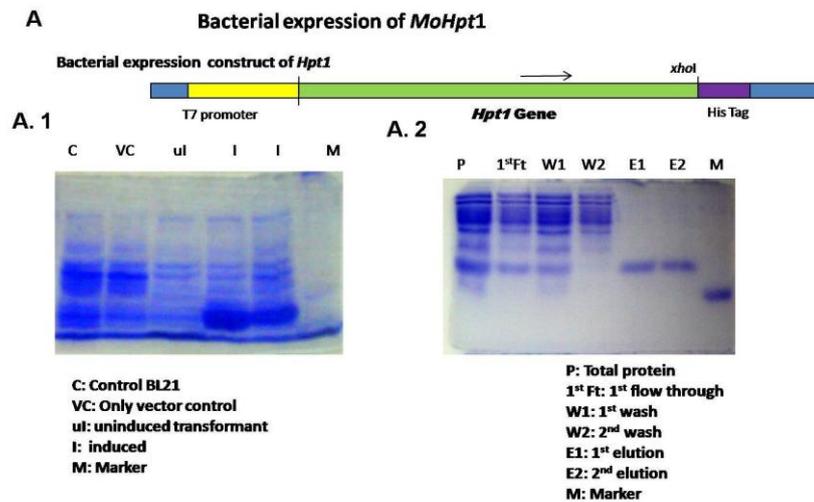
Ni-NTA resin was used for immunisation of rabbit for raising antibody. The serum collected after 2 boosters was purified. The predicted molecular weight of MoHPT1p was 17.4 kD, while single band of MoHPT1p detected by western blot analysis was around 30 kD (Fig. 11B). The size of native MoHPT1p was confirmed by western blot of HA-MoHPT1 using HA antibody.

### **3.2.5 Complementation of Yeast *YPD1* heterozygous deletion mutant with *Magnaporthe MoHPT1***

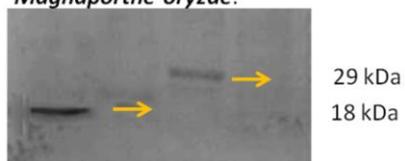
The complemented yeast transformants containing the pYES-*MoHPT1* construct were selected on 4% galactose ura dropout plates with 200 mg/l Geneticin. The selection assured only the growth of the transformed *MoHPT1* complemented BY4743 YPD1 heterozygous deletion mutants (Fig. 12A). The mating type assay further confirmed that these selected ones are the complemented haploid strains (Fig. 12B). The restoration of the mutant viability of the transformed haploids showed that *Magnaporthe MoHPT1* could complement the mutation in yeast, suggesting the conservation of function.

### **3.2.6 The functional expression of histidine phosphotransferase in *M. oryzae***

Initially the expression of *MoHPT1* in *M. oryzae* was confirmed at both transcriptional and translational level. The expression analysis of *MoHPT1* in B157 was done under osmotic stress conditions (Fig. 13A). The wild type fungus BI57 was treated with 0.8 M NaCl and 1.2 M Sorbitol. RNA was isolated from these samples and qRT-PCR was done. The NaCl treated sample showed about 4.6 fold induction in *MoHPT1*



**B Evidence of *MoHPT1* protein expression in *Magnaporthe oryzae*.**



Lane 1- *MoHPT1* expressed in pET 30-a  
 Lane 2- B157 (WT) *MoHPT1*

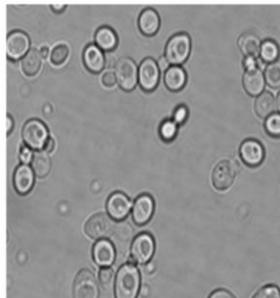
## Complemented haploids on Ura dropout plates

A



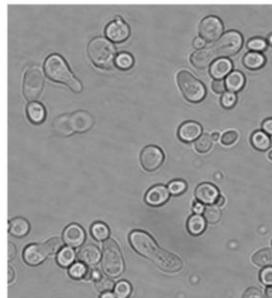
B

Diploid strain with Haploid 'a' strain



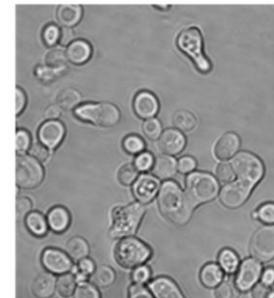
Negative control for shmoo formation 100x

Test strain with Haploid 'a' strain



Complemented Haploid 100x

Haploid alpha strain with Haploid 'a' strain



Positive Control for shmoo formation 100x

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expression and the Sorbitol treated samples showed about 1.8 fold induction. The expression of MoHPT1 protein in the wild type strain B157 was analysed under different stress conditions to evaluate the inducibility of the protein in response to stress. The western blot of MoHPT1 and Hog1 under different stress conditions showed that MoHPT1 expression is higher in oxidative stress and sorbitol stress conditions, while the Hog1 expression remained the same (Fig. 13A2).

### 3.2.7 Indirect immunolocalisation of MoHPT1

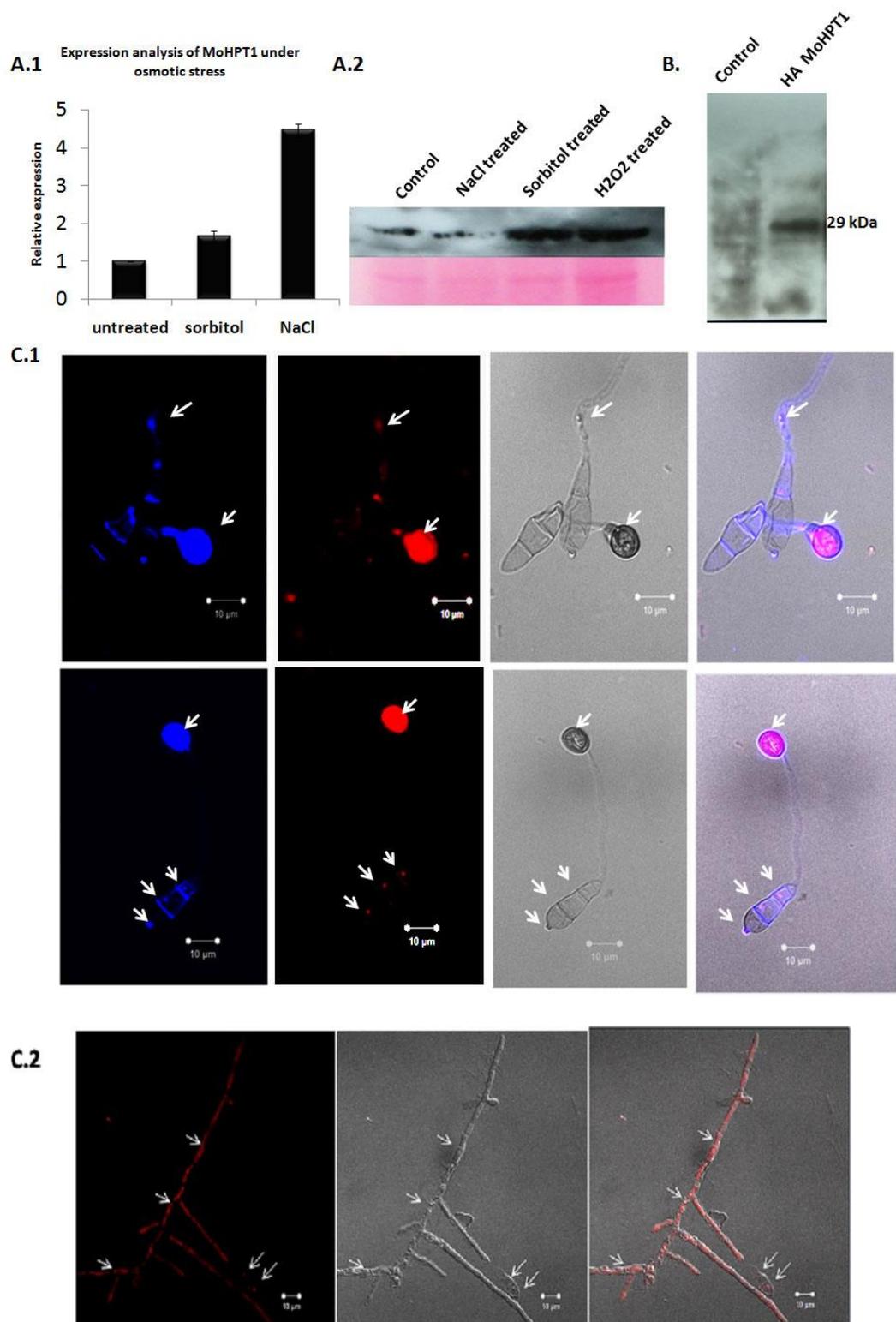
Immunolocalisation experiments were carried out using MoHPT1 primary antibody and TRITC-conjugated secondary antibody. The samples were counter stained with Calcofluor White for staining chitin and DAPI for visualising the nucleus. The wild type showed the presence of MoHPT1 in the spore nucleus. Where as, in the developing appressoria (Fig.13C.1) and vegetative hyphae it was also observed in the cytoplasm (Fig. 13C.2).

