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ORIGINAL PAPER

The HOG signal transduction pathway in the halophilic fungus *Wallemia ichthyophaga*: identification and characterisation of MAP kinases WiHog1A and WiHog1B

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Abstract The high-osmolarity glycerol (HOG) pathway is one of the several MAP kinase cascades in fungi. It is the main signal transduction system that is responsible for cellular stress responses, and has primarily been studied in the context of osmotic stress. In the present study, we provide the first insights into the HOG pathway of the obligatory halophilic basidiomycetous fungus *Wallemia ichthyophaga*, with the characterisation of its two Hog1-like kinases: WiHog1A and WiHog1B. These share high similarity to Hog1 kinase from *Saccharomyces cerevisiae* (ScHog1) at the level of amino-acid sequence. While WiHog1A could not optimally complement the function of ScHog1, WiHog1B was a fully functional Hog1-like kinase and could improve the halotolerance of the yeast, compared to the wild-type or the ScHog1-expressing *hog1Δ* strain. In *W. ichthyophaga* cells, Hog1 was constitutively phosphorylated under optimal osmotic conditions and dephosphorylated when the cells were challenged with hypo-osmolar or hyperosmolar stress. This pattern of phosphorylation kinetics is opposite to that of yeast. Transcriptional analysis of these two kinases in *W. ichthyophaga* shows that *WiHOG1B* is more responsive to changes in NaCl concentrations than *WiHOG1A*. Our identification and characterisation of these Hog1-like kinases from *W. ichthyophaga* confirm the existence of the HOG signalling pathway and its role in osmosensing in this halophilic fungus.

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Abbreviations

HOG	High-osmolarity glycerol
ScHog1	*Saccharomyces cerevisiae* Hog1
HwHog1	*Hortaea werneckii* Hog1
WiHog1A	*Wallemia ichthyophaga* Hog1 isoform A
WiHog1B	*Wallemia ichthyophaga* Hog1 isoform B
EXF	Culture collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana
YNB	Yeast nitrogen base
WT	Wild type
EV	Empty vector
LG model	Le-Gascuel model

Introduction

To assure normal physical and chemical conditions for intracellular processes, the cell must maintain optimal water potential and turgor. Responding to changes in osmolarity is, therefore, of essential importance, especially if the organism inhabits environments that can have fluctuating osmolyte concentrations (e.g., ripening fruit, solar salterns) (Gunde-Cimerman et al. 2000; Heermann and Jung 2004). In *Saccharomyces cerevisiae*, there are several MAPK pathways (Gustin et al. 1998; Chen and Thorner 2007), the most studied of which is the high-osmolarity glycerol (HOG) pathway (Westfall et al. 2004; Hohmann et al. 2007). The HOG pathway is also defined as a stress-

activated protein kinase cascade (de Nadal et al. 2002). The upstream part of the HOG pathway consists of two functionally redundant, but structurally distinct, branches. These two branches, known as the *SHO1* and *SLN1* branches, converge at Pbs2 MAP2K, which is an activator of Hog1 (ScHog1) MAPK (Brewster et al. 1993). When the *S. cerevisiae* cell shrinks upon osmotic shock (Petelenz-Kurdziel et al. 2011), ScHog1 is doubly phosphorylated and acts on different cellular targets (Westfall et al. 2008; Mas et al. 2009; Sole et al. 2011). As a result of HOG pathway activation, also the glycerol production is upregulated. Once the cell volume and turgor are restored, ScHog1 is dephosphorylated by phosphatases Ptp2, Ptp3 and Ptc1 (Hohmann et al. 2007). The ScHog1 MAPK contains defined sites, motifs and domains that are important for MAPK function and interactions: the active site (Westfall and Thorner 2006), the phosphorylation sites (Bell and Engelberg 2003), the common docking domain (Tanoue and Nishida 2003) and the Pbs2-binding domain 2 (PDB-2) (Murakami et al. 2008).

In recent years, much attention has been given to the interplay between the HOG pathway and other signalling routes in yeast. A number of studies have investigated the mechanisms of signal specificity, where multiple signalling pathways share the same protein components. It is now apparent that ScHog1 kinase is also involved in the inhibition of interacting pathways (O'Rourke and Herskowitz 1998; Saito 2010).

Other fungi have similar signalling pathways that are homologous to HOG (Krantz et al. 2006b), which show distinctive structural and functional features. For example, *Aspergillus nidulans* (Furukawa et al. 2005) and *Aspergillus fumigatus* (Reyes et al. 2006) have two Hog1 homologues, known as MpkC and SakA (HogA), which are similar in sequence, but do not respond in the same way to environmental stress. In *Cryptococcus neoformans* serotype A, Hog1 is constitutively phosphorylated under 'normal' conditions, while in *C. neoformans* serotype D it is not (Bahn et al. 2005). In the extremely salt tolerant fungus *Hortaea werneckii,* phosphorylated HwHog1 kinase can be detected when the cells are grown in ≥3 M NaCl, but not when they are grown at lower salinity (Turk and Plemenitas 2002). The transcription of HwHog1 is also salt dependent (Lenassi et al. 2007). Interestingly, almost all of the HOG pathway components that have been studied in *H. werneckii* are represented by two gene copies (Lenassi and Plemenitas 2007; Fettich et al. 2011).

Our model organism is the recently discovered halophilic fungus *Wallemia ichthyophaga*. This is a basidiomycetous fungus from the xerophilic genus *Wallemia* (Wallemiomycetes and Wallemiales, cl. et ord. nov), which also includes *Wallemia sebi* and *Wallemia muriae* (Zalar et al. 2005). *W. ichthyophaga* forms multicellular meristematic clumps and, in contrast to the other two Wallemia species, has almost no hyphal growth in liquid cultures. Its surface is covered with an extracellular polymeric substance matrix in a salt-dependent manner (Gunde-Cimerman et al. 2009). Recently, the draft sequence of the *W. ichthyophaga* genome became available (Gostincar, unpublished results). With its small size of 9.7 Mbp, it is similar to the genome of *W. sebi*, where the expansion of several gene families was observed, with the most significant of these being amino-acid transporter, Heat shock protein *HSP20*, and stress responsive A/B barrel domain. This might represent the background for the xerophilic nature of *W. sebi* (Padamsee et al. 2012). On the other hand, *W. ichthyophaga* is the most halophilic eukaryote known to date, as it grows in 1.7 to 5.1 M NaCl. The addition of salt to the growth medium of *W. ichthyophaga* is mandatory, and it has been shown that the salt concentration affects its morphology (Kralj Kuncic et al. 2010). The mRNA transcript of the glycerol-3-phosphate dehydrogenase, the enzyme involved in the production of the glycerol (the main compatible solute in *W. ichthyophaga*), is induced upon hyperosmotic shock (Lenassi et al. 2011).

In the present study, we present the first insights into the HOG pathway of the extremely halophilic *W. ichthyophaga*, with the identification and characterisation of its two Hog1 homologues: WiHog1A and WiHog1B.

Materials and methods

Strains and growth conditions

W. ichthyophaga (EXF 994) was isolated from the saline waters of the Sečovlje solar saltern and is preserved in the culture collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (EXF). *W. ichthyophaga* was grown at 28 °C in a rotary shaker (180 rpm) in yeast nitrogen base (YNB) medium, which contained: 0.08 % (w/v) complete supplement mixture (CSM; Formedium), 0.16 % (w/v) YNB (Formedium), 2.0 % (w/v) glucose (Mallinckrodt Baker), 0.5 % (w/v) ammonium sulphate (Carlo Erba Reagents), and 3.4 M NaCl (Carlo Erba Reagents), in deionised water, at pH 7.0. Prior to the experiments with the hypo-osmotic and hyperosmotic stress, *W. ichthyophaga* cells were grown to the mid-exponential growth phase (when the medium pH had decreased to 4.0) in the above defined YNB medium containing 3.4 M NaCl, and then aliquoted into two parts, and harvested by centrifugation at 4000×*g* for 10 min. The application of the hypo-osmotic and hyperosmotic stress was carried out by replacing the supernatant medium of these aliquots with the same volume of the YNB medium at pH 4.0 (to avoid the pH stress) containing 1.7 or 5.1 M

