ThHog1 controls the hyperosmotic stress response in Trichoderma harzianum

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INTRODUCTION

Eukaryotes use different mitogen-activated protein kinase (MAPK) cascades to control the gene expression required by a plethora of biological processes (Gustin et al., 1998). Changes in the environment that provoke stress are sensed via a MAPK member of the family of stress-activated protein kinases (SAPKs) (Roux & Blenis, 2004). In yeasts, the MAPK that controls stress response is Hog1 (Hohmann, 2002), and homologue proteins have been also found in other fungi such as Schizosaccharomyces pombe (Degols et al., 1996), Aspergillus nidulans (Han & Prade, 2002; Kawasaki et al., 2002), Cryptococcus neoformans (Bahn et al., 2005), Magnaporthe grisea (Dixon et al., 1999), Candida albicans (Alonso-Monge et al., 2003), Cryphonectria parasitica (Park et al., 2004), Neurospora crassa (Zhang et al., 2002) and Colletotrichum lagenarium (Kojima et al., 2004). In humans, a related group of MAPKs with three different families (SAPK, ERK and p38) also controls stress response and inflammation (Kyriakis & Avruch, 2001).

To adapt to stress conditions, different strategies involving diverse regulatory mechanisms have been developed by fungi. In Saccharomyces cerevisiae, Hog1 is activated by hyperosmotic shock (Hohmann, 2002), heat (Winkler et al., 2002) and citric acid (Lawrence et al., 2004). Moreover, Hog1 and other proteins are required to develop stress resistance (Bilsland et al., 2004). Sty1, the Hog1 homologue in fission yeast, is activated when cells are exposed to a wide variety of stress conditions (Degols et al., 1996; Shieh et al., 1997) and stress genes are mostly controlled by Sty1 (Chen et al., 2003). As a result, in S. cerevisiae, hog1Δ mutants are mainly impaired in the high-osmolarity stress response, and the oxidative stress response in some conditions (Bilsland et al., 2004). In fission yeast and Aspergillus nidulans, MAPK mutants are highly sensitive to high osmolarity, heat shock and oxidative stress (Kawasaki et al., 2002). The set of genes that is highly induced under different stress conditions has defined the common environmental stress response, CESR (Alonso-Monge et al., 2003; Causton et al., 2001; Chen et al.,...
2003; Gasch et al., 2000; Rep et al., 2000; Smith et al., 2004). Cells exposed to a low dose of one stress factor become resistant to a low dose of a second unrelated stress. This phenomenon is called cross-resistance (Hohmann & Mager, 1997).

The mechanism by which Hog1 activates the stress response has been thoroughly studied in yeasts (Hohmann, 2002). Two osmosensors, localized in the plasma membrane, transduce the signal perceived through two different MAPKKK inducing the phosphorylation of the MAPKK Pbs2, and, subsequently, Hog1. Emerging cues point to a similar situation in other fungi (Han & Prade, 2002; Motyoyama et al., 2005).

Trichoderma has been identified as a fungal genus with a high ability to colonize diverse environments. This versatile genus antagonizes other fungal cells (mycoparasitism), which is the basis for its use in agriculture to control fungal plant pathogens. Furthermore, increasing evidences point to a symbiotic mycorrhiza-like relationship of Trichoderma with plants (Harman et al., 2004; Lu et al., 2004). Finally, a variety of hydrolytic enzymes are used by Trichoderma to obtain nutrients from soil and plant material (Kubicek & Penttila, 1998), which makes this genus a unique source to identify polymer-degrading enzymes with industrial value (Penttila, 1998). Elucidating the role of the Hog1 protein homologue in T. harzianum may help to understand how this fungus adapts to stress and a changing environment and develops its parasitic, symbiotic and saprophytic lifestyles.

