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MOLECULAR CLONING OF *Ganoderma boninense* HOG1-TYPE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cDNA AND TRANSCRIPTIONAL RESPONSE TO SALINITY STRESS

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ABSTRACT

The white rot fungus, Ganoderma boninense *is the main causal agent of basal stem rot disease in oil palm plantations in South-east Asia especially Malaysia and Indonesia. Despite serious attention given the fungus, knowledge on the pathogen-oil palm interaction especially from the molecular and biochemical aspects is still inadequate to provide a better understanding on the disease. In this study, an 1161 bp of full-length cDNA encoding a Hog1-type MAPK was obtained from* G. boninense*. Based on the multiple sequence alignment, the conserved motif TGY (novel activation loop motif), N-terminal-conserved domain, HRDLKPN and the C-terminal-conserved domain, TRWYRAP were found in* G. boninense *Hog1 MAPK. Results of salinity stress assay indicated that* G. boninense *growth was slower on media containing 0.4 M NaCl and could not survive on media containing 1.0 M NaCl. Real-time quantitative PCR analysis showed that GbHog1 MAPK transcript was consistently upregulated to nearly 4.0 folds after 2 hr of exposure to 0.4 M NaCl. This study provides a preliminary understanding on the involvement of GbHog1 MAPK in salinity stress response. Identification of GbHog1 MAPK could also lead to understanding of the involvement of* G. boninense *MAPK in pathogenicity as reported in several plant pathogenic fungi.*

Keywords: Mitogen-activated Protein kinase (MAPK), high osmolarity glycerol response Protein 1 (HOG1), *Ganoderma boninense*, salinity stress, basal stem rot (BSR).

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INTRODUCTION

Oil palm is one of the major contributors to the world's vegetable oils supply for both food and non-food industries (USDA, 2017). However, the perennial oil crop is vulnerable to several diseases including basal stem rot (BSR) caused by *Ganoderma* spp. (Turner, 1981). *Ganoderma boninense* has been identified as the primary pathogen for oil palm BSR disease because this fungus was frequently found in the areas with moderate and high disease incidences (Idris *et al*., 2000). Other species of *Ganoderma* that are associated with BSR disease include *G. miniatocinctum*, *G. zonatum* and *G. tornatum* (Idris, 2011). BSR disease gains serious attention in South-east Asia (SEA) especially Malaysia and Indonesia as the disease causes significant agricultural and economic losses to oil palm plantations by reducing the oil yield (smaller and less fruit bunches) and shortening the productive life span of the infected palms (Singh, 1991; Roslan and Idris, 2012; Asis *et al*., 2016).

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Despite that the threat of BSR disease is growing in SEA, knowledge on the *Ganoderma* biochemical processes and interaction between *Ganoderma* and oil palm especially at the molecular level is still inadequately generated. Such information is vital to give a better understanding on the disease, which is essential for the design of effective prevention or control measures against the disease. Several efforts to gain such knowledge were initiated recently including cloning of cDNA and gene expression studies of putative pathogenicity genes from *G. boninense* (Rasid *et al*., 2014; Lim *et al*., 2017), *de novo* transcriptome analyses of *Ganoderma* in oil palm root tissues (Ho *et al*., 2016) and insight study of interaction between *G. boninense* and oil palm using scanning electron microscopy (Alexander *et al*., 2017).

Plant pathogenic fungi have evolved and developed various modes of infection and nutritional strategies, but the signalling pathways still remain conserved (Turra *et al*., 2014). One of the key signalling pathways is Mitogen-activated Protein kinases (MAPK) pathway. MAPK cascades are three-tiered protein kinase modules where the MAP kinase kinase kinase (MAPKKK) phosphorylates the MAP kinase kinase (MAPKK), which in turn activates the MAPK by dual phosphorylation of a pair of conserved threonine and tyrosine residues. In the budding yeast, *Saccharomyces cerevisiae*, at least five MAPK pathways (designated as FUS3, KSS1, HOG1, SLT2 and SMK) have been identified. They are respectively involved in mating, filamentous growth, high-osmolarity response, cell integrity and ascospore formation (Gustin *et al*., 1998).

One of the well-studied MAPK pathways in fungi is the high osmolarity glycerol (HOG) pathway. Earlier study in *S. cerevisiae* showed that osmosensing mechanism in yeast involved both a two-component signal transducer (Sln1p, Ypd1p and Ssk1p) and a MAPK cascade (Ssk2p/Ssk22p,Pbs2p, and Hog1p) (Posas *et al*., 1996). The transmembrane protein Sln1p contains a sensor domain, cytoplasmic histidine kinase and receiver domains, while the cytoplasmic protein Ssk1p contains a receiver domain. Ypd1p binds to both Sln1p and Ssk1p and mediates the multistep phosphotransfer reaction (phosphorelay). When the osmotic stress build up in the media, the Hog1p will be localised in the cell nucleus and activates specific transcription factors to promote an initial response and adaptation (Reiser *et al*., 1999). Studies on other fungi showed that the HOG1 homologous are involved in response to osmotic and oxidative stresses, heat shock and tolerance to a phenylpyrrole fungicide (Kawasaki *et al*., 2002; Kojima *et al*., 2004; Zhang *et al*., 2002).