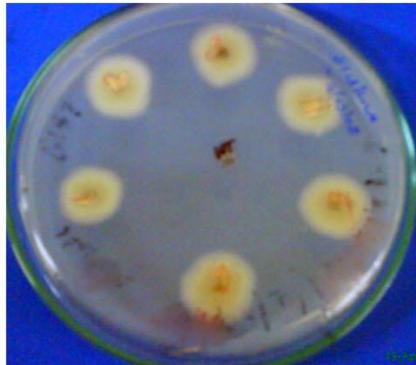
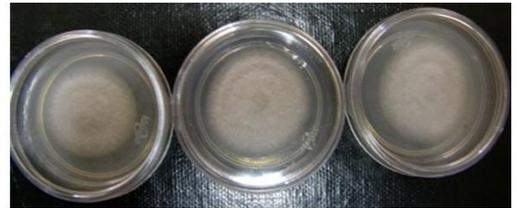
### 3.2.8 Null mutant of *MoHPT1* is probably in-viable in B157

Disruption of *MoHPT1* was carried out to explore the biological role of *MoHPT1* in *M. oryzae*, The *MoHPT1* disruptant construct was used for transformation of the B157 *M. oryzae* strain to generate *MoHPT1* mutant. The transformants obtained were selected on hygromycin followed by F2DU (5-fluoro-2'-deoxyuridine) (Fig. 14).

Selected transformants were subjected to locus PCR to screen for true transformants.

None of the transformants were disruptants of the gene because none those selected



**Disruption construct of *MoHPT1*****A.** Transformants selected on Hygromycin**B.** Transformants selected on F2DU

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gave the PCR amplification pattern expected for a disrupted locus . The experiment was repeated multiple times.

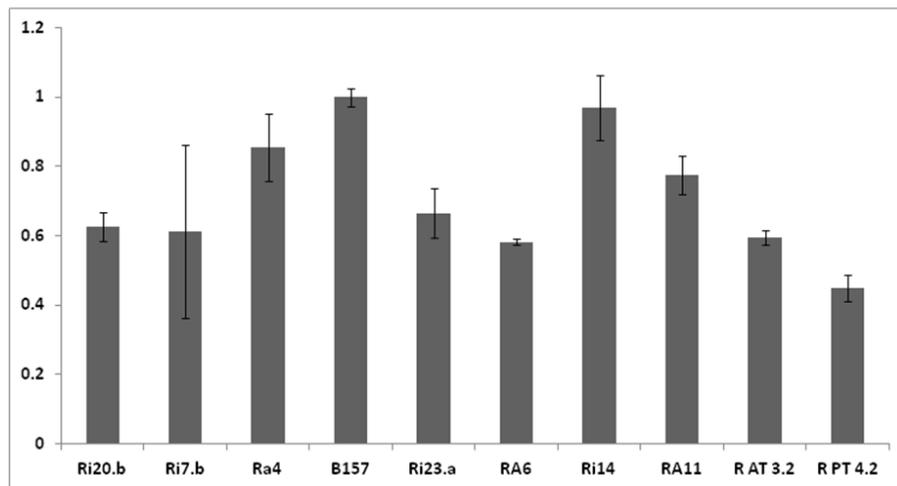
### **3.2.9 Knock-down of *MoHPT1* in *M. oryzae***

When repeated attempts failed to obtain a disruptant, we assumed that the gene might be indispensable for B157 *M. oryzae*. Silencing of *MoHPT1* was initially carried out using an antisense construct. Since the antisense transformants didn't show consistent down regulation of *MoHPT1*, we adopted RNAi technology. Knock-down transformants were then generated using a pSilent silencing vector to functionally analyse the gene. The transformants were screened for reduced expression of *MoHPT1* and stress sensitivity. Among 123 transformants obtained, fifteen showed reduction in *MoHPT1* expression by 40-60% as compared to the wild type (Fig. 15A). These selected transformants showed stable growth on hygromycin selection plates. Three of the knock-down transformants RA6, RA11 and Ri14 were used in initial studies (Fig. 15B). The knock-down transformant RA6 showed 40% silencing of *MoHPT1*.

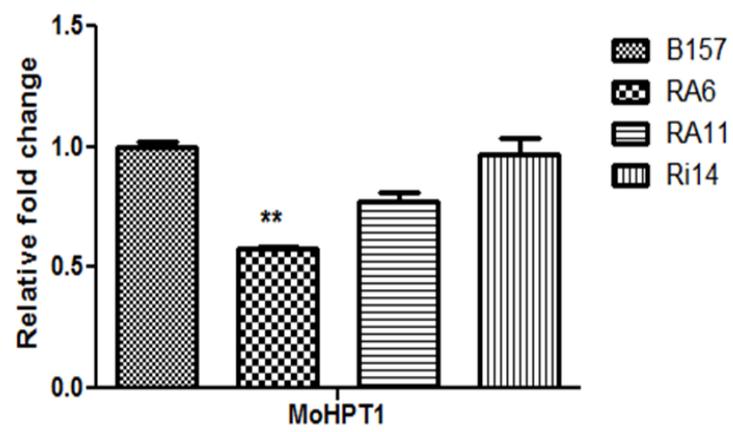
### **3.2.10 Tail PCR of *MoHPT1* Knock-down transformant RA6**

Genomic DNA of the wild type B157 and the RNAi transformant RA6 was isolated (Dellaporta *et al.*, 1983.) and tail PCR was performed as described in a previous report (Mullins *et al.*, 2001) for the identification of site of integration. The amplicon obtained was sequenced and the results suggested a possible integration of the construct

A



B



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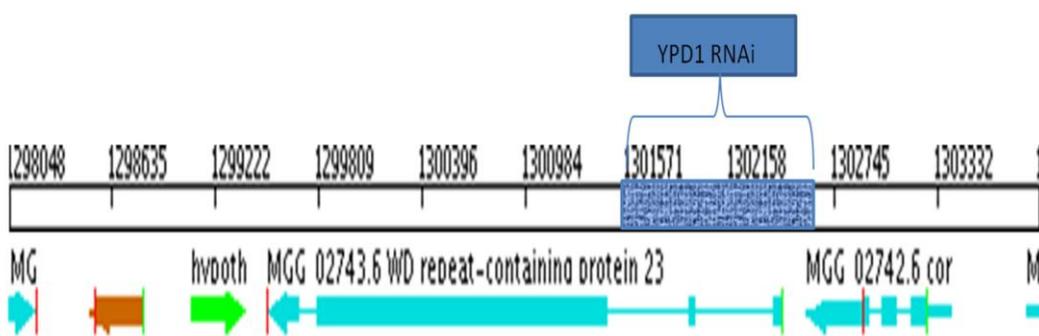
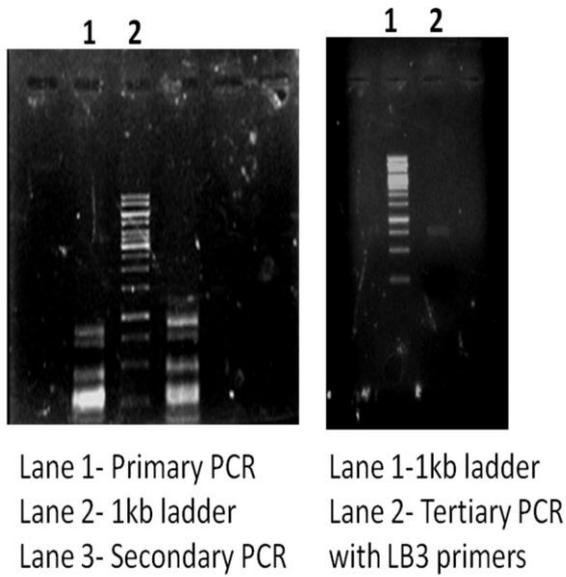
in a region between the WD23 repeat protein MGG\_02743 and Sec protein MGG\_02742 (Fig. 16) (Appendix 3). Later, we also analysed differential expression of these two genes to confirm that the integration of the construct at this site was not interfering with the expression of these genes. The expression levels of both transcripts (MGG\_02743 and MGG\_02742) in the wild type as well as in RA6 were not significantly different in oxidative stress treated samples and the control.