**METHODS**

**Strains, media, growth and transformation conditions.** Trichoderma harzianum CECT 2413 was obtained from the Colección Española de Cultivos Tipo (Burjasot, Valencia, Spain). Colletotrichum acutatum and Phoma betae were provided by Dr. E. Monte (University of Salamanca, Salamanca, Spain). Yeast strains were kindly provided by Dr. G. Yaakov (Hebrew University of Jerusalem, Israel). T. harzianum was maintained on potato dextrose agar (2 %, w/v), commercial dehydrated potato flakes; 2 %, w/v, glucose; 2 %, w/v, agar). Plates for drop assay were prepared using minimal medium (MM) supplemented with 2 % (w/v) glucose and 0 % (w/v) ammonium sulphate (Penttila et al., 1987). When indicated, 10 μg cyclosporin A ml⁻¹ (Calbiochem), 1-2 M sorbitol, 1 M NaCl, 10 mM H₂O₂, 40 mM CaSO₄ or 5 mg l-tryptophan ml⁻¹ was added. In some instances, carbon, nitrogen and phosphorus depletion conditions were used. In 1 % (w/v) chitin-containing medium, glucose was not added. When indicated, the pH of MM was adjusted to 10. Plates were incubated at 30 °C. For drop assay, the plates for drop assay were prepared using minimal medium (MM) supplemented with 2 % (w/v) glucose and 0 % (w/v) ammonium sulphate were used as carbon and nitrogen sources. Liquid cultures were grown at 28 °C and 200 r.p.m. in a rotary shaker.

**Vectors and strains.** Cloning of the T. harzianum hog1 gene was performed as follows: The ESTs (expressed sequence tags) database from T. harzianum developed within the TrichoEST project (www.trichoderma.org) was screened. EST L027340.26202431 contained the complete ORF from the hog1 gene; it was fully sequenced, and used as a probe to screen a genomic library prepared in λGEM11. Genome walking was performed to complete promoter sequencing using the Genome Walker kit (Clontech). The specific primer 5’-CTCGTAAAGATGAGGATCGACGCGG-3’ and commercial adaptor primer were used, and the band obtained was re-amplified using the nested specific primer 5’-GGATCGACGCGG-3’. One contig of 4-2 kbp was obtained and fully sequenced.

 Yeast phenotypic assays were carried out using a hog1Δ strain of S. cerevisiae (MATa his3 leu2 ura3 hog1::1-TRP ade8) kindly provided by Dr. G. Yaakov, Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem (Bell et al., 2001). As well as pES86HOG1 and pES86HOG1’ (with yeast HOG1 wild-type and mutant allele, respectively). This vector contains the ura3 marker and adh1 promoter. To clone the hog1 gene from T. harzianum into pES6 vector, the hog1 cDNA was amplified using primers 5’-GGAAGCTTTTACCATGTCGCCTAATACGATCC-3’ and 3’-GGC-GCGCTCGGCTGTTTTCCATCTCAAA-5’, and cloned in pES6 using HindIII and NotI enzymes (sites underlined). Using the resulting pES86-hog1 as a template, pES86-hog1’ was obtained using the QuickChange Mutagenesis kit (Stratagene). Primers used in mutagenesis were 5’-GGGATGAGGATGCTGAACTGGAGAG-3’ and its complementary. Mutation was checked by sequencing. Transformation and standard techniques used in yeast were performed as described by Sambrook & Russell (2001). Stress sensitivity of yeasts was assayed in appropriately supplemented YNB medium.

 Vectors for T. harzianum transformation were derived from pLMR3 (Mach et al., 1994). The genomic clone of hog1 was amplified using primers 5’-GGGTCTAGAGCCCTAGAGCTCAATACGATCC-3’ and 5’-GGATCGATGGTTCGGCTGTTTTCCATCTCAAA-5’, which introduced XbaI and NsiI sites respectively (underlined). Using the resulting pLMR3-hog1 as a template, pLMR3-hog1’ was obtained using the QuickChange Mutagenesis kit (Stratagene) with primer 5’-GGC-GGGATGAGGATGCTGAACTGGAGAG-3’ and its complementary. Mutation was checked by sequencing. T. harzianum CECT 2413 was transformed; one strain which had integrated only one copy of the vector was used in further experiments and named FS12, although other strains obtained in the same transformation experiment showed identical results.