In plant pathogenic fungi such as *Mycosphaerella graminicola*, Hog1 MAPK is required for mating and switching from a yeast like form to filamentous growth (Mehrabi *et al*., 2006). It has been shown to be needed for penetration through plant tissues in *Botrytis cinerea* (Segmuller *et al*., 2007). In *Fusarium graminearum*, Hog1 MAPK was essential for sexual development and invasive growth on maize and wheat (Van Thuat *et al*., 2012). In a study on necrotrophic fungus which causes the southern corn leaf blight, *Cochliobolus heterostrophus* Hog1 MAPK mutants produced smaller appressoria and caused weaken disease symptoms on maize (Igbaria *et al*., 2008). In a recent study, mutant complementation of *Melamspora larici-populina* Hog1 MAPK (MlpHog1) suggested that the protein could be involved in various environmental stresses and infectious growth (Yu *et al*., 2016). However, the absence of Hog1 MAPK did not perpetually affect the fungal pathogenicity as shown in the studies on *Colletotrichum orbiculare* (Kojima *et al*., 2004) and *Bipolaris oryzae* (Moriwaki *et al*., 2006). These results suggested that Hog1 MAPK are essential for virulence of some but not all of the phytopathogenic fungi. The reasons for this difference are still unclear but it has been suggested that this condition could be related to the disparity of fungal infection strategies and/or ability to overcome the host counter defences (Zhao *et al*., 2007).

The fungal Hog1 MAPK was found to play essential role in response to osmotic and oxidative stresses and required for mating, growth or pathogenicity process. Some plant pathogenic fungi Hog1 MAPK has been well characterised but there is no related study on *G. boninense* Hog1 (*Gb*Hog1) MAPK being reported yet. Hence, this study was aimed to identify and isolate full-length cDNA encoding Hog1-type MAPK from *G. boninense*. Prediction of the function of this conserved protein was made based on determination of the novel activation loop motifs and conserved domains. Salinity stress assay was carried out and the transcript profil of *Gb*Hog1 MAPK was investigated. Identification of the *Gb*Hog1 MAPK could lead to understanding of the fungal regulatory mechanism in response to environmental stresses and fungal pathogenicity.

MATERIAL AND METHODS

Preparation of *Ganoderma boninense* Culture

G. boninense Pat. PER71 culture was provided by the *Ganoderma* and Diseases Research for Oil Palm (GanoDROP) Unit, Malaysian Palm Oil Board (MPOB), Selangor, Malaysia. The culture was maintained on complete yeast medium (CYM) containing 20.0 g litre^{-1} of dextrose, 2.0 g litre^{-1} of peptone, 2.0 g litre^{-1} of yeast extract, 0.5 g litre^{-1} of $MgSO_4$, 1.0 g litre^{-1} of K_2HPO_4, 0.46 g litre^{-1} of KH_2PO_4 and 15.0 g litre^{-1} of agar and incubated at 28°C prior to further usage.

Total RNA Extraction

The *G. boninense* mycelium was grown in CYM at 28°C, 150 rpm for seven days. Approximately 100 mg of mycelium was washed with distilled water and ground into fine powder using liquid nitrogen. Total RNA was extracted using RNeasy® Mini Kit (QIAGEN, Germany). The RNA quantity and quality were determined using gel electrophoresis and Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA).

Molecular Cloning of Partial-length cDNA Encoding MAPK from *G. boninense*

A few plant pathogenic fungi MAPK genes were identified from GenBank database including *Botryotinia fuckeliana* (AAG23132), *Gaeumannomyces graminis* (AAG44657), *Laccaria bicolor* (XP_001881046), *Postia placenta* (XP_002469816) and *Ustilago maydis* (AAF15528). The amino acid and DNA sequences of these genes were aligned using Vector NTI software (Invitrogen, USA). The fungal MAPK conserved regions were determined and degenerate primers were designed based on these regions.

First-strand cDNA was synthesised from *G. boninense* total RNA using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA). Polymerase chain reaction (PCR) amplification was performed using AccuPrime™ *Taq* DNA Polymerase System (Invitrogen, USA). The PCR mixtures were prepared in a total volume of 50 µl containing 50 ng of cDNA, 10 µM of each degenerate primer, 1X AccuPrime™ PCR Buffer I and 1.0 µl of AccuPrime™ *Taq* DNA Polymerase. The thermocycler was programmed for 1 min of pre-heating at 94°C followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 68°C for 2 min, then a final extension for 5 min at 68°C. For amplification of 5′ and 3′ end cDNA of *G. boninense* MAPK, specific primers were designed based on the partial length cDNA sequence. PCR amplifications using combination of specific primers and Universal Primer Mix (UPM) (Clontech, USA) were performed according to the PCR protocols above. List of primers used in the amplification of full-length cDNA encoding *Gb*Hog1 MAPK was designed using Primer3 webpage (http://bioinfo.ut.ee/primer3/) and presented in *Table 1*.

The PCR products were separated by gel electrophoresis. Potential PCR products were extracted using QIAquick® Gel Extraction Kit (QIAGEN, Germany). The purified fragments were cloned into *pCRII® TOPO®* vector (Invitrogen, USA) and potential clones were sent for sequencing.