Table 1 Primers used in this study

Name of primer[a]	Sequence (5′–3′)[b]
p*WiHOG1A*.f	TTCAACGACGCTGATCT CCCTACTGAT
np*WiHOG1A*.f	GGAAGGTGATGATGTA CTCTGAGATA
p*WiHOG1A*.r	GCCTGGGGAAATGAAA ACGTCTAACAA
np*WiHOG1A*.r	GGAAATGATGTTCTC ATGGCGGAGGTGTT
WiHOG1A.f	ATGTCCCAAGAAGACAGCAGTTT
WiHOG1A.r	CTAGTGAGCACCAGCGGTGGTA
WiHOG1B.f	ATGGCGGATTTCGTCAACGCTAGCAT
WiHOG1B.r	TTAAACGGTGAAGGACGAATCACT
(*EcoRI*)*WiHOG1A*.f	TAGAATTCATGTCCCAAGAAGACAGCAGTTT
(*BamHI*)*WiHOG1A*.r	TAGGATCCCTAGTGAGCACCAGCGGTGGTA
(*BamHI*) *WiHOG1A*.r.MYC	TAGGATCCCTACAAATCTTCTTCAGAAATCAATTT TTGTTCGTGAGCACCAGCGGTGGTA
(*EcoRI*)*WiHOG1B*.f	TAGAATTCATGGCGGATTTCGTCAACGCTAGCAT
(*SmaI*)*WiHOG1B*.r	TTCCCGGGTTAAACGGTGAAGGACGAATCACT
(*SmaI*) *WiHOG1B*.r.MYC	TTCCCGGGTTACAAATCTTCTTCAGAAATCAATTTTTGTTCA ACGGTGAAGGACGAATCACT
(*BamHI*)*ScHOG1*.f	TAGGATCCCATGACCACTAACGAGGAATTCATTA
(*HindIII*)*ScHOG1*.r	TTAAGCTTTTACTGTTGGAACTCATTAGCGTACTG
(*HindIII*) *ScHOG1*.r.MYC	TTAAGCTTTTACAAATCTTCTTCAGAAATCAATTTTTGTTCCTG TTGGAACTCATTAGCGTACTG
qPCR*WiHOG1A*.f	ATGTCATCAACACCATCACA
qPCR*WiHOG1A*.r	TGTCTCTTTGGTAAAGCGTCTA
qPCR*WiHOG1B*.f	ACGACGTCATCCAGACTATTT
qPCR*WiHOG1B*.r	GCTTTTTAGGCAAGCTTTGA

[a] In primer names, f stands for forward, r for reverse primer, and MYC for MYC-tag sequence

[b] Restriction sites are underlined in primer sequences

NaCl for the hypo-osmotic and hyperosmotic stress, respectively. Aliquots of the cell suspensions were removed at the indicated times before and after the replacement of the medium, and then fast filtered through 1.2-µm-pore-size MCE filters (Sartorius) and frozen in liquid nitrogen.

The *S. cerevisiae* auxotrophic wild-type (WT) (BY4741; MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0; ura3Δ0*) and *hog1Δ* mutant (BY4741; MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YLR113w*::*kanMX4*) haploid strains were obtained from the Euroscarf Yeast deletion strain collection (Frankfurt, Germany). For the growing conditions of *S. cerevisiae*, see "Sequence, domains and phylogenetic analysis".

DNA and RNA isolation, and cDNA synthesis

Highly purified fungal genomic DNA was isolated from mid-exponential-phase cells grown in the YNB medium with 3.4 M NaCl using the modified phenol/chloroform/isoamyl alcohol method described previously (Rozman and Komel 1994). Genome Walker Universal kits (Clontech) were used to construct pools of uncloned, adaptor-ligated genomic DNA fragments. The RNA was extracted from mid-exponential phase cells of *W. ichthyophaga* grown in the YNB medium containing 1.7, 3.4 and 5.1 M NaCl, using the TRI reagent (Sigma Aldrich). Possible DNA contamination was removed using DNAse I (Fermentas), and the integrity and purity of the RNA were evaluated spectrophotometrically and by capillary electrophoresis (Agilent 2100 Bioanalyser). The cDNA was synthesised using SuperScript III first-strand cDNA synthesis kits (Invitrogen) and random hexamer primers (Promega), or with SMARTer race cDNA amplification kits (Clontech), according to the manufacturer instructions. The DNA from the *S. cerevisiae* WT strain was also isolated according to the method described above.

Identification, amplification, cloning and sequencing of the *HOG1*-like genes from *W. ichthyophaga*

In our previous study (Lenassi et al. 2007), a partial sequence of the *HOG1*-like gene from *W. ichthyophaga* was PCR amplified from genomic DNA using degenerate primers. Based on the partial sequences of this *HOG1*-like gene, we designed gene-specific primers (Table 1) to

amplify sequences upstream (5′) and downstream (3′) of the known fragment, using cDNA or gDNA as template and prepared with SMARTer race cDNA amplification kits and genome walker universal kits, respectively. Primary touchdown PCR and the following secondary nested PCR (annealing for 5 cycles at 72 °C, 5 cycles at 70 °C and 25 cycles at 68 °C, for both) were performed with high fidelity polymerase mix (Fermentas), using 50 ng cDNA template for 20 µL reactions and p*WiHOG1A*.f/p*WiHOG1A*.r and np*WiHOG1A*.f/np*WiHOG1A*.r primers for the touchdown and nested PCR, respectively. The products were purified using EZNA gel extraction kits (Omega Bio-Tek) and cloned into the pJET cloning vector (Fermentas). The cloned fragments were sequenced (Macrogen) and the new forward and reverse primers *WiHOG1A*.f/*WiHOG1A*.r were subjected to PCR amplification of the whole coding sequence, again with 50 ng template cDNA, high fidelity polymerase mix, and annealing for 30 cycles at 60 °C. A bioinformatic analysis using local BLAST searches of the *W. ichthyophaga* draft genome that became available later during this study (Gostincar, unpublished results) identified another copy of a *HOG1*-like gene. This gene was amplified from 50 ng template cDNA with high fidelity polymerase mix and annealing for 30 cycles at 60 °C, using the forward and reverse primers *WiHOG1B*.f and *WiHOG1B*.r (Table 1).

Sequence, domains and phylogenetic analysis

The protein sequences of ScHog1 (GenBank: NP013214), WiHog1A (GenBank: JX573532) and WiHog1B (GenBank: JX573533) were aligned using M-Coffee with the default settings (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee; August 2012). This programme combines outputs of different multiple alignment algorithms (Notredame et al. 2000). The sequences were visualised with the Jalview 2.7 software (Waterhouse et al. 2009). The domain annotation was based on data from the UniProt database (the Uniprot Consortium, 2012; http://www.uniprot.org/; August 2012) and the literature (Murakami et al. 2008; Maayan et al. 2012). The ProtParam ExPasy programme (http://web.expasy.org/protparam/; August 2012) was used to analyse the physicochemical parameters of the amino-acid sequences (Wilkins et al. 1999). Sequence similarity searches using the BLAST algorithm were conducted with the *Aspergillus* Genome Database (http://www.aspgd.org/; August 2012), with the non-redundant database at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi; August 2012), and with the local BLAST programme for the *W. ichthyophaga* genome (version 2.2.25; http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download; November 2010).

The alignment for the maximum likelihood phylogenetic tree of the deduced Hog1-like proteins was carried out with M-Coffee. The phylogeny was reconstructed with the PhyML 3.0 software (Guindon et al. 2010), with aLRT implementation for the calculation of χ^2 based support (Anisimova and Gascuel 2006). The analyses were run using a Le-Gascuel (LG) model of amino-acid substitution, the optimised proportion of the invariable sites, and the six substitution rate categories with an optimised gamma distribution parameter.