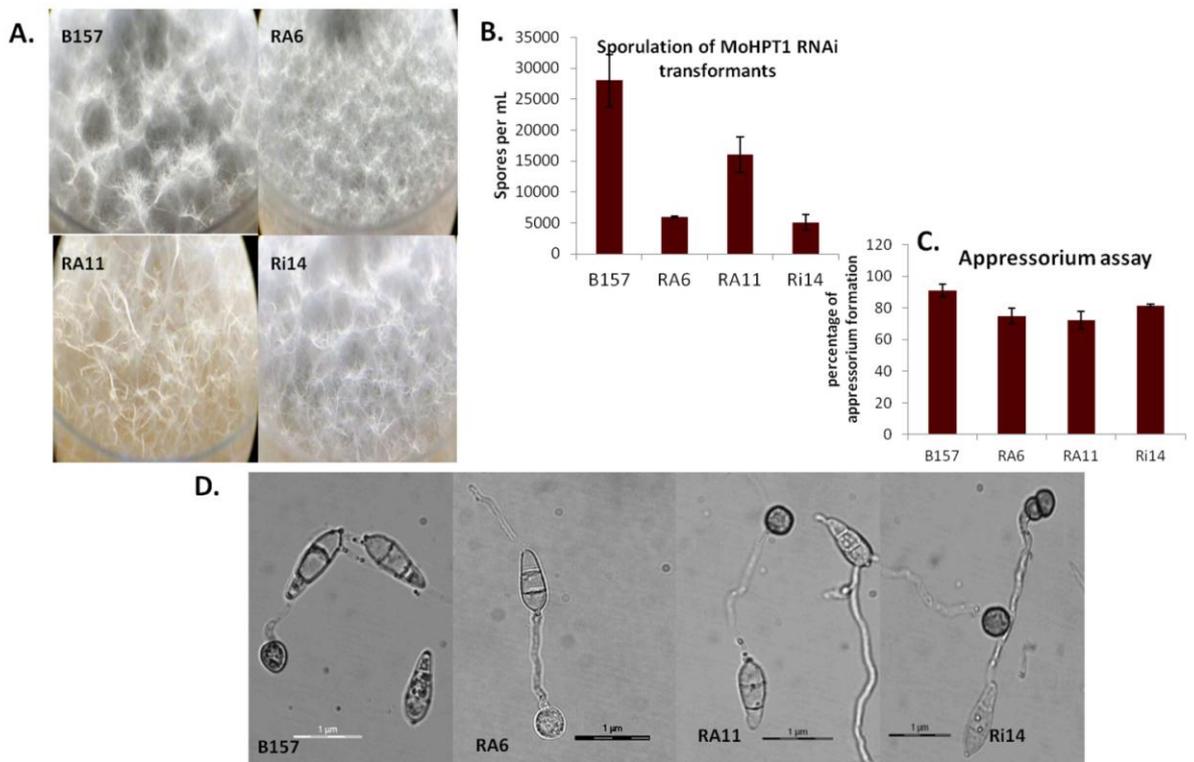
### **3.2.11 *MoHPT1* gene knock-down highly affected sporulation and pathogenicity**

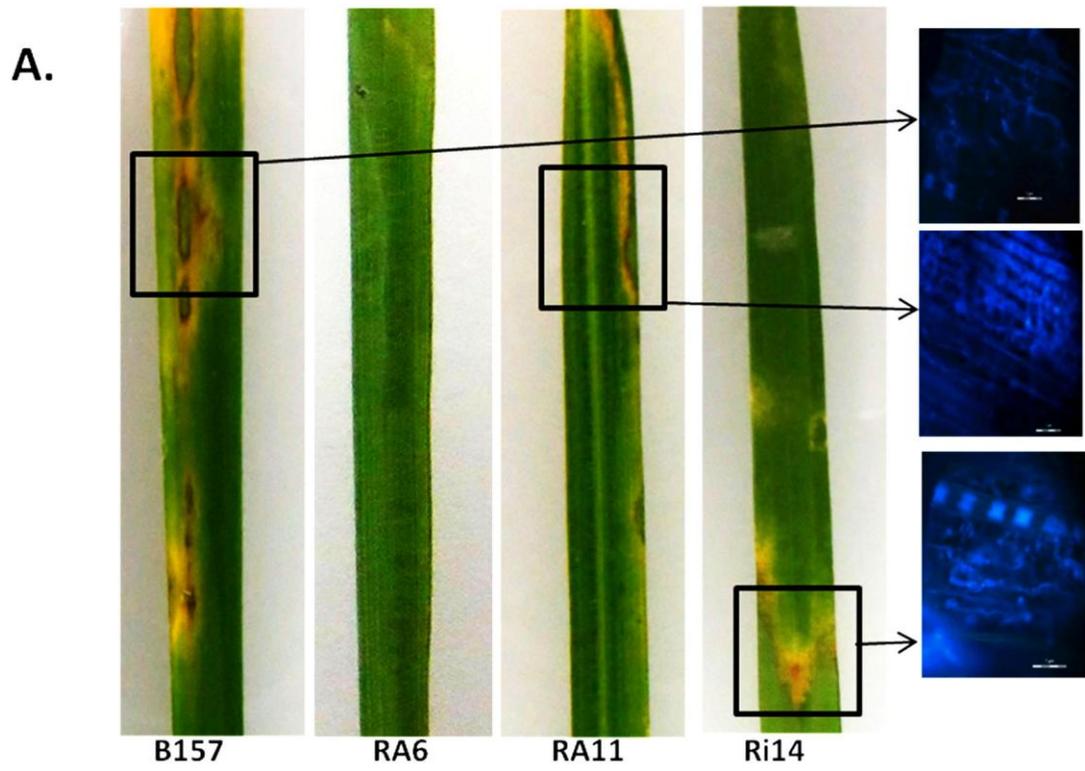
The knock-down transformants produced reduced numbers of spores when compared to the wild type. While reduced number of spores decreased infection, the efficiency of appressoria formation from the existing spores was not affected (Fig. 17). However, none of the more strongly silenced transformants (RA6, RA11) could establish infection within the host (Fig. 18A). Even wounded rice leaves inoculated with the hyphal plugs did not show significant infectivity. The infection assays on rice leaf showed that the transformants could penetrate the wounded host, but could not produce profuse growth and attain necrotrophy (Fig. 18B)

### **3.2.12 Knock-down of *MoHPT1* significantly affects the light induced expression of Histidine kinases**

The expression of Histidine kinases containing the PAS domains was analysed under light induced conditions in B157 as well as in RA6. All the PAS domain histidine