Sequence Analysis and Determination of Novel Activation Loop Motifs and Conserved Domains of *G. boninense* MAPK

DNA sequence of the representative clones was determined by sequencing using Applied Biosystems 3730*xl* DNA Analyser. Multiple sequence alignment of fungal Hog1 MAPK was performed using Multalin (http://multalin.toulouse.inra.fr/multalin/) to identify the novel activation loop motifs and conserved domains (Corpet, 1988). A total of 10 Hog1 MAPK originated from different fungal species including basidiomycetes *Heterobasidion annosum* sensu lato (JGI ID: 153508, phytopathogenic), *L. bicolor* (JGI ID: 639980, mutualist), *Puccinia graminis-tritici* (JGI ID: 655, phytopathogenic), *U. maydis* (JGI ID: 2357, phytopathogenic) and ascomycetes *B. cinerea* (JGI ID: 1429, phytopathogenic), *Fusarium oxysporum* (JGI ID: 24542, phytopathogenic), *Magnaporthe oryzae* (JGI ID: 120984, phytopathogenic), *Tuber melanosporum* (JGI ID: 3007, mutualist), *S. cerevisiae* (JGI ID: 4133, none phytopathogenic), *Trichoderma virens* (JGI ID: 83666, none phytopathogenic) were obtained from MycoCosm database, Joint Genome Institute (DOE JGI), USA. The parameters used for the multiple alignment were as follow: protein weight matrix: Blosum62-12-12, gap penalty at opening: default, gap penalty at extension: default, gap penalties at extremities: none, one iteration

TABLE 1. LIST OF PRIMERS FOR AMPLIFICATION OF FULL-LENGTH cDNA ENCODING *G. boninense* HOG1-TYPE MAPK

Amplification of partial-length cDNA	
GbHog1-1	5′-GHGCBTAYGGDGTBGTCTG-3′
GbHog1-6	5′-GAGCATCTCAGCSAGRATGC-3′
Amplification of 5′ and 3′ end of cDNA	
UPM (Clontech, USA)	5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3' 5'–CTAATACGACTCACTATAGG GC –3'
5GbHog1-1	5′-CGAGGAGCCTGTCAACTGAT-3′
3GbHog1-1	5′-CTTGCCCGCATACAGGAC-3′
End-to-end PCR	
GbHog1F-5	5′-GCATACCTCTCGGCTCTTCTC-3′
GbHog1F-2	5′-TCACAAGTCCGACGGAAAC-3′

Note: IUPAC code: Meaning. B: C/G/T; D: A/G/T; H: A/C/T; R: A/G; S: C/G; Y: C/T.

only: no, high consensus value: 90% (default), low consensus value: 50% (default), maximum line length: 130, and graduation step: 10.

Phylogenetic Analysis of *G. boninense* Hog1 MAPK

To construct the fungal MAPK phylogenetic tree, the deduced amino acid of *Gb*Hog1 MAPK was determined and amino acid sequences of 36 MAPK from seven fungal species (four from Ascomycetes and three from Basidiomycetes) were obtained from MycoCosm database, Joint Genome Institute (DOE JGI), USA. Construction of the fungal MAPK phylogenetic tree was performed using Phylogeny. fr (http://www.phylogeny.fr). Parameters used to construct the phylogenetic trees were as follows. Input data: protein; statistical tests for branch support: approximate likelihood-ratio test (aLRT): SH-like; substitution model: default; number of substitution rate categories: 4; gamma distribution parameter: estimated; proportion of invariable sites: estimated; remove gaps from alignment: yes.

G. boninense Salinity Stress Assay

In the first experiment, *G. boninense* mycelium was sub-cultured on the CYM plates containing 0 M, 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M NaCl. The plates were maintained at 28°C for 14 days. The colony diameters for each treatment were measured every day until Day 14 or the colony has achieved full plate growth. In the second experiment, *G. boninense* mycelium was initially sub-cultured in liquid CYM and incubated at 28°C, 150 rpm for seven days. The mycelia were then harvested by filtration using sterile miracloth and transferred into flasks containing 50 ml of CYM, CYM with 0.4 M NaCl or CYM with 0.8 M NaCl. All the flasks were incubated at 28°C on a shaking incubator and each mycelium sample was harvested at 10, 20, 30, 60 and 120 min post exposure period. The mycelia were quickly frozen in liquid nitrogen and kept at -80°C prior to total RNA extraction.

Transcript Analysis of *G. boninense* Hog1-MAPK

Total RNA was extracted from the *G. boninense* samples collected from the second salinity stress assay using RNeasy® Mini Kit (Qiagen, Germany). The RNA quality and quantity were determined using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA). The integrity of total RNA was determined via Plant RNA Nano Assay using Agilent 2100 Bioanalyser (Agilent Technologies, USA). The total RNA samples were converted into cDNA using High-Capicity cDNA Reverse Transcription Kit (Applied Biosystem, USA) according to manufacturer protocols. A pair of real-time qPCR primers for *Gb*Hog1 MAPK were designed using Beacon Designer 7.91 software (PREMIER Biosoft International, USA). Efficiency test for this primer set was performed using two-fold diluted cDNA template (*G. boninense* mycelium maintained in CYM for seven days) started from 1 ng to 16 ng.