 Silencing was obtained using a hairpin construct as follows. pSIL vector constructed by S. Sousa (Sousa, 2004) was used. This vector contains a strong promoter from T. harzianum (tsl1), an intron sequence and the cbh2 terminator. The final construct showed two direct inverted sequences of hog1 separated by the intron. A 545 bp hog1 fragment including the 5’ end of the ORF was amplified using primers 5’-GGGAAGCTTTTACCATGTCGCCTAATACGATCC-3’ and 5’-GGGAATTCGGAATTCGCCTGTTTTCCATCTCAAA-5’, which introduced HindIII and SpeI sites and EcoRI and BsmHI sites, respectively (underlined and italicized). The fragment in direct orientation was cloned using SpeI and BsmHI sites, whereas an inverted repeat was introduced using EcoRI and HindIII sites. This vector, named pSILHOG, was used to transform T. harzianum CECT 2413. One of the transformed strains only showed one copy of the vector and was named SHM1.
Array experiments

**Macro-array building.** The DNA to be spotted was obtained by PCR using universal primers from the library vector and cDNA clones isolated from libraries prepared from *T. harzianum* CECT 2413 (www.trichoderma.org). Those libraries were obtained from mycelia growing in different cultures which covered a wide range of conditions (glucose or chitin as carbon sources, nitrogen and carbon starvation, fungal cell walls, plant cell walls, and acid pH). One-hundred-microlitre PCRs were performed in 96-well microtitre plates, with standard amplification parameters. One microlitre of the PCR products was separated on an agarose gel to check the product quality and quantity. Before spotting, the DNA was denatured in 50 % DMSO. The DNA was spotted with a BIOMEK 2000 robot (Beckman) on Hybond-N+ membranes (Amersham Biosciences). After deposition, DNA was cross-linked to the membranes by UV irradiation (125 ml) in a Vilber-Lourmat cross-linker. Membranes were stored at room temperature until use. A set of filters was prepared containing all unique sequences found at the time of the analysis (M. Rey & F. González, unpublished). Twenty-six microtitre plates containing 96 clones each (2496 total spots) were spotted with duplicates. Additionally, different controls were included in the membranes: positive (ESTs corresponding to known *T. harzianum* genes), negative (library vector, PCR buffer) and charge controls. Membranes were prehybridized in 50 % formamide gels, blotted onto nylon membranes, and hybridized with specific genes. RNA was isolated as described under ‘DNA and RNA hybridizations’. For macroarray hybridization, the probe was prepared by reverse transcription using 12 U–32P-dCTP and 1 µl PowerScript reverse transcriptase (Clontech) per membrane (9 x 12 cm), following the manufacturer’s indications. Reaction efficiency was quantified by means of a scintillation counter.

**Hybridization and image acquisition.** Prehybridization and hybridization were performed at 65 °C. Membranes were prehybridized in Church buffer (0·5 M sodium phosphate buffer, pH 7·2; 10 mM EDTA; 7 %, w/v, SDS) for 1 h and then hybridized using 10–15 ml fresh buffer per 9 x 12 cm membrane. The radioactive probe was added appropriately diluted to obtain 5 x 106 c.p.m. per ml hybridization buffer. After overnight hybridization, membranes were washed three times with washing buffer (0·04 M sodium phosphate buffer, pH 7·2; 0·1 %, w/v, SDS) at 65 °C. Then membranes were wrapped with one layer of transparent film and exposed for 3 h on a Packard Multipurpose MP intensifying screen and the image was obtained using a Cyclone Storage Phosphor System (Perkin Elmer). Three independent hybridizations were performed using different probes and membranes.

**Data analysis.** The image of the hybridized membrane was analysed using Phoretix Array v3.1 (Nonlinear Dynamics). Two main procedures were applied to this image: first a background control subtraction of the median spot edge average, and then normalization of dot intensities on the basis of the intensity of some internal controls. Dots with intensity values below the mean intensity of negative controls were discarded for further analysis. Each EST was dotted in duplicate, and each membrane was hybridized three times. Dot and replicate mean and standard deviation were calculated for the whole membrane to determine the accuracy of the experiment. Finally, the ratio intensity between two different experiments (test and control conditions) was calculated. An arbitrary threshold of twofold difference in intensity levels was established to identify genes as differentially expressed.