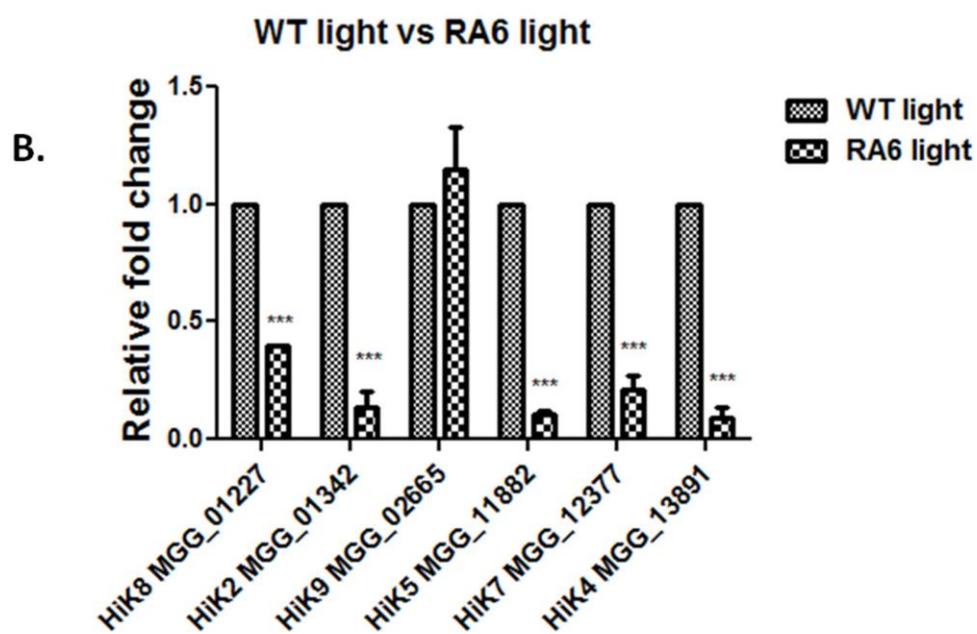
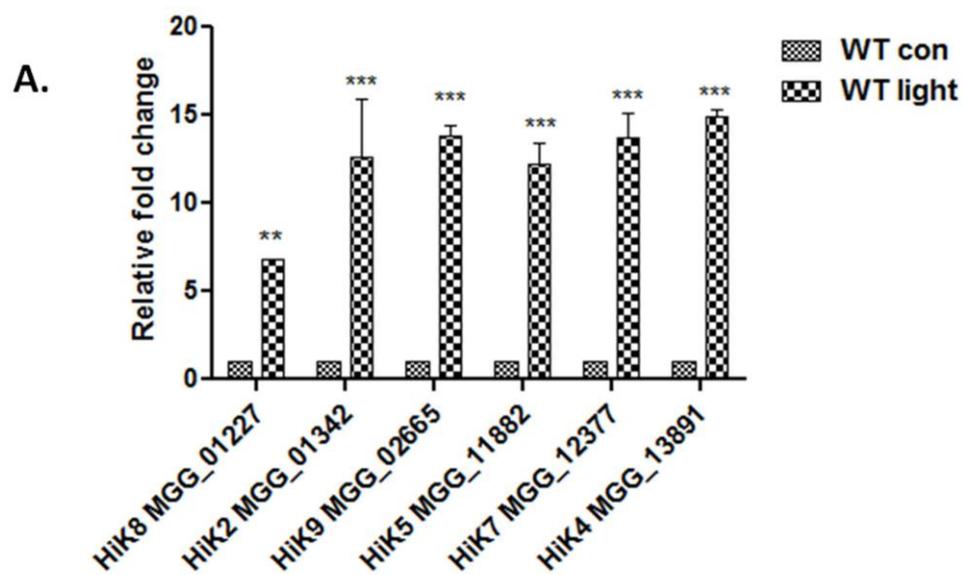


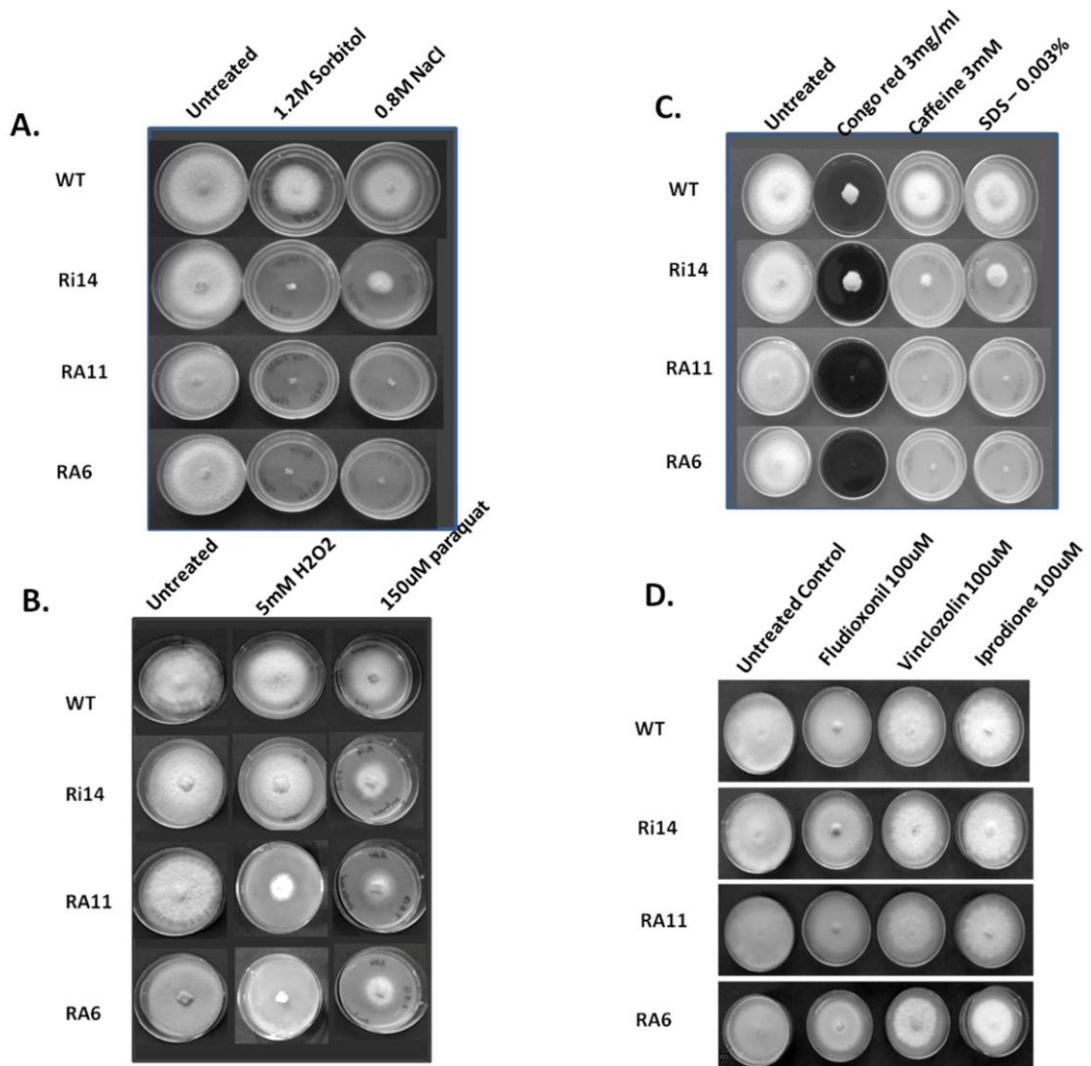


kinases were found to be highly up-regulated by about 6 to 15 fold in light induced conditions. The most strongly induced among the HKs were MGG\_13891 (LuxQ), MGG\_12377 (Phytochrome HK) and MGG\_02265. When RA6 was compared to B157, the PAS domain containing HKs, except for MGG\_02665, were seen to be highly down-regulated to about 10-40% even in the light induced conditions (Fig.19). The results suggest that *MoHPT1* influences the expression of PAS domain containing histidine kinases.

### **3.2.13 Stress sensitivity and expression of *MoHPT1* under stress in knock-down transformants**

The role of *MoHPT1* in stress adaptation was analysed. The wild type B157 and *MoHPT1* knock-down strain RA6 were exposed to different conditions on solid media including osmotic stress (NaCl and sorbitol), oxidative stress (H<sub>2</sub>O<sub>2</sub> and Paraquat), cell wall stress and cell membrane stress. The transformants were highly sensitive to all the kinds of stress studied as compared to the wild type but with varying degrees of sensitivity depending on the type of perturbant used (Fig.20). The expression of *MoHPT1* in the knock-down transformant RA6 under stress conditions was analysed. RA6 showed only about 60% of *MoHPT1* expression as compared to wild type under normal conditions and could not induce its expression to higher levels under stress conditions as compared to wild type (Fig. 21A). The knock-down transformants with a maximum of 40-60% reduction in *MoHPT1* expression showed higher sensitivity to different stress inducers, suggesting its role in stress response and survival.