The expression study of *Gb*Hog1 MAPK using samples collected from the second salinity stress assay was performed in the 96 well-plate in CFX Connect™ Real-Time System (Bio-rad, USA). A total of 10 µl real-time quantitative PCR (qPCR) reaction mixture containing 8 ng of cDNA template, 10 mM of each forward and reverse primer and 1X of iTaq™ Universal SYBR® Green Supermix (Biorad, USA) was prepared. The primers used in the qPCR were listed in *Table 2*. All qPCR reactions were prepared in triplicate (n=3). The thermal profile of the qPCR reaction was 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, and 55°C for 10 s. The quantification cycle (Cq) was automatically captured during the annealing stage and the data were analysed using Bio-Rad CFX Manager Software (Livak and Schmittgen, 2001). The expression data were first normalised to three reference genes, *α-tubulin*, *β-tubulin* and *eEF2* as suggested by Lim *et al*. (2014). The relative expression fold changes of the salinity stressed and control samples were calculated by comparing to the control *G. boninense* sample maintained in CYM for 10 min (Calibrator).

TABLE 2. LIST OF PRIMERS FOR REAL-TIME qPCR GENE EXPRESSION ANALYSIS

Gene	Primer	Sequence	Product length (bp)
Reference genes (Lim *et al*., 2014)			
α-Tubulin	GTR 7	GCACCGACTCTGGTGATGCT	
	GTR 8	GATAGGCTATGGTCGCGAAG	100
β-Tubulin	GBR 3	GAGTTCACTGAGGCCGAGTC	
	GBR 4	TGCAACACGCTTATTCTTCG	130
eEF2	GER 1	TGG TCA AGA ACA TCC GTA T	
	GER 2	CGC TAA CAA AGA CAA GGG	173
Target gene			
*Gb*Hog1 MAPK	GHR 1	GGACTTCAAGCACCTAGA	
	GHR 2	TCGTGTTATACAGTGATGATT	200

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of Full-length cDNA Encoding *G. boninense* Hog1 Type MAPK

PCR amplification using a combination of two degenerate primers has successfully produced a partial-length cDNA sequence of 539 bp. Specific primers were designed based on 5′ and 3′ ends of the partial cDNA sequence to amplify the full-length cDNA. A 232 bp and 924 bp cDNA were obtained from the amplification of 5′ and 3′ end cDNA, respectively. End-to-end PCR was performed and a single PCR product (about 1200 bp) was obtained indicating that all these three partial cDNA sequences were originated from the same gene sequence. Basic logic alignment search tool (BLASTX) analysis showed that the *G. boninense* MAPK encodes 375 deduced amino acid residues and shared 97% similarity to MAPK from other fungi (data not shown).

Determination of *G. boninense* Hog1 MAPK Novel Activation Loop Motifs and Conserved Domains

Based on the alignment result, a TGY motif is present in *Gb*Hog1 MAPK as in Hog1 MAPK from other fungi (*Figure 1*). The presence of this motif is required for the activation of the MAPK via threonine/tyrosine phosphorylation and it is a hallmark motif for this stress-induced MAPK in fungi and animals (Kultz, 1998). The alignment result also showed that the *Gb*Hog1 MAPK is highly identical to Hog1 MAPK from other 10 fungal species. The MAPK are characterised based on the presence of motifs such as TEY, TDY, TGY and TPY in their activation loop region. This region is important for regulating various cellular responses in eukaryotic organisms (Hamel *et al.*, 2006; Morrison, 2012). Some of the motifs are only present in certain groups of organisms. For example, the activation loop TEY motif is found mainly in plants, animals and fungi, whereas the TGY motif is only found in fungi and animals.