**Phosphorylation assay.** For analysis of the phosphorylation state of p38 MAPks, mycelia grown as indicated above were frozen, ground in a mortar and suspended in phosphatases inhibition buffer (10 %, w/v, glycerol, 50 mM Tris/HCl, pH 7-5, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 0·1 % SDS, 50 mM NaF, 5 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 25 µg ml-1 each of tosylphenylalanine chloromethyl ketone, tosylslyline chloromethyl ketone, pepstatin-A, antipain, leupeptin and aprotinin) (Delgado-Jarana et al., 2005). Samples were homogenized and centrifuged to pellet the cell debris. Protein concentration of the supernatant was determined using the Lowry assay. Fifty micrograms of total protein was separated by SDS-12 % PAGE and blotted onto Immobilon-P membranes (Millipore), following the manufacturer’s instructions. Membranes were blocked using 2 % non-fat skimmed milk for 1 h. p38 MAPK was detected using the PhosphoPlus p38 MAPK antibody kit (Cell Signalling Technology), following the manufacturer’s instructions. Coomassie-blue-stained gels were used as loading control.

**Fluorescence microscopy.** Localization of the nuclear green fluorescent protein (GFP)-tagged Hog1 protein was determined by fluorescence microscopy. A chimeric hog1-gfp gene was obtained as follows. A hog1 cassette containing promoter and gene was amplified from genomic DNA by PCR using primers 5’-GTTGAGCCCTGTGTC-CCTCATCCACGC-3’ and 5’-CACTATGGTTGTTGGAATTGC-TCC-3’, which added SaI and Spel sites, respectively (underlined). These enzymes were used to clone the fragment into pZEGAI vector (Zeilinger et al., 2003) previously cut with Xhol and XbaI. The resultant vector PHog1-GFP was used to transform *T. harzianum* using a PEG-based protocol (Penttila et al., 1987). Of the transformed strains, two showed only one copy of pHog1-GFP vector and were chosen for microscopy analysis. Transformed strains were grown in MM for 24 h. Hyperosmotic shock was achieved by mixing the culture with an equal volume of 2 M sorbitol. Oxidative stress was assayed by adding 10 mM H2O2. Cultures were grown for 1 h whereas untreated cultures were maintained as control. Formaldehyde was then added up to a final concentration of 4 % (w/v) and maintained for 10 min at room temperature. Cells were then centrifuged, suspended in PBS/formaldehyde (PBS containing 4 %, w/v, formaldehyde) and maintained for 1 h at room temperature. Finally, cells were washed twice with PBS and treated with 1 µg 4′,6-diamidino-2-phenylindole (DAPI) ml-1. DAPI and GFP fluorescence were captured by exciting cells with 365 and 450–490 nm wavelengths, respectively, by using a Olympus BX60F5 microscope with a 40 x objective, a Leica DFC300 Fx camera and an IM50 Leica imaging system. DNA and RNA hybridizations. Standard molecular techniques were performed as described elsewhere (Sambrook & Russell, 2001). For nucleic acid isolation, mycelia harvested from different culture conditions were frozen in liquid nitrogen, lyophilized and ground to a fine powder. Genomic DNA extractions were done according to previously described procedures (Raeder & Broda, 1985). Total RNA extractions were performed with Trizol reagent (Invitrogen), following the manufacturer’s indications. For Northern experiments, 10 µg total RNA from each sample was separated on 1·2 % agarose-formaldehyde gels, blotted onto nylon membranes, and hybridized (Sambrook & Russell, 2001). Blots were probed with the indicated DNA fragments. Probes were labelled with [32P]dCTP by using the Ready-To-Go Oligolabelling kit (Amersham Biosciences). The loading of Northern blots was checked using radish 18S rRNA as a probe.