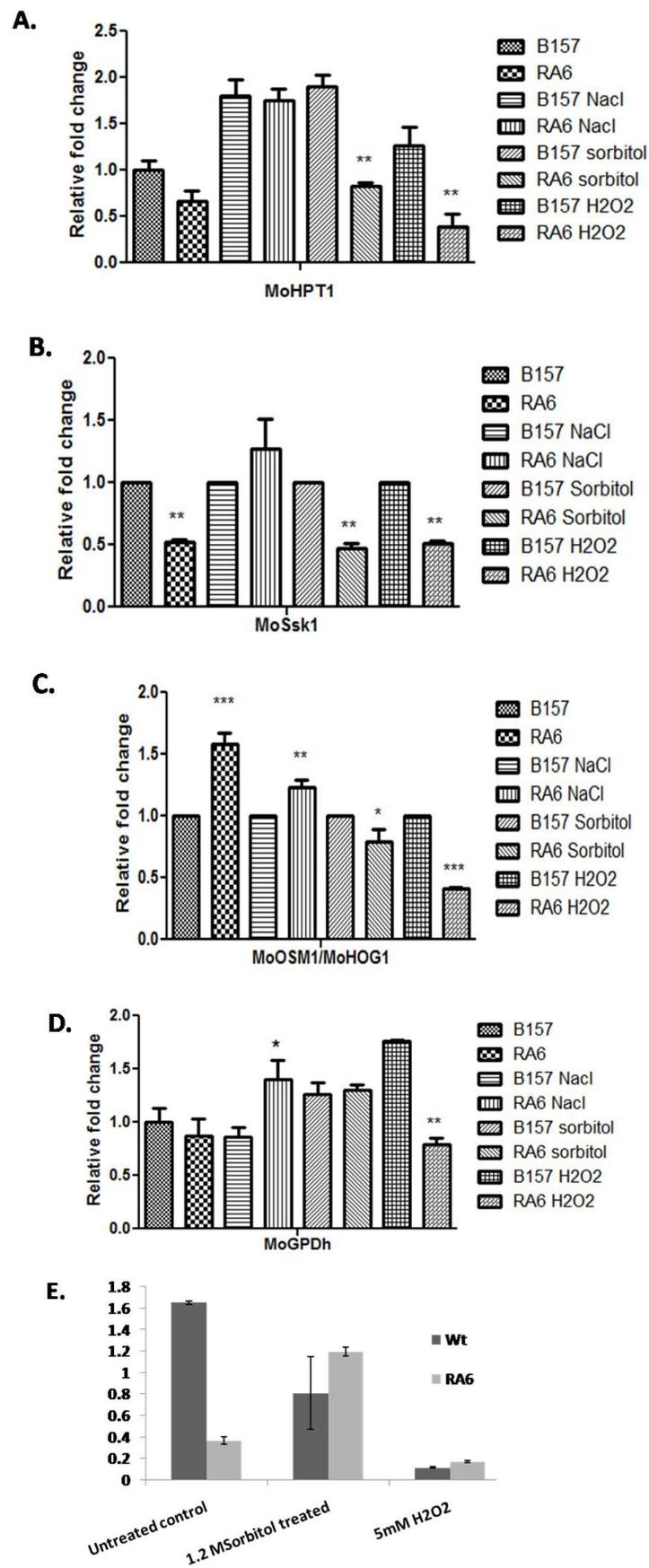


### 3.2.14 Resistance to phenylpyrrole and dicarboximide antifungal agents

The two component system mutants are generally attributed to phenylpyrroles and dicarboximides resistance through HOG1 pathway (Tanaka and Izumitsu, 2010). Our B157 itself was found to be resistant to fludioxonil, viclozolin and iprodione. The *MoHPT1* RNAi transformants were checked for resistance to phenylpyrroles and dicarboximides. The phenotype of the *MoHPT1* RNAi transformants also did not change in the presence of these compounds (Fig. 20D). Our results the lowered transcript levels of *MoHPT1* had no effect on its resistance to phenylpyrrole and dicarboximide anti fungals. The protein expression of the knock-down transformants in presence of phenylpyrroles antifungals also showed little difference as compared to wild type suggesting little effect of the antifungals on *M. oryzae*.

### 3.2.15 Expression of *MoHPT1* regulated genes under stress in knock-down mutant

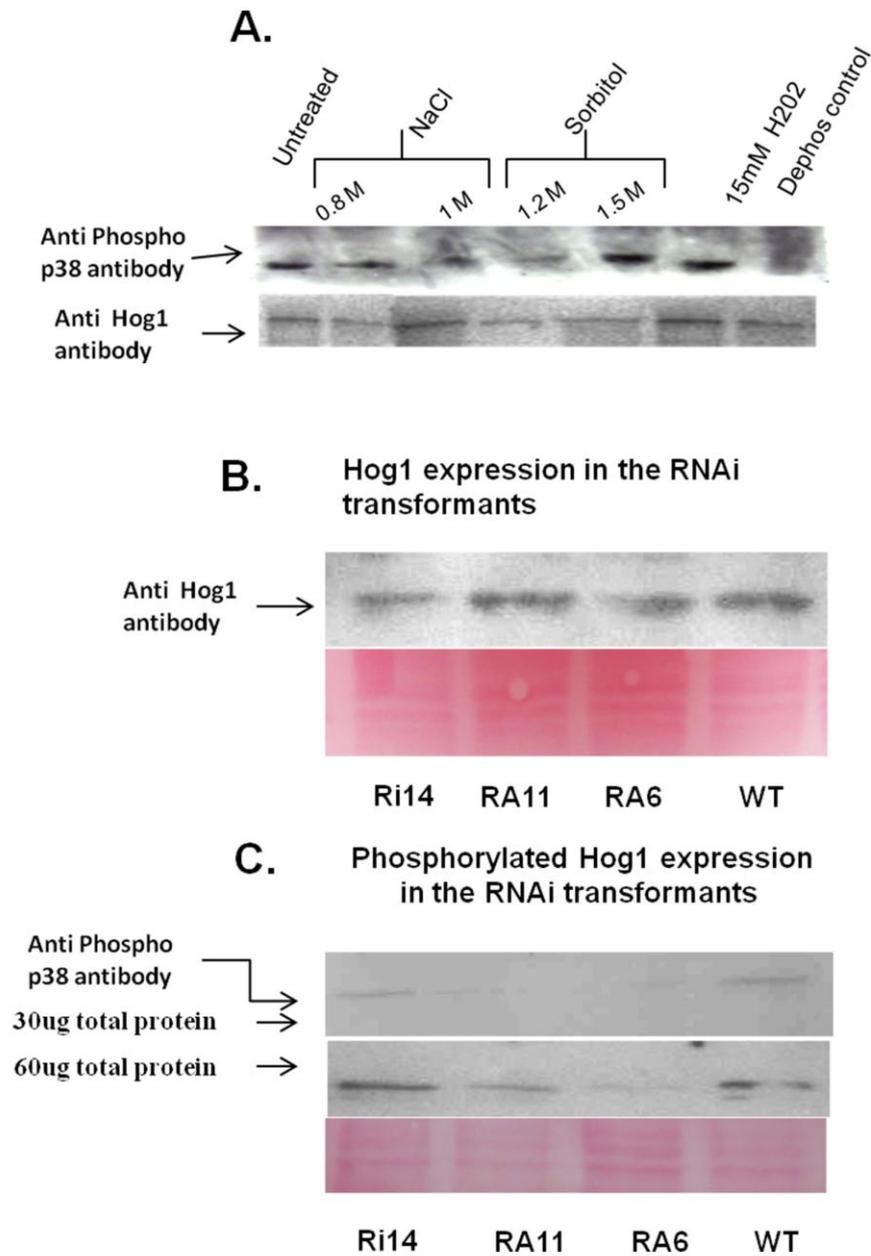
The expression of the response regulators *MoSSK1*, stress activated MAPK *Hog1/osm1* and the glycerol 6-phosphate dehydrogenase *G6PDH* was analysed in the knock-down transformant under stress conditions. The expression of *MoHPT1* and *MoSSK1* in the knock-down transformant was most significantly down-regulated under sorbitol stress and oxidative stress (Fig. 21A and 21B). The transcript levels of *HOG1* were induced under NaCl stress even in the *MoHPT1* knock-down transformants while; *HOG1* expression was decreased most under oxidative stress conditions (Fig. 21C). In the wild type, while MAPK Hog1 protein expression is not significantly affected under



these stress conditions, the phosphorylated form of Hog1 was altered in different stress conditions. The phosphorylation levels of Hog1 showed a decrease under NaCl stress, whereas increased phosphorylation was seen in sorbitol treated as well as H<sub>2</sub>O<sub>2</sub> treated samples (Fig. 22A). The level of Hog1p was examined in both phosphorylated and non phosphorylated form in RNAi transformants to determine whether the silencing of *MoHPT1* does affect the downstream signalling. Phosphorylated Hog1p was highly reduced in the RNAi transformants; however the total Hog1 protein concentration remained the same in all the samples (Fig. 22C and 22D). Thus total Hog1p levels was observed to be independent of *MoHPT1* expression in the knock-down transformants under stress conditions, while the reduction of phosphorylation on Hog1p in the knock-down indicates that MoHPT1 is involved in the downstream Hog1 MAPK signalling (Fig. 22B and 22C). These immunoblots show that the silencing of *MoHPT1* has effectively influenced the downstream MAP kinase. However, the glycerol content of cells in the knock-down transformant was unaffected and the expression of mitochondrial *GPDH* which accounts for the maximum glycerol levels in the cytosol was also not affected in the *MoHPT1* knock-down (Fig. 21D and 21E).