Expression of ScHog1, WiHog1A and WiHog1B in the *S. cerevisiae hog1Δ* mutant, and the halotolerance assay

For the expression of *W. ichthyophaga* genes in *S. cerevisiae*, the corresponding open reading frames were amplified from the pJET cloning vectors (see "Identification, amplification, cloning and sequencing of the *HOG1*-like genes from *W. ichthyophaga*") with primers containing restriction sites (Table 1). *WiHOG1A* was amplified using the forward primer (*EcoRI*)*WiHOG1A*.f and the reverse primer (*BamHI*)*WiHOG1A*.r or (*BamHI*)*WiHOG1A*.r.MYC. *WiHOG1B* was amplified with the forward primer (*EcoRI*)*WiHOG1B*.f and the reverse primer (*SmaI*)*WiHOG1B*.r or (*SmaI*)*WiHOG1B*.r.MYC. The *ScHOG1* gene amplified from *S. cerevisiae* gDNA with the forward primer (*BamHI*)*ScHOG1*.f and the reverse primers (*HindIII*)*ScHOG1*.r or (*BamHI*)*ScHOG1*.r.MYC served as the positive control in these experiments. The resulting untagged or MYC-tagged products were cloned into the corresponding restriction sites of the low-copy-number plasmid pYX142 (CEN, LEU, TPI, Amp^r), which contained the constitutive triose-phosphate isomerase promoter. The *S. cerevisiae hog1Δ* mutant cells, where the complete Hog1 open reading frame is knocked-out, were transformed with an empty vector pYX142 or with the constructs pYX142 + *ScHOG1*, pYX142 + *WiHOG1A* or pYX142 + *WiHOG1B*, to create transformants *hog1Δ* + EV, *hog1Δ* + *ScHOG1*, *hog1Δ* + *WiHOG1A* and *hog1Δ* + *WiHOG1B*, respectively. The WT strain was transformed with the empty vector pYX142 to give the transformant WT + EV. The transformations were performed using the quick and easy (Gietz and Schiestl 2007) LiAc/SS carrier DNA/PEG method, and the cells were selected on YNB plates with leucine (LEU) drop-out selection, with the presence of the plasmids confirmed by colony PCR.

For the halotolerance assays, the positive transformants were grown to mid-exponential phase in YNB-LEU medium containing: 0.08 % (w/v) CSM-LEU (Formedium), 0.16 % (w/v) YNB (Formedium), 2.0 % (w/v) glucose (Mallinckrodt Baker), 0.5 % (w/v) ammonium

sulphate (Carlo Erba Reagents), and NaCl (Carlo Erba Reagents) added to different concentrations, at pH 7.0 and 30 °C in a rotary shaker (180 rpm). Next, the OD_{600} was adjusted to 0.5, and 3.5 µL of 10-fold serial dilutions (1–10^3 dilutions) was spotted onto the YNB-LEU plates (YNB-LEU medium with 2 % (w/v) agar) that contained the indicated NaCl concentrations. The plates were incubated at 30 °C for 4 days, and then scanned. For the generation of growth curves, the transformed cells were inoculated at OD_{600} 0.05 into 96-well plates containing 100 µL YNB-LEU medium in each well, without NaCl, and with 0.4, 0.8 and 1.6 M NaCl. The 96-well plates were incubated at 30 °C in a Synergy 2 (Biotek) microplate reader, and the OD_{600} was measured for the media containing 0, 0.4, 0.8 and 1.6 M NaCl at 32, 48, 48 and 70 h, respectively. The graphs were generated using the GraphPad Prism 5 software. We used two positive controls: WT + EV and *hog1Δ* + *ScHOG1*.

The HOG-specific β-galactosidase assay

For analysis of the induction of *GPD1–lacZ* reporter expression by HOG pathway activation, the positive transformants *hog1Δ* + EV, *hog1Δ* + *ScHOG1*, *hog1Δ* + *WiHOG1A*, *hog1Δ* + *WiHOG1B* and WT + EV were co-transformed with the YCplac33 + *GPD1lacZ* plasmid, which contained 800 bp of the *GDP1* promoter that controls the expression of the *lacZ* gene. The cells were grown to mid-exponential phase in YNB-URA-LEU medium, aliquoted into two parts, and the medium of one aliquot was replaced with fresh YNB-URA-LEU medium containing 1.2 M NaCl and incubated for 4 h. For the β-galactosidase assays, first, 5 mL cells was harvested in the mid-exponential phase and resuspended in the same volume of Z buffer (60 mM $Na_2HPO_4 \times 7H_2O$, 40 mM $NaH_2PO_4 \times H_2O$, 10 mM $MgSO_4 \times 7H_2O$, 50 mM 2-mercaptoethanol, pH 7.0), after which the OD_{600} was measured. Then, the cells were lysed with 4 freeze–thaw cycles (liquid nitrogen, 30 °C water bath), and 30 µL aliquots of these lysed cells was diluted with 470 µL Z buffer containing 100 µL 4 mg/mL ortho-nitrophenol-β-D-galactopyranoside in Z buffer. Following incubations for 15–30 min at 37 °C, the reactions were stopped by addition of 250 µL 1 M Na_2CO_3, and the OD_{420} and OD_{550} were recorded using a Synergy 2 microplate reader (BioTek). The β-galactosidase values were calculated in Miller units, according to the formula $(A_{420} \times 1000 - 1.75 \times OD_{550})/(\text{time}_{(\text{min})} \times V_{(\text{mL})} \times OD_{600})$ (Miller 1972; Murakami et al. 2008). The β-galactosidase values were measured in triplicate, and the mean ± SD values were calculated.

Activation of the cross-talk mating response was assessed in the same way, except that the transformants *hog1Δ* + EV, *hog1Δ* + *ScHOG1*, *hog1Δ* + *WiHOG1A*, *hog1Δ* + *WiHOG1B* and WT + EV were co-transformed with the reporter plasmid YCplac33 + *FUS1lacZ*, which contained 834 bp of the *FUS1* promoter that controls *lacZ* gene expression. The β-galactosidase values were calculated as above.

Quantitative real-time PCR (qPCR) analysis of *WiHOG1A* and *WiHOG1B* transcription in *W. ichthyophaga*

The levels of *WiHOG1A* and *WiHOG1B* transcripts were determined with the qPCR experiment using the cDNA from the cells grown at denoted salinities. The reaction mixture was prepared with the SYBR green PCR master mix (Applied Biosystems), according to the manufacturer instructions. The gene-specific qPCR primers (Table 1) were used to generate 65-bp-long amplicons. The forward primer qPCR*WiHOG1A*.f and the reverse primer qPCR*WiHOG1A*.r were used for the *WiHOG1A* amplicon, and the forward primer qPCR*WiHOG1B*.f and the reverse primer qPCR*WiHOG1B*.r for the *WiHOG1B* amplicon. None of the primers formed observable dimers at the optimal final concentration of 200 nM. Approximately, 5 ng of template cDNA was used in the reaction with the thermal profile of 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a dissociation curve. The experiment was performed in an ABI 7500 real-time PCR system, and analysed with the 7500 system SDS software (Applied Biosystems). The expression levels of *WiHOG1A* and *WiHOG1B* transcripts were normalised to the amount of the *28S* rRNA transcript, the expression levels of which remain unchanged under these different environmental conditions (Rizner et al. 1999).

Western blotting of cell lysates

In *S. cerevisiae*, the ScHog1 protein is phosphorylated upon osmotic shock (Brewster et al. 1993). To determine whether the ScHog1, WiHog1A and WiHog1B kinases expressed in the *S. cerevisiae hog1Δ* mutant cells are also phosphorylated under such conditions, the transformants *hog1Δ* + *ScHOG1* (positive control), *hog1Δ* + *WiHOG1A* and *hog1Δ* + *WiHOG1B* expressing the MYC-tagged kinases were harvested before and 5 min after application of 0.6 M NaCl shock. For the negative control, *hog1-Δ* + EV cells were used. The cell lysates were prepared by disruption with glass beads in protein extraction buffer (100 mM Tris–HCl, pH 6.8, 20 % glycerol, 2 % 2-mercaptoethanol, 4 % SDS) containing a cocktail of fungal protease inhibitors (Sigma) and phosphatase inhibitors (10 mM NaF, 0.1 mM Na_3VO_4). The protein

concentrations were measured spectrophotometrically using the BCA protein assay (Pierce), at 562 nm. Equal concentrations of proteins were boiled for 10 min in 5× protein-loading buffer (0.313 M Tris–HCl, pH 6.8 at 25 °C, 10 % SDS, 0.05 % bromophenol blue, 50 % glycerol; Fermentas) before loading. The proteins were separated by SDS-PAGE in 12 % acrylamide gels and blotted onto Immobilon PVDF membranes (Millipore). Immunodetection with anti-phospho-p38 antibodies (anti-P-p38; cell signalling technology) and anti-rabbit secondary antibodies (cell signalling technology) conjugated with horseradish peroxidase (HRP) was performed using the rapid step ECL reagent (Calbiochem). An anti-MYC-tag antibody (Abcam) that recognises the tagged kinases independent of the phosphorylation state, together with the anti-rabbit-HRP antibody, was used as controls. A LAS-1000 camera (Fuji) was used for visualisation of the signals on the membranes.