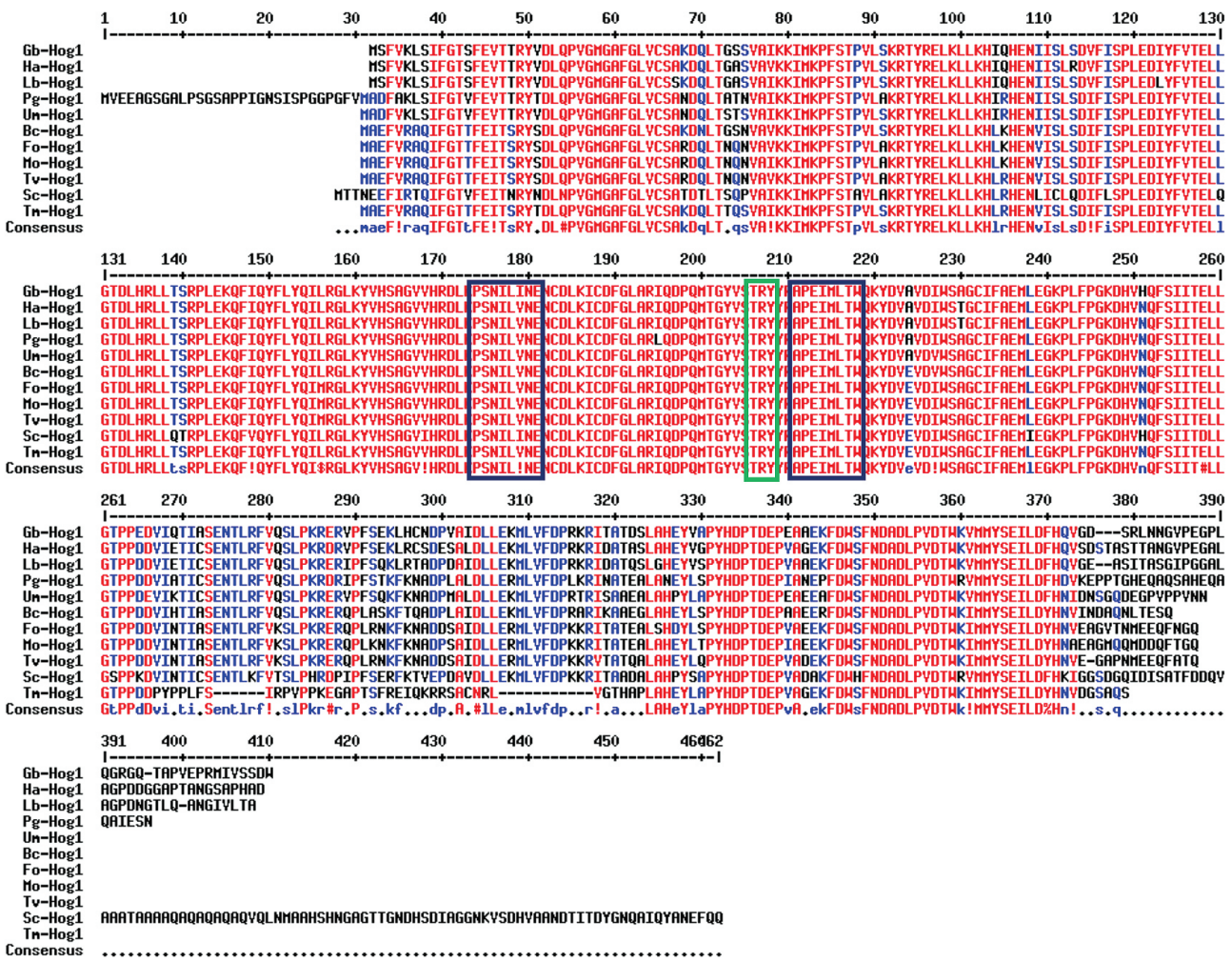

Figure 1. G*bHog1 Mitogen-activated Protein kinases (MAPK) deduced amino acid alignment and determination of novel activation loop motifs and conserved domains. Highly conserved regions are highlighted in red. Residues with greater than 50% identities are highlighted in blue. The MAPK activation loop motif is shown in green box and the conserved domains are shown in black boxes.*

Additional analysis of the sequence indicated that N-terminal-conserved domain, HRDLKPN and C-terminal-conserved domain, TRWYRAP were found in *Gb*Hog1 MAPK. The N-terminal-conserved domain is located before the activation loop motif, TGY and the C-terminal-conserved domain is located after the activation loop motif. This observation concurs to the conserved domains found in the Hog1 MAPK from other fungi (*Figure 1*).

Phylogenetic Analysis of *G. boninense* Hog1 MAPK

Based on the fungal MAPK phylogenetic analysis, *Gb*Hog1 MAPK was clustered together with the Hog1 MAPK from the fungi included in the analysis. The fungal MAPK family generally can be segregated into four different clades including Kss1 / Fus3-type, Slt12 type, Hog1 type and Ime2 type. The GbHog1 MAPK was found phylogenetically closer to Hog1 MAPK from *F. oxysporum, M. oryzae, B. cinerea, H. annosum sensu lato, P. graminis-tritici* and *U. maydis* as compared to Hog1 MAPK from *S. cerevisiae* (*Figure* 2). The analysed Hog1 MAPK did not segregate according to division indicating the conservation of this protein across the divisions (Ascomycota and Basidiomycota).

***G. boninense* Salinity Stress Assay**

Fungi display different tolerant level to different salt concentrations. For example, *H. annosum* was able to tolerate less than 0.5 M of salt concentration (Raffaello *et al*., 2012). Other fungi species such as