### 3.2.16 *MoHPT1* in cell wall stress signalling

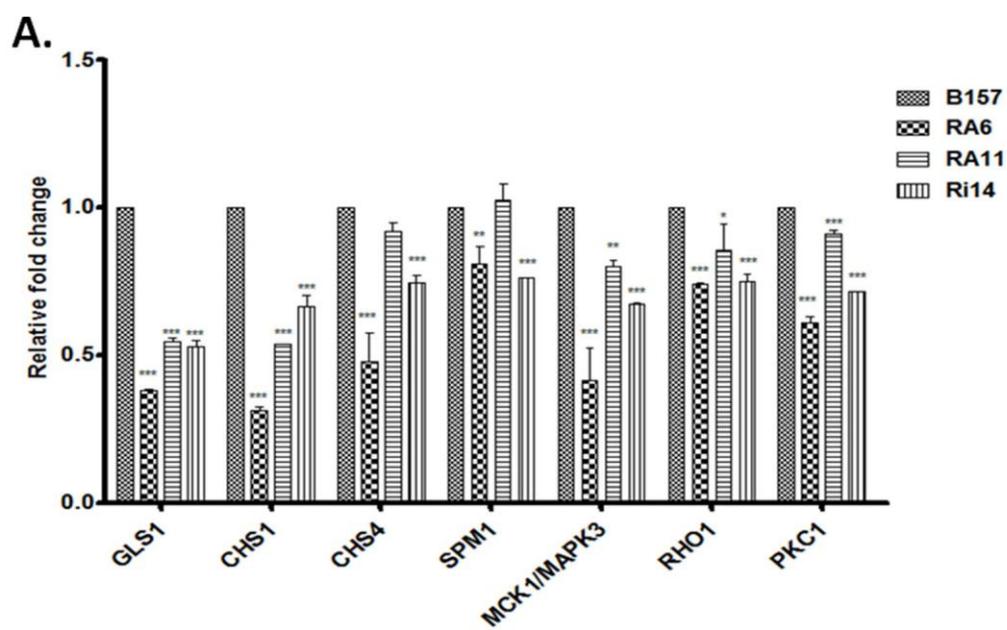
Since the *MoHPT1* knock-down transformant was hypersensitive to oxidative and osmotic stresses, we suppose that it may result from defects in cell wall composition and function. To determine this possibility, we tested the integrity of cell walls and membranes of the *MoHPT1* knock-down transformants. Mycelial plugs were cultured on CM agar with 0.003% SDS, 3 mM caffeine and 3 mg/ml Congo red (CR), which inhibit



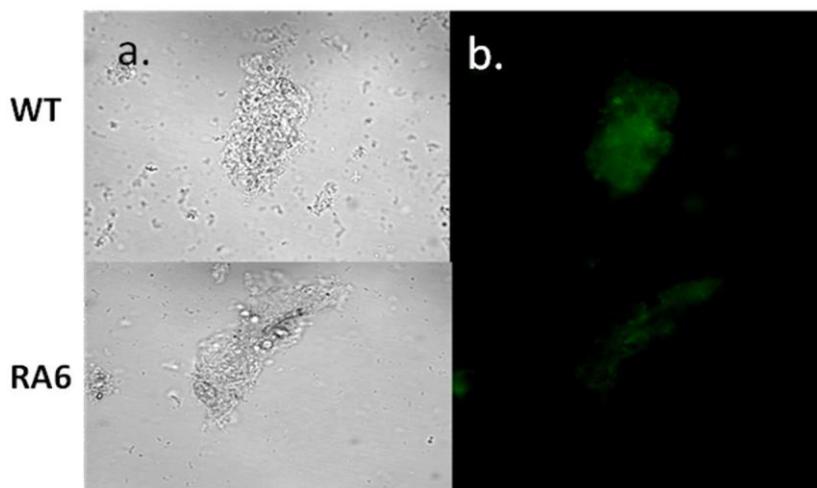
fungal cell wall assembly by binding chitin and 1, 4-glucans, respectively (Ram *et al.*, 1994; Wood and Fulcher, 1983). Mycelial growth rates of *MoHPT1* mutants on SDS and caffeine was severely affected. However, differences in growth on Congo red media were not distinguishable because the wild type itself showed sensitivity at 3 mg/ml concentration of Congo red. The knock-down transformants were highly sensitive to these cell wall integrity perturbing agents. The expression analysis of cell wall integrity signalling genes including *PKC*, *RHO1*, *MCK1* and *SPM1/MPS1*, in *MoHPT1* knock-down transformants was carried out (Fig. 23). The genes involved in the cell wall integrity pathway including the CWI MAP Kinase gene *SPM1/MPS1* were found to be significantly down regulated. *MoHPT1* knock-down also showed a lower level of chitin synthases (CHS genes) and *GLS1*. Immunohistology with *MoHPT1* antibody of B157 and RA6 treated with lysing enzymes revealed lower expression of *MoHPT1* in RA6.

### 3.2.17 Laccase expression in *MoHPT1* RNAi transformants

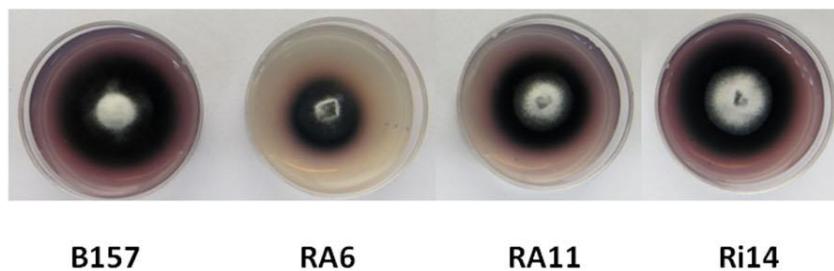
During the first few hours of spore germination and infection, *M. oryzae* secretes a number of laccases (Iyer and Chattoo, 2003). In *C. neoformans* two laccases *Lac1* and *Lac2*, are involved in oxidative stress response (Missall *et al.*, 2005). Laccases may play an important role in the initial infection process. The qualitative plate tests of the silencing transformants showed lower laccase levels on ABTS plates (Fig. 24A). Investigation of the role of laccases in this context was examined by the expression analysis of twelve different laccases in the RNAi transformants compared to the wild type (Fig. 24B). Except for one laccase (MGG\_09139) which is very highly expressed



**B.**

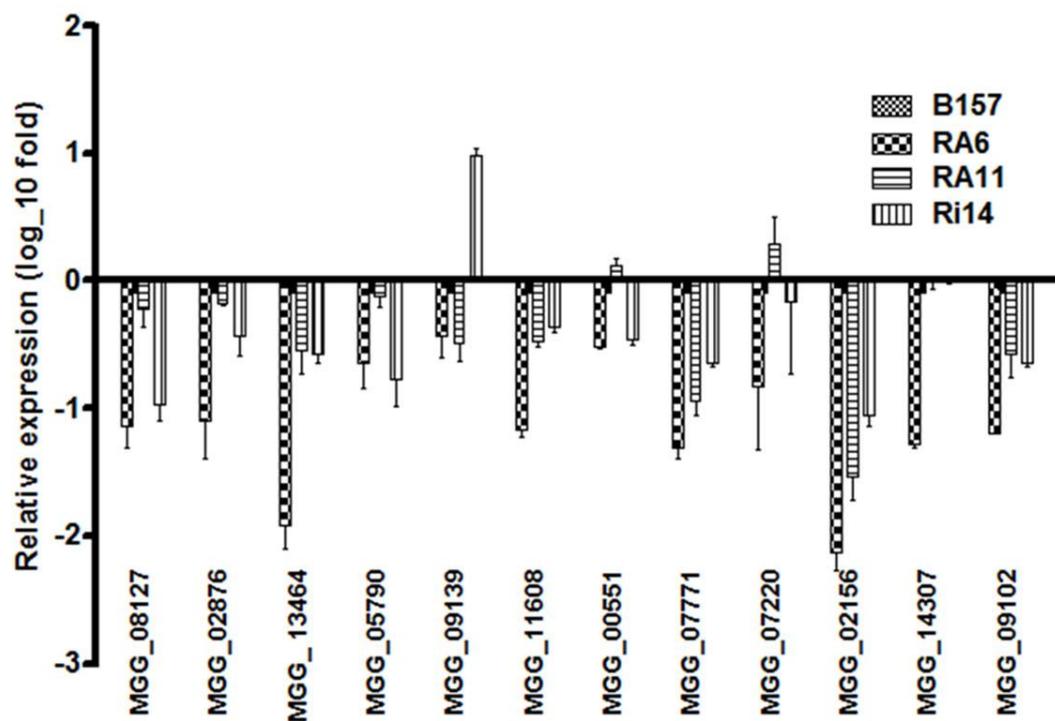


A.