The protein extracts of *W. ichthyophaga* were prepared from cells grown and stressed as described in "Strains and growth conditions". The cells were separated from the medium before (time 0) and 2, 10 and 30 min and 24 h after the hypo-osmotic and hyperosmotic NaCl stress was applied, and frozen in liquid nitrogen. The cells harvested at 24 h were considered as adapted to the stress conditions. For protein extraction, the cells were first ground in a pestle and mortar under continuous cooling with liquid nitrogen, and then the extraction was carried out in the protein extraction buffer and with glass beads, as described for *S. cerevisiae*. The protein concentrations were determined and the samples were loaded onto SDS-PAGE gels, and then blotted as above. The phosphorylated WiHog1 kinases were probed and visualised using anti-P-p38 and anti-rabbit-HRP secondary antibodies. As we could not detect WiHog1A or WiHog1B with any of the commercially available antibodies against ScHog1 or p38, the membranes were stained with 0.1 % (w/v) Ponceau S (Carl Roth) in 0.5 % (v/v) acetic acid (Carl Roth), and scanned. The images of the visualised proteins were analysed densitometrically, using the ImageJ software (Schneider et al. 2012), to normalise the phospho-WiHog1 (P-WiHog1) bands according to the amount of protein loaded. Only the area of Ponceau S-stained protein in the range from 70 to 25 kDa was used for normalisation.

Results

Two *HOG1*-like genes in *W. ichthyophaga*: *WiHOG1A* and *WiHOG1B*

We constructed gene-specific primers (Table 1) and amplified the 5′ and 3′ ends of the *WiHOG1A* gene from SMARTer RACE cDNA and Genome Walker genomic DNA. The *WiHOG1A* gene amplified from the genomic DNA was intronless, as the sequence was identical to the cDNA sequence (GenBank: JX573532). From the 1098-bp-long gene, a 365-amino-acid protein was deduced (Fig. 1),

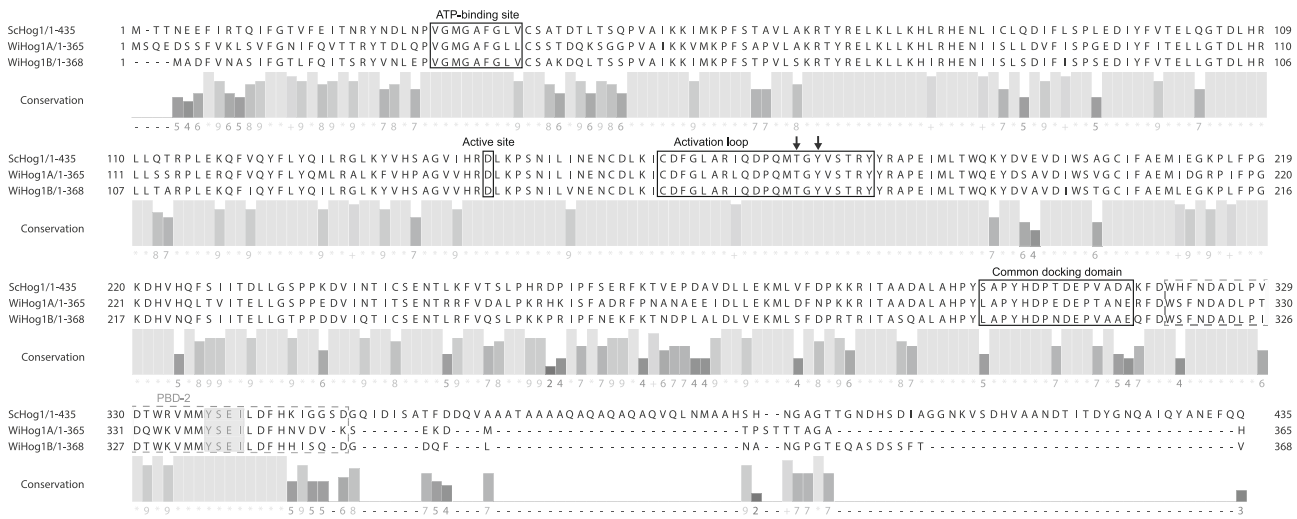

Fig. 1 Protein alignment of ScHog1 (GenBank: NP013214), WiHog1A (GenBank: JX573532) and WiHog1B (GenBank: JX573533). The deduced amino-acid sequences of the kinases were used to create an alignment with M-Coffee. Conservation of amino-acid sites is demonstrated by *grey columns*, where higher and lighter-coloured columns represent greater conservation. Important regions and sites are indicated with *framed boxes*: ATP-binding site; active site; activation loop containing the TGY motif with the T and Y phosphorylation site residues marked by *black arrows*; common docking domain. The annotations are based on *S. cerevisiae* Hog1 UniProt entry P32485 (The Uniprot Consortium 2012; http://www.uniprot.org/; August 2012). *Grey box* with dashed frame: Pbs2 binding domain PBD-2 (Murakami et al. 2008). *Grey box* inside the PBD-2 domain: motif necessary for ScHog1 autophosphorylation (Maayan et al. 2012)

with a predicted molecular weight of 41.63 kDa and a theoretical isoelectric point (pI) of 5.56.

In the draft genome sequence of *W. ichthyophaga* (Gostincar, unpublished results), we recently identified another *HOG1*-like gene using local BLAST searches, which we named *WiHOG1B*. The gene was amplified from cDNA as described in "Identification, amplification, cloning and sequencing of the *HOG1*-like genes from *W. ichthyophaga*". This 1516-bp-long gene contains eight introns that range in size from 43 to 56 bp, and it transcribes to a 1107-bp-long coding sequence (GenBank: JX573533). The deduced protein has 368 amino acids (Fig. 1), with a predicted molecular weight of 41.84 kDa and a theoretical pI of 5.49.

WiHog1A and WiHog1B kinases show high similarity to ScHog1 kinase

M-Coffee multiple protein sequence alignment revealed high conservation of the Hog1 kinase domains and motifs in both of the WiHog1A and WiHog1B isoforms (Fig. 1). In all of these Hog1 kinases (i.e., also ScHog1; Fig. 1), the conserved serine/threonine kinase domain starts very close to the N-terminus, at five residues upstream of a conserved ATP-binding site. The aspartic acid in the active site remains unchanged, as does its surrounding amino acids. The activation loop that contains the TGY motive is also highly conserved, with the only difference being at position 8, which in WiHog1A is leucine, whereas in ScHog1 and WiHog1B it is isoleucine. The CD domain is not as highly conserved as the activation loop, although the important negatively charged residues at positions 6, 9 and 10 (Tanoue and Nishida 2003) remain unchanged. Following the CD domain, there is the PBD-2 region. As can be seen from the alignment in Fig. 1, WiHog1A and WiHog1B also have relatively conserved PBD-2 regions. Murakami et al. (2008) confirmed the role of PBD-2 in the binding to Pbs2 kinase and Ptp2 phosphatase in *S. cerevisiae*. All of the residues that are important for the function of the PBD-2 domain in ScHog1 are conserved in WiHog1A and WiHog1B. Two new regions were recently identified in ScHog1: a motif that resides inside the PBD-2 domain and is responsible for ScHog1 autophosphorylation (Fig. 1); and the domain which extends from the residue 341 to the C-terminus and inhibits this autophosphorylation (Maayan et al. 2012). However, from the PBD-2 domain further towards the C-terminus, there are only a further 15 and 22 residues in WiHog1A and WiHog1B, respectively, while in ScHog1, 85 residues follow the PBD-2 region (Fig. 1).

Gene phylogeny reconstruction

To construct a maximum likelihood phylogenetic tree and to place the WiHog1A and WiHog1B proteins in the fungal kingdom, the newly identified sequences were compared to homologous sequences of other fungi. The fungal homologues were retrieved from the *Aspergillus* Genome Database for *Aspergilli*, and from the National Center for Biotechnology Information for the other fungi (for details, see "Sequence, domains and phylogenetic analysis"). Figure 2 shows the phylogenetic tree that was arbitrarily rooted to *Rhizopus delemar*. The two deduced kinases of *W. ichthyophaga* are positioned apart from each other. *W. ichthyophaga* Hog1A is placed together with *W. sebi* Hog1 at the base of the Basidiomycota, which indicates that these two kinases separated from the common ancestor a long time ago. The other isoform, *W. ichthyophaga* Hog1B, is closely related to *W. sebi* SakA and is phylogenetically more similar to other Hog1-like kinases from basidiomycetous fungi.