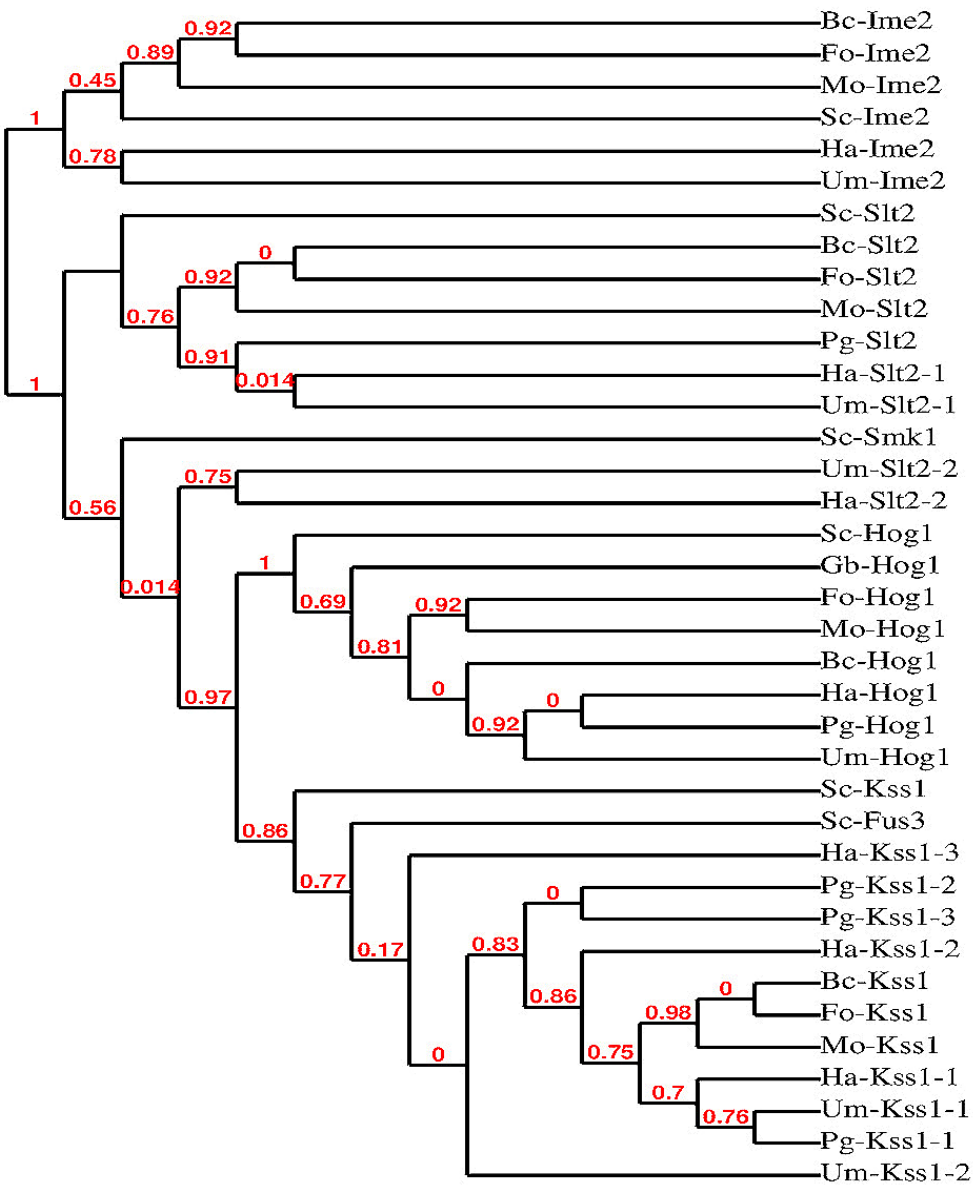

Figure 2. Phylogenetic analysis based on Mitogen-activated Protein kinases (MAPK) from seven fungal species and Gb*Hog1 MAPK. Construction of the fungal MAPK phylogenetic tree was performed using Phylogeny.fr (http://www.phylogeny.fr). The numbers highlighted in red indicate the display branch support values.*

C. albicans, *C. glabrata* and *Debaryomyces hansenii* could accommodate higher salt concentration compared to *H. annosum* (Raffaello *et al*., 2012). The phytopathogenic fungus, *B. cinerea* could survive on the media containing 1.5 M NaCl (Segmueller *et al*., 2007) and *Cochliobolus heterostrophus*, the causal of southern corn leaf blight, was able to tolerate up to 0.75 M of KCl (Igbaria *et al*., 2008).

In the first salinity stress assay, the presence of 0.2 M NaCl has promoted the *G. boninense* mycelium growth as compared to the control (*Figure 3a*). It took seven days to achieve full plate growth as compared to the control plates (eight days) (*Figure 3b*). For some fungi such as *Pinus thunbergii* and *Rhizopogon rubescens*, there is no significant difference on the fungi growth rate when exposed to the concentrations of NaCl between 0 M and 0.2 M (Matsuda *et al*., 2006). In the same study, the presence of NaCl at low concentration (0.025 M) has promoted the hyphal growth of several species of ectomycorrhizal fungi. In another separate study, improvement of fungal biomass growth was observed in some basidiomycete fungi such as *Dacrypionax elegans* SXS323 (up to 90%) with the presence of 0.5 M NaCl in the growing media, suggesting that the basidiomycetes strains required salt for optimal growth and consequently could be considered as moderately halophilic (Arakaki *et al*., 2013).

The growth rates for *G. boninense* sub-cultured on CYM containing 0.4 M and 0.6 M of NaCl were slower compared to the control. The *G. boninense* maintained on 0.4 M NaCl took 10 days to achieve full plate growth while the *G. boninense* maintained on 0.6 M NaCl did not achieve full plate growth after 14 days of observations. Total growth inhibition was observed when *G. boninense* was maintained on the CYM containing 1.0 M of NaCl. Based on the first salinity stress assay, *G. boninense* growth was slower when exposed to 0.4 M of NaCl and could tolerate not more than 0.8 M of NaCl. These two concentrations were then used for subsequent salinity stress and expression studies.