**Stress cross-protection experiments.** Cells from wild-type, FS12 and SHM1 strains were grown in 40 ml TSB cultures (10° conidia ml-1) for 9 h. Cultures were treated for 1 h with the following conditions: untreated control, oxidative stress (0·4 mM H2O2) and osmotic stress (0·3 M NaCl). Mycelia were collected by centrifugation, washed in pre-warmed TSB medium, and resuspended in 10 ml TSB medium. Aliquots of 2 ml were taken in triplicate and treated for 1 h as follows: untreated control, 10 mM, 25 mM and 50 mM
H₂O₂ at 28 °C and 200 r.p.m. Finally, cells were diluted and plated at 30 °C for 2 days. Survival was expressed as percentage of viable cells at zero time. The experiment was repeated three times.

RESULTS

Genomic analysis of the response of T. harzianum to osmotic shock

In order to study the stress response of T. harzianum, we performed an array analysis comparing RNA samples of T. harzianum growing under standard growth conditions (minimal medium, MM) versus hyperosmotic shock (MM containing 1·2 M sorbitol) conditions. We analysed membranes with 2496 unique ESTs arrayed. These sequences were obtained under the EST sequencing project TrichoEST (www.trichoderma.org) (Rey et al., 2004). The probes were obtained using 16 h germlings that were grown in MM (control) or MM containing 1·2 M sorbitol for 2 h. RNA was extracted, labelled and used to hybridize array membranes. Those ESTs that showed a twofold induction level in the sorbitol sample were considered to be induced by hyperosmotic stress. Around 70 ESTs were identified using this criterion (Table 1). Although a number of entries without identifiable similarity were detected, some genes encoding proteins putatively involved in redox reactions and sugar metabolism, as well as genes involved in defence, development and signal transduction, were induced in high-osmolarity medium. The gene that showed the highest induction level was highly homologous to the NRC-2 kinase from N. crassa, which seems to repress conidiation (Kothe & Free, 1998). A gene related to the yeast CKS1 protein was also induced. Interestingly, cks1 null mutants showed high salt sensitivity (Yu & Reed, 2004). Several oxidoreductases were also induced (cytochrome P450 oxidoreductase, NADP-specific glutamate dehydrogenase, alternate oxidase, quinone oxidoreductase, glutaryl-CoA dehydrogenase). This high expression of oxidoreductases may counteract the oxidative stress and provide NADPH for detoxification, or be involved in metabolism of oxidized molecules (Causton et al., 2001; Chen et al., 2003; Hohmann, 2002). Genes putatively implicated in acting against unrelated stress factors were also identified, such as one 30 kDa heat-shock protein also described in yeasts (Chen et al., 2003; Rep et al., 2000), and one putative ABC transporter. Some of the upregulated genes seem to be related to energy-providing pathways. This phenomenon has also been described in yeasts (Gasch et al., 2000). One putative hexose transporter, one glucoamylase precursor, the glycolytic enzyme fructose-1,6-bisphosphatase, genes encoding proteins involved in the TCA cycle (succinyl-CoA synthetase) and respiration (mitochondrial 2-oxidadicarboxylate carrier, cytochrome c oxidase), or an ATPase can be identified in this group. The energy requirement is higher under osmotic shock due to the demand of ATP by chaperones to maintain the intracellular pH and to produce solutes and cell protectants (Causton et al., 2001). Taken together, these results indicate that T. harzianum develops a broad response under osmotic stress, covering different metabolic areas.

Characterization of the hog1 gene

Since the pivotal enzyme in response to osmotic stress in yeasts is the MAPK Hog1 we tried to identify HOG1 gene homologues in T. harzianum. We performed an in silico screening over the described EST database using the TBLastN algorithm (Altschul et al., 1997). Several ESTs which putatively coded for Hog1 MAP kinase were isolated. The cDNA clone was 1·3 kbp long, with 102 bp before an ORF of 1068 bp and a short 3′ end sequence of 216 bp before the polyadenosine tail. A genomic clone of 4·2 kbp was fully sequenced and analysed. No 5′-TATAAA sequence was found, but three 5′-CCAAT sequences were located at −328 bp, −425 bp and −622 bp with respect to ATG.