WiHog1A and WiHog1B rescue the osmosensitive phenotype of *S. cerevisiae hog1Δ*

To demonstrate experimentally that the *WiHOG1A* and *WiHOG1B* genes are indeed functional homologues of the *ScHOG1* gene, functional complementation assays were performed with the *S. cerevisiae hog1Δ* strain expressing the MYC-tagged kinases WiHog1A and WiHog1B. Along with the expressed ScHog1 kinase, WT cells also served as the positive control. The transformants were plated on YNB-LEU medium with the indicated NaCl concentrations. The growth was recorded after 4 days, with representative data shown in Fig. 3a. On the YNB-LEU plate without NaCl (Fig. 3a, YNB control), the cells showed identical growth patterns, confirming the uniform cell spotting. The growth of the WiHog1A-expressing cells was reduced in comparison to the cells expressing ScHog1, WiHog1B and the WT cells, even at 0.4 M NaCl, although these cells grew better than the negative control (Fig. 3a, *hog1Δ* + EV); this demonstrates that the WiHog1A kinase can partially complement the function of ScHog1. At 0.8 and 1.6 M, there was better growth of the WiHog1B-expressing cells, compared to all of the other transformants (Fig. 3a). In parallel with the MYC-tagged genes, transformations were also carried out with the untagged *WiHOG1A*, *WiHOG1B* and *ScHOG1* genes. In functional complementation assay, there were no differences when the untagged kinases were expressed in transformants (data not shown) and, therefore, we excluded these untagged kinases from the further studies. It has also been shown previously that the function of GFP-tagged ScHog1 remains the same as that for untagged ScHog1 (Reiser et al. 1999).

To more precisely monitor the growth patterns of these transformants, the cells were inoculated into liquid YNB-LEU media and the OD_{600} was followed over the indicated

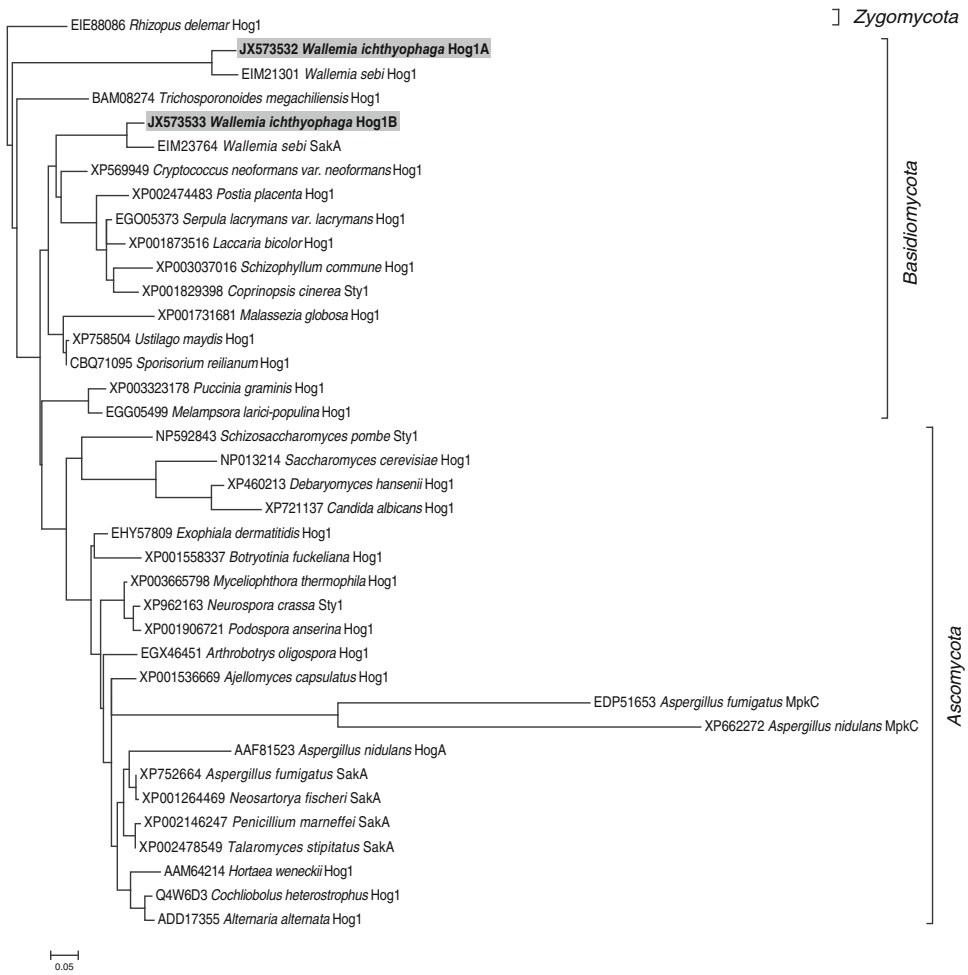

Fig. 2 Maximum likelihood phylogenetic tree of deduced Hog1-like proteins arbitrarily rooted to *Rhizopus delemar*. The protein alignment was carried out with M-Coffee (Notredame et al. 2000), and PhyML (Guindon et al. 2010) was used for construction of the tree (settings described in "Sequence, domains and phylogenetic analysis"). *Grey highlighted W. ichthyophaga* Hog1A and *W. ichthyophaga* Hog1B, positioned apart on the tree

time course in a 96-well microplate reader. The growth curves presented in Fig. 3b show that growth on the YNB-LEU medium alone (Fig. 3b, YNB control) was similar for all transformants. At 0.4 and 0.8 M NaCl, the *hog1Δ* cells expressing WiHog1A did not reach as high an OD_{600} as the ScHog1-expressing and WiHog1B-expressing cells and the WT strain (Fig. 3b). More importantly, the WiHog1B-expressing cells grew significantly better than the other transformants at 1.6 M NaCl (Fig. 3b). These data further support the results of the functional complementation assay on the YNB-LEU medium on plates, where the *hog1Δ* cells expressing WiHog1B showed the greatest tolerance to high salinity (Fig. 3a, 1.6 M NaCl).

To determine whether the WiHog1 kinases are phosphorylated upon osmotic shock in *S. cerevisiae*, the cell lysates were prepared from the transformants carrying the empty vector and from those expressing the MYC-tagged kinases ScHog1, WiHog1A and WiHog1B. The cells were harvested before and after 0.6 M NaCl stress for 5 min. Immunoblotting of the lysates was carried out with the anti-MYC-tag or anti-P-p38 antibodies to probe for the MYC-tagged and phosphorylated kinases, respectively. Figure 3c shows representative data where these kinases were expressed (Fig. 3c, anti-MYC). There was no phosphorylation of the WiHog1A and WiHog1B kinases before the NaCl stress (Fig. 3c, no salt, anti-P-p38); in contrast, all kinases were phosphorylated upon application of the salt shock (Fig. 3c, 0.6 M NaCl, anti-P-p38). Lower phosphorylation of the WiHog1A kinase is in agreement with the lower halotolerance of this transformant (Fig. 3a, b).

WiHog1B kinase activates the HOG pathway and inhibits the cross-talk more efficiently than WiHog1A in *S. cerevisiae hog1Δ*

Quantitative measures of HOG pathway activation was examined using the β-galactosidase assay (Miller 1972). The *hog1Δ* cells carrying the empty vector, the *hog1Δ* cells expressing ScHog1, WiHog1A, WiHog1B, and the WT cells were co-transformed with the plasmid carrying the *GPD1–lacZ* reporter gene. The activity of the β-galactosidase enzyme was determined before and after the