B.

Relative expression of Laccases



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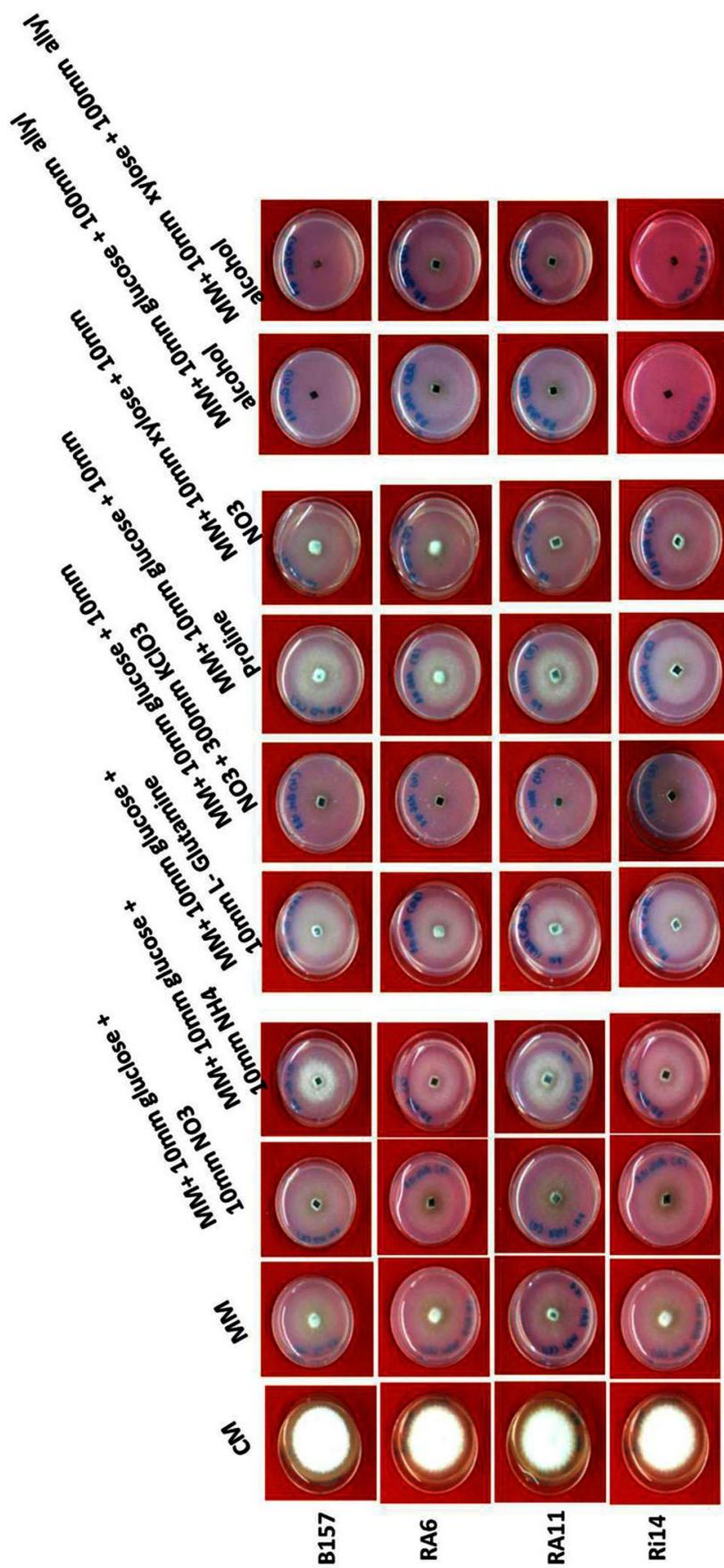
in Ri14, most of the laccases in the knock-down transformants showed lower expression.

### **3.2.18 Role of *MoHPT1* in Nitrogen catabolite repression or Carbon catabolite repression**

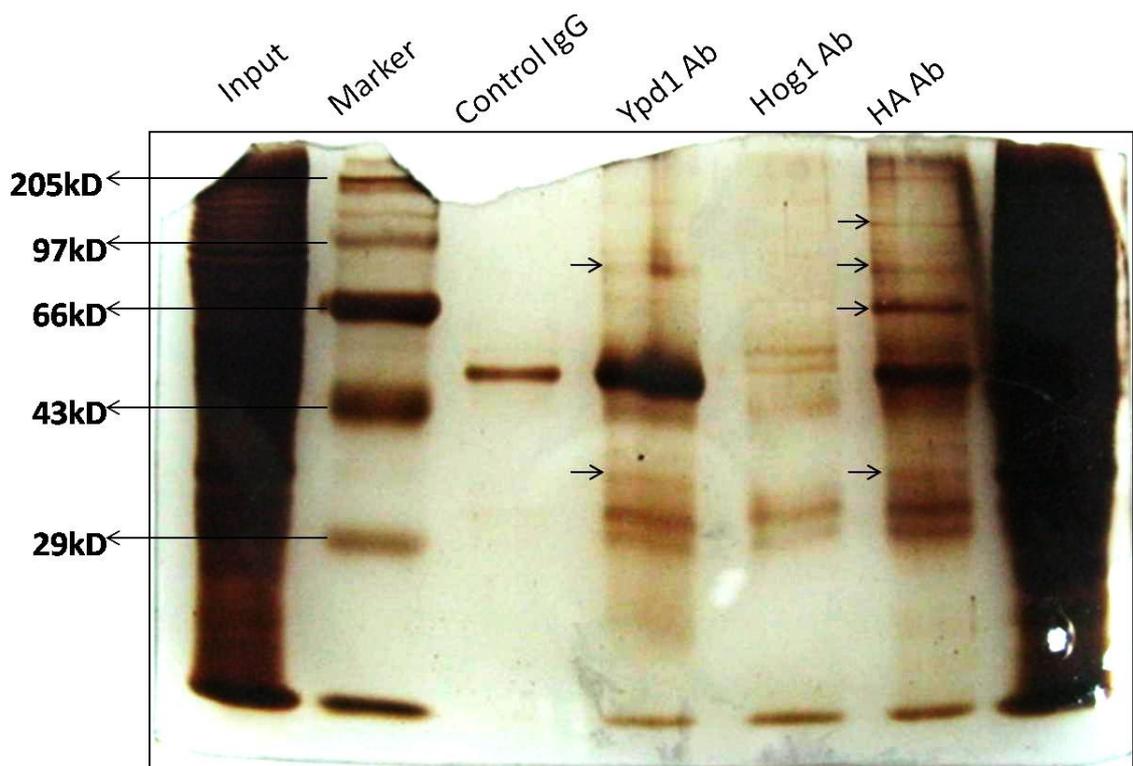
Nitrogen catabolite repression and carbon catabolite repression play an important role in plant pathogenesis. Since the knock-down mutants were sensitive to stress and are less pathogenic, plate assays were performed to investigate the role of *MoHPT1* in NCR or CCR (Fig. 25). The *MoHPT1* knock-down transformants did not show any significant difference in growth when compared to wild type, except in minimal medium with xylose as the sole carbon source. The wild type as well as Ri14 was not able to grow in presence of allyl alcohol, while RA6 and RA11 were able to grow on allyl alcohol xylose plates, suggesting a defect in xylose utilisation in the knock-down transformants.

### **3.2.19 Co-immunoprecipitation of MoHPT1**

Co-immunoprecipitation of MoHPT1 was attempted using the HA tagged *MoHPT1* transformant and HA monoclonal antibody as well as the polyclonal antibody of MoHPT1 raised in rabbit. Co-immunoprecipitation was also performed with control IgG, and HOG1 Antibody as negative controls (Fig. 26). However, may be due to the transient interaction, the protein that was co-immunoprecipitated was not enough to be purified for sequencing.