No polyadenylation sequence was detected as described by Ballance (1986). The start codon environment (5′-TCACAATG) matched the consensus described in T. harzianum: 5′-(T/C)CAA(A/C)ATG (Goldman et al., 1998). When comparing the cDNA and the genomic clone, nine exons were located to complete the ORF deduced from the cDNA sequence.

Translation of this ORF yielded a 356 aa putative protein. This protein was analysed using the InterProScan tool from EMBL-EBI (http://www.ebi.ac.uk/InterProScan/) and several domains were detected. Among them, protein kinase (IPR000719), comprising the ATP-binding domain between amino acids 26 and 50, and serine-threonine protein kinase (IPR002290), covering positions 20–299, produced the highest scores. The deduced protein also showed the TGY domain in position 171, which is involved in phosphorylation in the p38 family of MAP kinases (Widmann et al., 1999). Moreover, a phenylalanine residue (position 315) which can be mutated to serine to obtain hyperactive mutant alleles (Bell et al., 2001) was also identified. We concluded that the EST identified coded for a protein highly similar to the yeast MAPK HOG1 gene. The corresponding gene in T. harzianum was named hog1. The deduced protein (ThHog1) was highly homologous to p38 proteins cloned in filamentous fungi and yeasts. Lack of similarity is only observed at the carboxyl end, where Hog1p from S. cerevisiae showed a long terminal tail (344–439 residues) which is not present in proteins from filamentous fungi.

The hog1 gene from T. harzianum is able to complement the hog1Δ mutation in yeast

To confirm that the hog1 gene from T. harzianum was homologous to S. cerevisiae HOG1, we complemented the hog1Δ mutation in baker’s yeast. Yeasts transformed with the empty pES86 vector did not grow on YNB plates supplemented with 0·4 M NaCl (Fig. 1). As expected, yeasts transformed with vector carrying T. harzianum hog1 or yeast HOG1 were able to grow in YNB + 0·4 M NaCl, which confirmed that the two genes were homologues. However, when higher amounts of NaCl were used, yeasts transformed
with the *T. harzianum hog1* gene grew profusely when compared with those transformed with yeast *HOG1* (Fig. 1). This suggests that differences between the two proteins could reflect distinct functional characteristics.

In yeasts, overexpression of *HOG1* produces lethality in PBS2 cells (Hohmann, 2002). Hog1 depends on Pbs2 to be activated since *HOG1* overexpression does not enable PBS2 cells to grow on high-salt media (Bell et al., 2001). Taking advantage of this phenotype, a hyperactive *HOG1* allele was identified since it rescued ∆pbs2 cells on high osmolality medium (Bell et al., 2001). This allele, however, cannot rescue lethality of *hog1Δ* yeasts under high-salt conditions (Bell et al., 2001). We checked whether the F315S mutation in *T. harzianum hog1* (*hog1* 

*ThHog1* protein is phosphorylated under stress conditions

Hog1 protein deduced from *T. harzianum hog1* gene (ThHog1) shows the TGY domain which is phosphorylated in threonine and tyrosine residues in p38 MAPK proteins. To check whether the ThHog1 protein was phosphorylated, we used phosphorylation-state-specific antibodies for the p38 MAPK family (anti-phospho-P38), which can recognize specifically the TGY domain when the protein is phosphorylated. We tested metabolic stress (carbon, nitrogen and phosphorus deprivation, as well as chitin as sole carbon source), osmotic stress (1 M sorbitol), saline/osmotic stress (1 M NaCl), oxidative stress (10 mM H2O2), heavy metal stress (40 mM CuSO4) and high-pH stress (pH 10).

All the conditions led to a high level of phosphorylation where ThHog1 is highly phosphorylated. In yeast mutants, the overexpression of the wild-type allele is lethal under high salt concentration (Fig. 1).