Transcript Analysis of GbHog1 MAPK under Salinity Stress

Based on the first salinity stress assay, the concentration of NaCl at 0.4 M (mild salinity stress) and 0.8 M (extreme salinity stress) were used for the second salinity stress assay. The real-time qPCR analysis indicated that *Gb*Hog1 MAPK was differentially expressed in responses to different saline concentrations (*Figure 4*). Under the 0.4 M NaCl saline stress, the expression of *Gb*Hog1 MAPK was upregulated to more than 1.5 folds after 10 min as compared to the control sample. The *Gb*Hog1 MAPK transcript continued to show a moderate

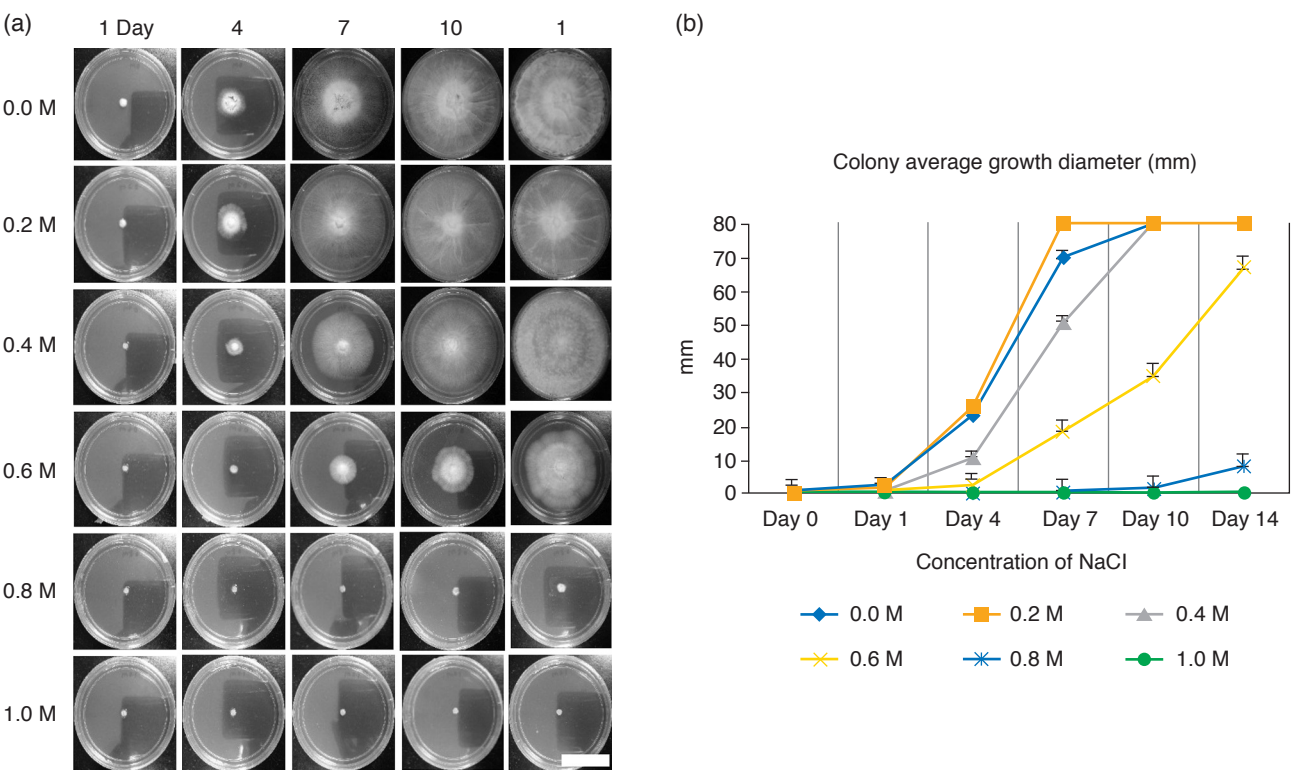

Figure 3. G. boninense *saline stress assay. a. The colony morphology of* G. boninense *grew on complete yeast medium (CYM) with different concentrations of NaCl. Bar: 4 cm. b. The average growth rate of* G. boninense *on CYM with different concentrations of NaCl.*

upregulation until the expression was achieved nearly 4.0 folds after 2 hr of exposure to 0.4 M NaCl. Similar expression profile was observed on *S. cerevisiae* Hog1 MAPK when exposed to the same concentration of saline (Posas *et al*., 2000). However, the transcript response of *Gb*Hog1 MAPK is slower compared to *S. cerevisiae* Hog1 MAPK under the same saline stress. *S. cerevisiae* Hog1 MAPK was upregulated to 6.4 folds in just a short exposure period of time (10 min) as compared to *Gb*Hog1 MAPK (3.9 folds after 2 hr). Under 0.4 M of NaCl, the expression of *Gb*Hog1 MAPK displayed a consistent increase pattern indicating the ability of the fungus to respond well to the saline stress.

The expression of *Gb*Hog1 MAPK did not see a sharp increase when exposed to a higher concentration of saline stress (0.8 M NaCl). The transcript was reduced by about 30% after 10 min of exposure to CYM containing 0.8 M NaCl and upregulated to 1.6 folds after 2 hr as compared to the control sample. This observation may be due to exposure of *G. boninense* to a higher concentration of NaCl and affected the cell regulations and fungal growth, as observed in the first saline stress assay. Some fungi such as *H. annosum* could only tolerate less than 0.5 M of salts as no fungal growth was observed when the fungus was exposed to 1.0 M of salts (Raffaello *et al*., 2012).