### Co-immunoprecipitation of *MoHPT1*



### 3.3 DISCUSSION

The role of *MoHPT1* histidine phosphotransferase in signalling by the TCS in *Magnaporthe oryzae* was investigated by silencing the only predicted histidine phosphotransferase in the fungus. Stress activated MAPK pathways and the SAPKs involved show considerable divergence in different fungi (Bahn, 2008; Enjalbert *et al.*, 2006). The HPT orthologues studied in different fungi also differ biologically (Fassler and West, 2013). The presence of *MoHPT1* in the spore nucleus, hyphae and appressorium suggests that *MoHPT1* has a vital role in the development of the fungus. We studied *MoHPT1* to analyse its diversified functions in *M. oryzae* B157 and also to investigate the different pathways in which it is involved. The sequence of *MoHPT1* available from the 8<sup>th</sup> version of [www.broadinstitute.org](http://www.broadinstitute.org) predicts two different transcripts for the same gene. However, in the earlier report of *M. oryzae* HPT the investigators were not successful in amplifying the longer transcript from the strain 70-15 (Jacob *et al.*, 2015). We demonstrate that the longer transcript of *MoHPT1* in B157 is expressed only under light induced conditions. Cloning and sequencing of the longer transcript revealed a unique stretch which is not found in any other fungal HPTs described so far, as predicted by the Broad database. *in silico* analysis of this transcript revealed a very strong Casein kinase binding site. Casein kinases are well known for their role in circadian rhythm and maintaining protein turn over. Casein kinase II is known for its role in phosphorylation of proteins leading to ubiquitin mediated degradation. Expression of *HPT1* and *OS-4* in *N. crassa* is required for the rhythmic expression of downstream targets of FRQ/WCC pathway (Lamb *et al.*, 2011). The light induced expression of the longer isoform of *MoHPT1* (T0) suggests that *MoHPT1*

has a wider role in the biology of the fungus and is possibly involved in some of the necessary elements of light regulated response. The presence of Casein kinase binding sites at the N- terminal stretch of *MoHPT1* T0 isoform suggests a light induced signalling of MoHPT1 which might also involve kinases other than *Osm1*. In *Neurospora*, the clock complex is known to regulate the rhythmicity of *Os-4* as well as *HPT1* independent of osmotic stress, where WCC directly binds to the *Os-4* promoter but *HPT1* is indirectly regulated by the clock (Lamb *et al.*, 2011). Our analysis shows that the PAS domain containing Histidine kinases are highly induced in the presence of light in the wild type B157. However, the knock-down transformant RA6 did not show induction of these histidine kinases under light. This decrease in expression of the PAS Histidine kinases in the *MoHPT1* knock-down implies a strong role of *MoHPT1* in light induced expression of these genes. This is the first study showing the role of *MoHPT1* in light response in *M. oryzae*.

The *MoHPT1* knock-down transformants produced reduced number of spores and also showed reduced infection compared to the wild type. The earlier reported *MoYPDI* disruption mutants (Jacob *et al.*, 2015) produced no spores and could not infect even the wounded plant tissue. The *MoHPT1* knock-down transformants were also unable to proliferate and expand inside the host. The phenotypes of *MoHPT1* knock-down transformants were similar to that reported in *M. oryzae* HPT knock-out mutants in 70-15 strain (Jacob *et al.*, 2015). The phenotypic defects of the *MoHPT1* knock-down in our study shows that even 50% reduction in expression is sufficient to affect the sporulation and infection. In another report, knocking out *MoSLNI*, the transmembrane histidine kinase in *M. oryzae*, resulted in a a broad effect on stress sensitivity and pathogenicity, but infectivity was not abolished (Zhang *et al.*, 2010). *MoSLNI* regulates osmotic stress

survival, growth, fungicidal resistance, cell wall integrity, nutritional stress, hyphal melanisation and oxidative stress (Zhang *et al.*, 2010). These effects were probably brought about through MoSSK1 and MoSKN7 signalling, since the knock-outs of these response regulators also showed similar phenotypes (Motoyama *et al.*, 2008). In our study, knock-down of *MoHPT1* rendered the fungus sensitive to osmotic stress and oxidative stress, and to cell wall perturbing agents. The expression pattern of *MoSSK1*, *OSMI* and *GPDHI* in the knock-down transformant was seen strongest under oxidative stress conditions, suggesting that MoHPT1 is a crucial player under oxidative stress. Since the phenotypes and stress sensitivities of *MoHPT1* knock-down resembles those of *MoSLN1*, *MoSSK1* and *MoSKN7* mutants, it is likely that *MoHPT1* is a downstream component of MoSLN1 TCS signalling.

*MoHPT1* knock-down transformants in B157 did not show any response to phenylpyrrole and dicarboximide antifungal agents. It was observed that unlike the other *M. oryzae* strains, B157 itself was highly resistant to these antifungal compounds. Generally, the responses to these antifungal compounds are attributed to be a signalling feature of group III histidine kinase mediated Hog MAPK response in filamentous fungi (Motoyama *et al.*, 2005b). The group III histidine kinase *HiK1* in *M. oryzae* is not required for cell growth or pathogenicity but is key for sensitivity to phenylpyrrole antifungal compounds like fludioxonil (Motoyama *et al.*, 2005a). *M. oryzae* HPT mutants in the 70-15 strain of *M. oryzae* were also found to be resistant to fludioxonil (Jacob *et al.*, 2015). However, it was shown that mutations in the group III histidine kinases may lead to resistance against these antifungal compounds (Furukawaa *et al.*, 2012). The genome of B157 was recently sequenced (Gowda *et al.*, 2015) and we noticed that two of the HKs among the ten had variations; a single amino acid change in

MoHiK1 and a twenty-two amino acid deletion in MGG\_12530 (Appendix 3, Table 4). The HAMP domain of the group III Histidine kinases is known to be important for antifungal responses. In MoHiK1 of B157 the 84<sup>th</sup> amino acid Histidine is replaced by a Tyrosine. It is interesting that such an amino acid replacement in the first 260 nucleotide stretch is bringing about such a drastic effect. This strain variation might account for the resistance of B157 strain against these antifungal compounds.

Cross talk of Hog1 with other pathways especially the CWI pathway, has been extensively studied in fungi under different stress conditions like hyper-osmotic stress, oxidative stress and zymolase treatment (Fuchs and Mylonakis, 2009). In the current study, the observation like weak cell wall organisation of the transformants was derived by plate assays and expression analysis of cell wall remodelling genes. The expression analysis of the cell wall integrity signalling pathway elements including *Rho1*, *PKC1* and *MPS1* revealed that the pathway is highly impacted in the *MoHPT1* knock-down. Present findings also suggest that this cell wall integrity signalling affects downstream cell wall biogenesis genes like *GLS1* and *CHSes*. These results confirm previous observations in *HiK1* and *SLN1* mutants in *M. oryzae*, signifying the importance of these genes in cell wall maintenance (Motoyama *et al.*, 2005b; Zhang *et al.*, 2010). *MPS1* mutants in *M. oryzae* are also known to be involved in stress response (Xu *et al.*, 1998). *MoSwi6*, which interacts with *Mps1*, was found to be necessary for proper chitin synthesis and cell wall integrity. *MoSwi6* mutants showed a marked reduction in the expression of laccases MGG\_11608 and MGG\_13464 (Qi *et al.*, 2012). The same laccases are also found to be regulated by *MoSLN1* and *MoAPI* (Guo *et al.*, 2011). The marked decrease in the production of laccases in *MoHPT1* knock-down transformants indicates that the laccases including MGG\_11608 and MGG\_13464 may be regulated through the HKs and *MPS1*

MAP Kinase, suggesting the possibility of crosstalk between the *MoMPS1* pathway and histidine kinase regulated SAPK pathways. Thus we hypothesise that regulation of both the plant cell wall degrading enzymes as well as the fungal cell wall enzymes for cell wall maintenance may involve interaction of MoHPT1 with many more HKs. The cell wall stress response of the knock-down transformants as observed in the cell wall stress sensitivity assays signifies the importance of the two component system in cell wall maintenance.

Taking the observations together, *MoHPT1* histidine phosphotransferase in *M. oryzae* regulates downstream stress responses through TCS in multiple stress survival events. *MoHPT1* plays an important role in light regulated signalling and transcriptional processes. *MoHPT1* may regulate developmental and pathogenicity related processes through different HKs at different stages of infection by proper cell wall maintenance and oxidative stress response.