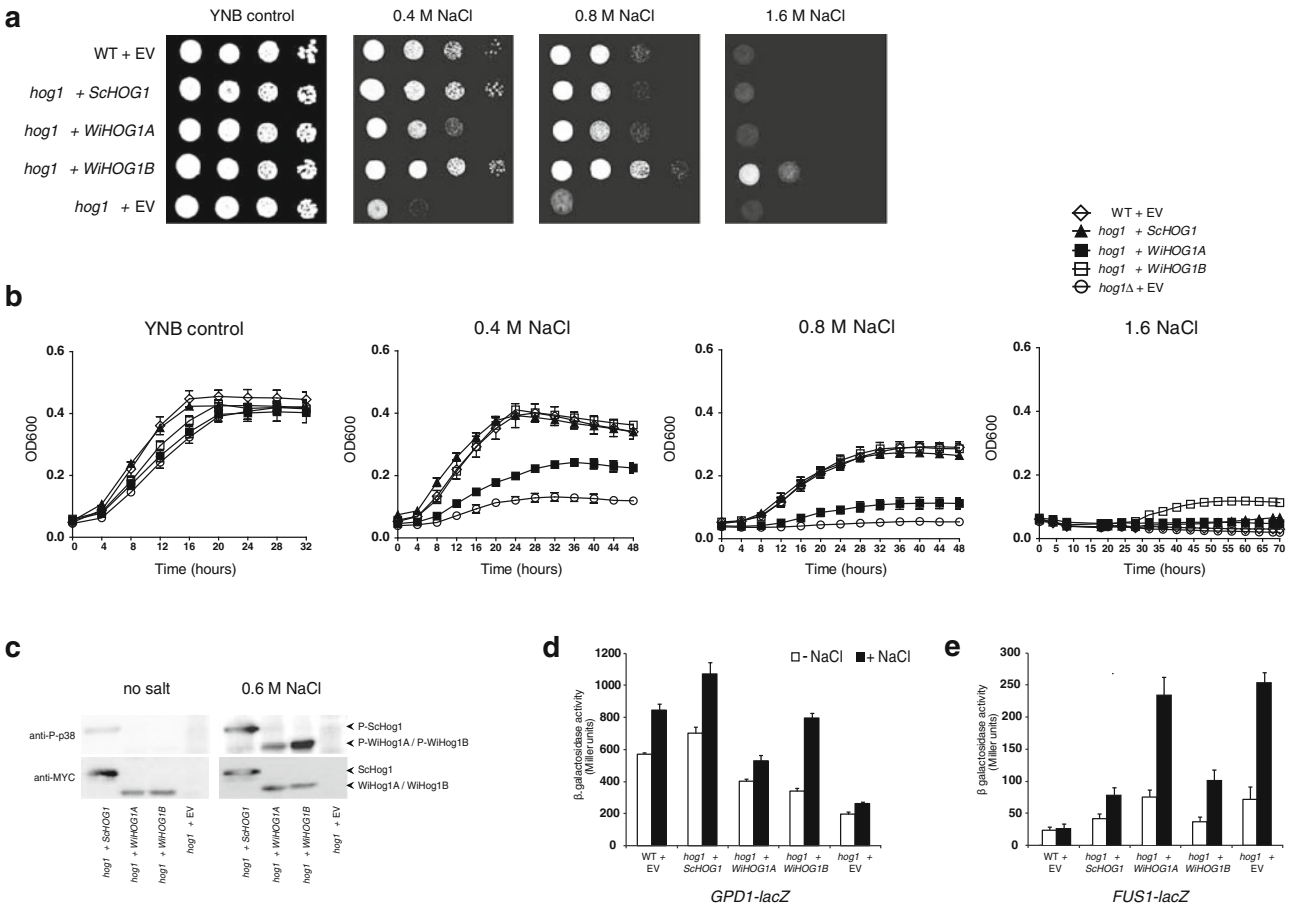

Fig. 3 Expression of the ScHog1, WiHog1A and WiHog1B kinases in yeast. *S. cerevisiae* wild-type (WT) strain (*positive control*) was transformed with an empty vector. The *hog1Δ* strain was transformed with an empty vector (*negative control*) or vectors containing the *ScHOG1* (*positive control*), *WiHOG1A* or *WiHOG1B* genes. **a** Functional complementation and halotolerance assay. Transformed cells were 10-fold serially diluted and spotted on YNB-LEU plates without salt (*YNB control*) or with 0.4, 0.8 or 1.6 M NaCl, as indicated. **b** Growth of transformed cells in liquid YNB-LEU medium without salt (*YNB control*) or with 0.4, 0.8 or 1.6 M NaCl, as indicated. Here, 96-well plates were incubated in a microplate reader and OD_{600} was measured at the indicated times. Data are mean ± SD of two experiments, each carried out in triplicate. **c** Immunodetection of expressed and phosphorylated kinases with anti-MYC-tag or anti-P-p38 antibodies, respectively, in transformants of *hog1Δ* cells carrying empty vector or vectors with the *ScHOG1* (*positive control*), *WiHOG1A* or *WiHOG1B* genes. The lysates were prepared from cells before and 5 min after application of the 0.6 M NaCl shock. *Arrows* positions of Hog1 MAPKs. **d**, **e** Activation of the HOG pathway (*GPD1–lacZ*) and cross-talk activation of the mating pathway (*FUS1–lacZ*) assayed by the β-galactosidase activity assay. Activity in cell lysates of transformants co-transformed with the *GPD1–lacZ* or *FUS1–lacZ* reporter plasmid was measured before (*white bars*) and after (*black bars*) application of 1.2 M NaCl stress for 4 h. Data are mean ± SD, carried out in triplicate

application of 1.2 M NaCl stress for 4 h. Figure 3d shows that in the WT and the WiHog1B-expressing cells, the induction of the *GPD1–lacZ* reporter (and thus the β-galactosidase activity) following this osmotic stress was comparable, while that for the WiHog1A-expressing cells was lower, as expected from the previous results above. In all of the cells, there was also high background enzyme activity independent of this salt stress, which might be the result of a leaking promoter. Indeed, taking this into account, it was the WiHog1B kinase that showed the highest relative induction of this β-galactosidase activity.

O'Rourke and Herskowitz (1998) described a phenomenon in yeast by which the mating pathway is activated during osmotic shock, when some components of the HOG pathway are deficient. This happens because the phosphorylation cascade cannot feed forward to ScHog1, which also negatively controls the cross-talk; instead, the phosphorylation/activation pathway proceeds through the mating pathway components via Ste11, to activate Fus3 or Kss1, which in turn activate *FUS1* expression via Ste12 transcription factor. Another way to test the function of these WiHog1 kinases in *S. cerevisiae* is thus to determine whether WiHog1A and WiHog1B can prevent this cross-talk. Therefore, the cells carrying the empty vector, the cells expressing ScHog1, WiHog1A and WiHog1B, and the WT cells were co-transformed with a plasmid carrying

the *FUS1–lacZ* reporter. Induction of β-galactosidase was measured in the lysates from these cell (as a measure of the cross-talk) before and after the application of 1.2 M NaCl stress for 4 h (as described for the *GPD1–lacZ* reporter). Figure 3e shows the results of this cross-talk activation of the mating pathway upon osmotic shock. As the negative control, the WT cells showed the lowest cross-talk activation. While levels of the cross-talk are almost the same in the cells expressing either ScHog1 or WiHog1, we observed higher cross-talk (as defined by the greater β-galactosidase activity) in the cells expressing WiHog1A, which is in agreement with the lower HOG pathway activation by WiHog1A (Fig. 3c, d, *hog1Δ* + *WiHOG1A*). As expected, there was also high cross-talk shown by the β-galactosidase activity of the *hog1Δ* cells used as positive control (Fig. 3e, *hog1Δ* + EV).

Expression of the *WiHOG1A* and *WiHOG1B* genes in *W. ichthyophaga* depends on environmental salinity

Despite the fact that the components of signalling pathways are regulated primarily at the level of posttranslational modifications, there are reported cases of mechanisms involving also transcriptional regulation (Han and Prade 2002; Lenassi et al. 2007). More importantly, transcriptional analysis is of interest in fungi with paralogous MAPKs (Reyes et al. 2006). Because two paralogous genes coding for Hog1-like kinases exist also in *W. ichthyophaga*, we preformed the qPCR analysis to check if their transcription was affected by salt levels. Following the growth of the cells under the optimal (3.4 M), hyperosmotic (5.1 M) and hypo-osmotic (1.7 M) conditions, the transcription of *WiHOG1A* and *WiHOG1B* genes was analysed. Results presented in Fig. 4 show that the transcript levels of both genes were upregulated in the hyperosmotic (5.1 M) and hypo-osmotic (1.7 M) medium. *WiHOG1A* gene expression increased less than 3-fold in 1.7 M and 5.1 M NaCl, while under these conditions *WiHOG1B* reached 6-fold that of the basal level. These results demonstrate that *HOG1*-like genes are differentially expressed and that *WiHOG1B* is more responsive to changes in environmental salinity.