The fungal MAPK Hog1 pathway plays important roles in regulating the cellular responses toward changes in external osmolarity. One of the well-studied Hog1 MAPK modules is from the Hog pathway in *S. cerevisiae*. Activation of the Hog pathway involves phosphorylation, activation, and nuclear translocation of the Hog MAPK. The activated Hog1 MAPK will subsequently activate multiple cellular responses such as induction or repression of gene transcription, regulation of protein translation, adjustments to cell cycle progression, and accumulation of compatible solutes to maintain the cellular turgor (Hohmann, 2002; Saito and Tatebayashi, 2004; Magan, 2001). Furthermore, fungal Hog1 MAPK was also found to be associated with pathogenicity in some, but not all the pathogenic fungi. For example, *M. graminicola* Hog1 MAPK mutant strain was found to be impaired in mating and unable to switch from a yeast-like form to filamentous growth (Mehrabi *et al*., 2006). Deletion of Hog1 MAPK in *B. cinerea* created strains that are unable to penetrate unwounded plant tissues (Segmüller *et al*., 2007). In another example, deletion of *M. oryzae* Hog1 MAPK produced strains that are highly sensitive to osmotic stress and showed severe morphological defects when grown under hyperosmotic conditions. However, the mutant strains could still generate normal appressorial turgor during infection stage and accumulation of glycerol is observed in their appressoria (Dixon *et al*., 1999). Based on the expression study, the *Gb*Hog1 MAPK has played an important role under hyperosmotic stress. Identification of the *Gb*Hog1 MAPK could provide an early clue on the involvement of *G. boninense* MAPK in fungal pathogenicity. This information will subsequently lead to the understanding of the fungal regulatory mechanism in response to environmental stresses and fungal pathogenicity.

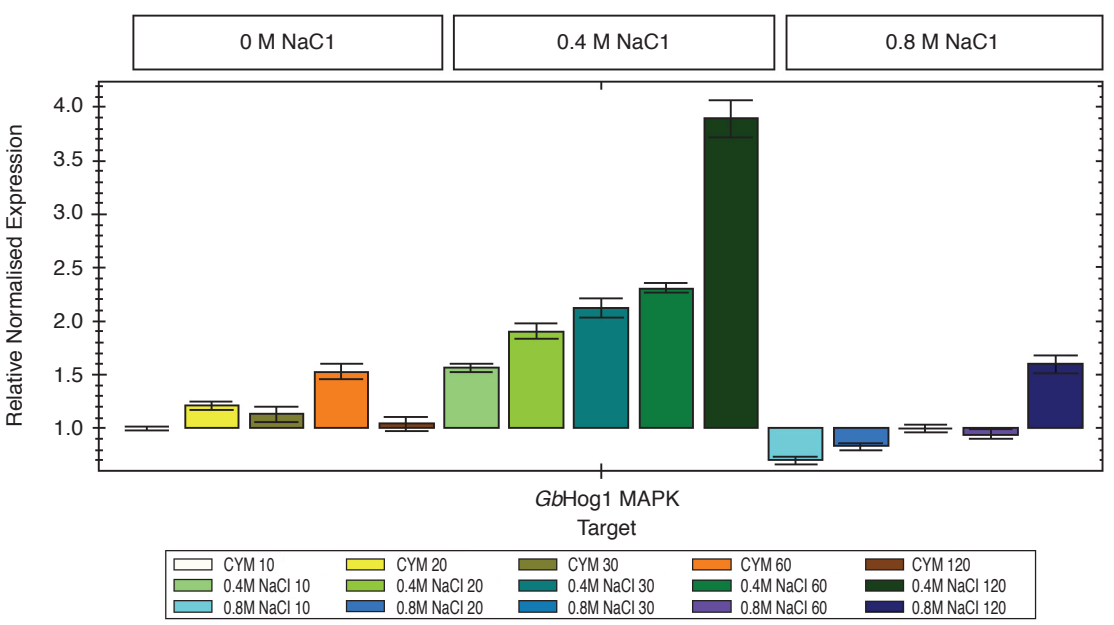

Figure 4. Expression profile of Hog1 Mitogen-activated Protein kinases (MAPK) in G. boninense *mycelia collected during the salinity stress assay. The relative normalised expression is presented in fold change. Standard deviations are shown by vertical bars. Complete yeast medium (CYM) represents the control samples, 0.4 M NaCl represents the samples from the flasks containing CYM+0.4 M NaCl and 0.8 M NaCl represents the samples from the flasks containing CYM+0.8 M NaCl. The numbers after the sample name indicate the exposure period in minutes. Expression at 10 min in CYM (CYM 10) was used as control (Calibrator).*

CONCLUSION

A full-length cDNA encoding Hog1 MAPK was successfully cloned from *G. boninense*. The *Gb*Hog1 MAPK shared a high similarity to other fungal Hog1 MAPK across the Ascomycota and Basidiomycota divisions. Based on the saline stress assay, the *G. boninense* mycelium growth was slower when exposed to 0.4 M NaCl and could not grow on media containing 1.0 M NaCl. Gene expression analysis showed that *Gb*Hog1 MAPK plays important role under hyperosmotic stress and could tolerate the hyperosmotic stress of 0.4 M NaCl.

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