Phosphorylation patterns of Hog1-like kinase in *W. ichthyophaga*

As *W. ichthyophaga* is a true halophile, which means that it cannot survive without NaCl and it shows optimal growth at 3.4 M NaCl, we examined its Hog1 phosphorylation patterns. To determine whether the Hog1-like kinases in *W. ichthyophaga* undergo phosphorylation kinetics that are similar to other fungi during osmotic changes, we probed the protein lysates of *W. ichthyophaga* with anti-P-p38

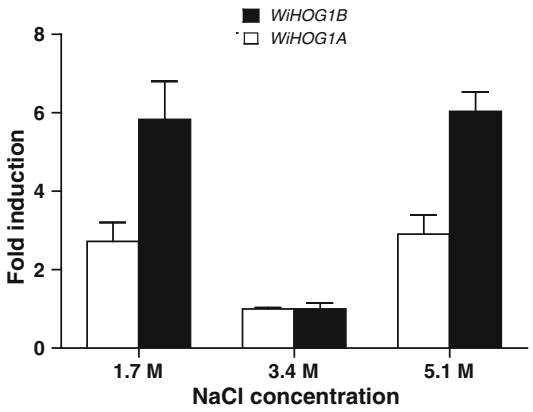

Fig. 4 Transcription of *HOG1*-like kinases in *W. ichthyophaga* analysed by qPCR. Expression of *WiHOG1A* (*white bars*) and *WiHOG1B* (*black bars*) under optimal (3.4 M), hyperosmolar (5.1 M) or hypo-osmolar (1.7 M) conditions. The fold-induction on cDNA levels is presented as mean ± SD of two real-time experiments each carried out in duplicate, relative to cDNA levels at 3.4 M NaCl

antibodies (Fig. 5). Here, we cannot discriminate between the two isoforms of WiHog1A and WiHog1B for the phosphorylation patterns that are detected. However, in the representative data shown in Fig. 5, the intensities of the WiHog1 phosphorylation bands (Fig. 5, P-Hog1) were normalised relative to the amounts of the loaded proteins (Fig. 5, Ponceau), as given in the histograms of Fig. 5. Following the growth of these cells under the optimal osmotic conditions (3.4 M), their time courses of WiHog1 phosphorylation under hyperosmotic and hypo-osmotic stress were followed. Under the hyperosmotic stress (5.1 M; Fig. 5a), the initial WiHog1 phosphorylation increased to a maximum within 2 min, followed by its dephosphorylation to below the starting level within 10 min, and a further decrease to 30 min. Under the hypo-osmotic stress (1.7 M; Fig. 5b), the initial Hog1 phosphorylation decreased immediately, and continued to decrease further to 30 min. Thus, under both hyperosmotic and hypo-osmotic stress, even 30 min after the cells were challenged, the Hog1 phosphorylation levels remained lower than at the start (Fig. 5a, b). With the continuation of the hyperosmotic and hypo-osmotic stress to 24 h (Fig. 5c), the cells grown at 5.1 M NaCl showed a return to the initial Hog1 phosphorylation level. However, in the cells grown at 1.7 M NaCl, even after 24 h, the Hog1 phosphorylation levels remained low, showing that these cells challenged with hypo-osmotic shock were still not adapted to this new condition.

Discussion

Through our studies of the HOG pathway of the obligatory halophilic fungus *W. ichthyophaga*, we identified two

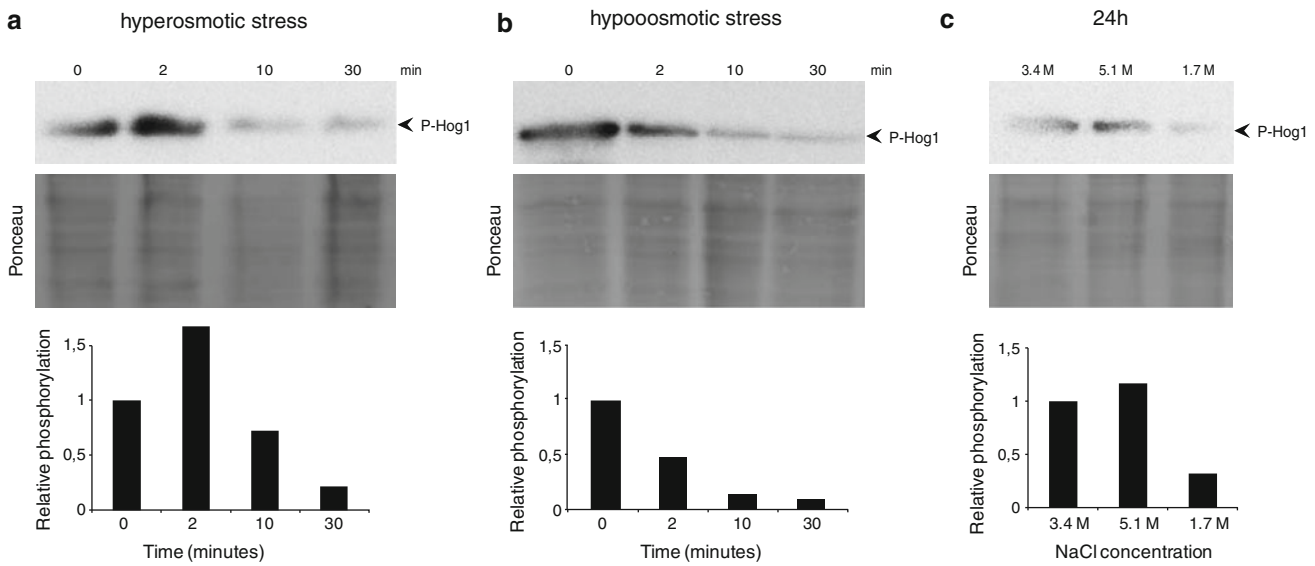

Fig. 5 Phosphorylation patterns of Hog1-like kinase in *W. ichthyophaga*. Cell lysates of *W. ichthyophaga* were probed with anti-P-p38 antibodies. The phosphorylated Hog1-like kinase (P-Hog1, *upper panels*) and images of Ponceau S staining (*middle panels*) are shown. The intensities of the phosphorylation bands were normalised to the amount of loaded protein for the histograms, for the phosphorylation relative to the normal state (*before stress*). **a**, **b** Cells grown under optimal osmotic conditions (3.4 M NaCl) with time courses for the application of hyperosmotic (5.1 M) stress (**a**) and hypo-osmotic (1.7 M) stress (**b**). **c** Cells grown under optimal osmotic conditions (3.4 M NaCl), and 24 h after the transfer to the medium containing 5.1 M and 1.7 M NaCl, as indicated

HOG1-like genes, *WiHOG1A* and *WiHOG1B*. The protein products of WiHog1A and WiHog1B deduced from these genes share 69 % identical amino acids with each other, and 59 and 66 % with ScHog1 kinase, respectively. Of note, the ScHog1 protein has 435 amino acids and is thus 70 amino acids longer than WiHog1A and 67 longer than WiHog1B.

Murakami et al. (2008) showed that deletion of the 77 C-terminal ScHog1 residues has no effect on its binding to Pbs2, while the study of Maayan et al. (2012) demonstrated an autophosphorylation-inhibiting role of these C-terminal residues. Furthermore, a hyperactive ScHog1 mutant was identified following the C-terminal N391D mutation (Bell et al. 2001). Our data show that although WiHog1A and WiHog1B lack a considerable portion of the C-terminal amino acids seen for ScHog1, they can still complement its function in the *S. cerevisiae hog1Δ* strain and they remain largely non-phosphorylated when expressed in unstressed cells. This thus confirms that the final 70 residues of ScHog1 are not essential for its function. This alanine-rich C-terminus of ScHog1 is also missing in all known filamentous fungi (Krantz et al. 2006a). However, WiHog1A is not fully functional when compared with WiHog1B and ScHog1, despite the most important residues being considerably conserved. Evidently, the lower phosphorylation levels, lower *GPD1–lacZ* induction, and greater cross-talk indicate that WiHog1A cannot interact optimally with the activating MAP2K Pbs2 or with its targets, which might be explained by small differences in the ATP-binding site, the activation loop, the common docking domain, and/or the PBD-2 region. On the other hand, WiHog1B can be phosphorylated, can efficiently bind to the *GPD1* promoter, and shows lower cross-talk activation, which demonstrates that in the *S. cerevisiae hog1Δ* background, WiHog1B is a fully functional kinase. Because of the efficient interaction between *GPD1* promoter and WiHog1B in *S. cerevisiae* and the induction upon hyperosmotic shock of the *GPD1* mRNA transcript in *W. ichthyophaga* (Lenassi et al. 2011), there is a strong indication that this kinase is involved in the induction of the glycerol synthesis in *W. ichthyophaga*. WiHog1B also improves the tolerance of the yeast strain to high salinity, which was not observed for the expression in *S. cerevisiae* of HwHog1, from the halotolerant *H. werneckii* (Lenassi et al. 2007).

It is not common for organisms to contain two copies of Hog1-like protein kinases (Krantz et al. 2006b). To date, the duplication of a *HOG1*-like gene had only been reported in the *Aspergillus* genus. In *A. nidulans*, the *hogAΔ* knock-out is osmosensitive, while deletion of the other Hog1-like kinase, AnMpkC, does not affect the phenotype (Furukawa et al. 2005). For AfSakA from *A. fumigatus*, as well as its involvement in osmotic stress signalling, it has been shown to take part in nitrogen sensing and germination (Xue et al. 2004). Furthermore, AfSakA shows different transcriptional responses to stress compared to AfMpkC, which in *A. fumigatus* is a MAPK that is required for the use of polyalcohol sugars (Reyes et al. 2006). The results of the BLAST searches of Hog1-like

kinases in the *Aspergillus* Genome Database indicated that *HOG1*-like genes in *Aspergilli* show high diversification. Our results from *W. ichthyophaga* (the present study) and the results from the *W. sebi* genomic data (Padamsee et al. 2012) show that duplication happened a long time ago also in the common ancestor of these two fungi. Most likely, this feature extends to the whole of the *Wallemia* genus. WiHog1A and WsHog1 share 93 % identity, while WiHog1B and WsSakA have 90 % identical residues. Usually core components of signalling pathways are under strong functional selection (Wu et al. 2010) and, therefore, a duplication of such a gene can release conservational pressure, to give the newly created gene the space to accumulate mutations more rapidly and to acquire new functions. Duplication of genes is also considered a mechanism of adaptation to extreme environments (Kondrashov et al. 2002), which was confirmed in our studies on the halotolerance of *H. werneckii* (Gostincar et al. 2011).

As *W. ichthyophaga* is a recently discovered species (Zalar et al. 2005), there are few molecular tools that have been developed to investigate this organism. Its slow growth, formation of cell clumps, and requirement for NaCl in the growth medium further aggravate its manipulation. In the present study, we performed transcriptional and post-translational analysis of Hog1-like kinases to investigate the mechanisms of the HOG pathway regulation in this fungus. We demonstrated that the two paralogous Hog1 kinases identified in *W. ichthyophaga*, WiHog1A and WiHog1B, responded differentially at the level of transcription. Data from the literature show that the response to increased osmolarity varies among fungi; when *S. cerevisiae* cells are exposed to osmotic shock, levels of *ScHOG1* mRNA remain unchanged (Brewster et al. 1993), while in *H. werneckii* transcription of *HwHOG1* gene is upregulated in such conditions (Lenassi et al. 2007). Levels of *AnHOGA* from *A. nidulans* (Han and Prade 2002) and *AfSAKA* from *A. fumigatus* (Reyes et al. 2006) also respond to osmotic shock, in contrast to the transcripts of the paralogous *MPKC* kinase gene, which in *A. nidulans* are not detected (Furukawa et al. 2005), and in *A. fumigatus* they increase in response to the carbon source and oxidative stress but not to osmotic stress (Reyes et al. 2006). Our data presented in Fig. 4 demonstrated that Hog1 paralogues in W. *ichthyophaga* are regulated at the level of transcription and that *WiHOG1B* is more responsive to changes in NaCl concentrations.

To investigate HOG pathway activation in *W. ichthyophaga*, cell lysates were prepared at different time points after the application of hyperosmotic and hypo-osmotic shock. In *S. cerevisiae*, ScHog1 is not phosphorylated under iso-osmotic or hypo-osmotic conditions. Upon hyperosmotic shock, ScHog1 is phosphorylated and translocates to the nucleus. After the cell adapts to the higher osmolarity, ScHog1 is dephosphorylated by phosphatases in a negative-feedback manner (Hohmann et al. 2007). However, here, *W. ichthyophaga* shows the opposite phosphorylation pattern: Hog1-like kinase in *W. ichthyophaga* remains phosphorylated under optimal osmotic conditions (3.4 M NaCl) and is dephosphorylated within 10 min of either hypo-osmotic or hyperosmotic shock. This suggests that phosphatases have an important role in the HOG pathway regulation in *W. ichthyophaga*. Indeed, after 30 min of both hyperosmotic and hypo-osmotic stress, the phosphorylation levels remained low, which indicates that the cells had not yet adapted to these new osmotic conditions. However, after 24 h of growth under hyperosmotic and hypo-osmotic shock, the phosphorylation levels remained lower with hypo-osmotic medium than in the cells grown at both optimal and the hyperosmolar salt concentrations. This suggests that under these hypo-osmotic conditions, the cells are still under acute stress, or that the HOG pathway from *W. ichthyophaga* shows a different feedback mechanism. Similar phosphorylation models have already been reported for *C. neoformans*, where serotype A has a unique, *W. ichthyophaga*-like patterns, while some isolates of serotype D regulate CnHog1 phosphorylation in a way parallel to that of *S. cerevisiae* (Bahn et al. 2005). The CnHog1 proteins from *C. neoformans* serotype A and D are identical at the amino-acid level and are functionally redundant, which demonstrates that their upstream and downstream signalling components are divergent and regulate the HOG pathway differently in *C. neoformans*. Similarly, by expression of the WiHog1A and WiHog1B proteins in *S. cerevisiae hog1Δ*, we have shown that *W. ichthyophaga* Hog1-like kinases can be functionally similar to ScHog1 in the yeast background, although they show completely different phosphorylation patterns in *W. ichthyophaga*. In a well-designed study, Bahn et al. (2007) provided evidence that Ssk2 MAP3K is a key element for such opposing regulation of HOG pathways in isolates of *C. neoformans*. Nevertheless, *W. ichthyophaga* needs a more elaborate examination to investigate and define further such hypotheses.

While in *S. cerevisiae* constitutive Hog1 phosphorylation is lethal (Maeda et al. 1994), in *W. ichthyophaga* and *C. neoformans* this appears not to be the case. In *W. ichthyophaga* and *C. neoformans*, the HOG pathway regulation follows a mechanism that is opposite to that of yeast. On the other hand, when the halotolerant *H. werneckii* is grown in medium containing $\geq$3 M NaCl, HwHog1 is constitutively phosphorylated, which is not the case if it is grown at lower osmolarity (Turk and Plemenitas 2002). As with organisms from hypersaline environments, the pathogens must also be resistant to different stresses when invading their host. This is evident by the ability of *H. werneckii* to cause tinea nigra, a mycotic skin infection that is seen in tropical regions (Gottlich et al. 1995). While the assumption has been made that a unique mechanism of

CnHog1 phosphorylation in *C. neoformans* is responsible for its higher stress resistance and the virulence of its serotype A (Bahn et al. 2007), in a similar way such HOG pathway regulation might be the reason for the high salt resistance of *W. ichthyophaga*. Halophiles are challenged with osmotic changes that are much greater than those of *S. cerevisiae* and, therefore, we propose halophilic and halotolerant models, like *W. ichthyophaga* and *H. werneckii* (Plemenitas et al. 2008), to investigate the complex responses to extreme changes in osmolarity that are governed by HOG signalling (Vaupotic and Plemenitas 2007; Lenassi et al. 2011). *S. cerevisiae* is a mesophilic organism, in which ScHog1 is not phosphorylated when the cells are adapted to the changed osmotic conditions. In *H. werneckii*, we observed a phosphorylation mechanism similar to that of *S. cerevisiae* only when the cells were grown in up to 3 M NaCl. When *H. werneckii* cells were grown at higher salinities, HwHog1 was phosphorylated constitutively (Turk and Plemenitas 2002). As this hybrid regulation might represent the background for the extreme halotolerance of *H. werneckii*, the constitutive phosphorylation of Hog1-like kinase from *W. ichthyophaga* might support its obligatory halophilic nature.

In the present study, we have evaluated the *W. ichthyophaga* Hog1-like kinases in the background of the *S. cerevisiae hog1Δ* mutant strain through three independent approaches. First, with complementation assays and growth on high-osmolarity medium, secondly, with immunodetection of phosphorylated Hog1, and finally with the transcriptional activation of the HOG-specific reporters *GPD1–lacZ* and *FUS1–lacZ*. WiHog1A and WiHog1B are functional homologues of ScHog1. From this study of phosphorylation in *W. ichthyophaga*, we can conclude that the HOG pathway regulation is similar to that of *C. neoformans* serotype A, and in contrast to that of *S. cerevisiae*.

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