Transformants carrying the *hog1* 

**Construction of hyperactive and silenced mutants**

In order to check whether ThHog1 protein is involved in the stress response in *T. harzianum*, two strains were constructed: a strain carrying the *hog1* 

We have not been able to obtain transformants overexpressing the wild-type *hog1* allele. Only a few colonies were isolated in several experiments, but none of them integrated this construct. This may be explained considering the phenotypes of yeast mutants. *T. harzianum* transformation is performed in medium containing 1-2 M sorbitol to stabilize the protoplasts osmotically; that is, a medium where ThHog1 is highly phosphorylated. In yeast mutants, the overexpression of the wild-type allele is lethal under high salt concentration (Fig. 1).

Transformants were checked by Northern blot analysis. As expected, strain FS12 showed higher levels of *hog1* mRNA both under basal growth conditions and under hyperosmotic shock (Fig. 4b). Strain SMH1 showed decreased, although detectable, levels of *hog1* mRNA. When this was quantified, strain SMH1 showed threefold lower *hog1* mRNA than the wild-type, whereas the FS12 strain showed two- to threefold higher levels than the wild-type. The levels of phosphorylated protein confirmed the mRNA analysis (Fig. 4c). Strain FS12 showed higher levels of phosphorylated ThHog1 protein under all conditions tested, although these levels were not constitutive. The same mutation in *S. cerevisiae* mimics the activated form of Hog1 without phosphorylation. Moreover, no signal was observed when strain SMH1 was analysed under basal and hyperosmotic shock conditions.

**ThHog1 protein is localized in nuclei under osmotic stress**

In *S. cerevisiae* (Reiser et al., 1999), *C. albicans* (Smith et al., 2004) and the filamentous fungus *Colletotrichum lagenarium* (Kojima et al., 2004) Hog1 protein accumulates in the nucleus in response to stress. To study whether this phenomenon also occurs in *T. harzianum*, we constructed a *hog1-gfp* fusion expressed from the *hog1* promoter. Strains transformed with the construct showed a diffuse fluorescence when growing in MM (Fig. 3). However, when cells were grown for 1 h under hyperosmotic shock (1 M sorbitol) fluorescence caused by GFP was localized in nuclei, which could be identified by DAPI staining (Fig. 3). Under oxidative stress (using 10 mM H2O2) ThHog1-GFP was also localized in the nuclei, although at lower levels than when using hyperosmotic shock. Hence, hyperosmotic and oxidative stress that caused phosphorylation also provoked nuclear localization of the ThHog1-GFP fusion, although at lower levels under oxidative stress.

**Phenotypic analysis of strains FS12 and SMH1**

The phenotypes of wild-type, FS12 and SMH1 strains were studied using a drop assay (Fig. 5). The two mutants grew, developed and conidiated like the wild-type. Strain SMH1 showed reduced resistance to hyperosmotic shock (1-2 M sorbitol) and salt stress (1 M NaCl). Lower effects were
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* Induction levels are indicated in percentage (%). bp: base pairs. Acc. no.: accession number. Score‡: score for BLAST analysis. E-value$: E-value for BLAST analysis.
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*The induction level is obtained by the ratio of the expression after 2 h 1·2 M sorbitol containing culture vs culture without sorbitol.
†BLAST: Basic Local Alignment Search Tool.
‡Given in ‘bits’. The bit score is defined as: \( S = \frac{\hat{N}}{\sqrt{\hat{N}} + \sqrt{K}} \), where \( \hat{N} \) and \( K \) are Karlin–Altschul parameters.
§Expected value. Estimates the statistical significance of a match, specifying the number of matches that are expected in a search of a database of the given size by chance.
observed under heavy metal stress (40 mM CuSO₄) and oxidative stress conditions (10 mM H₂O₂). No effect was detected when this strain was stressed by high temperature (37 °C) (Fig. 5a), the cell wall disturbing agent Congo red or 50 μM concentration of the oxidative stress agent paraquat (data not shown). No clear phenotypes were observed with strain FS12, which can be explained by the fact that activation of endogenous ThHog1 protein is sufficient to counteract stress conditions.

To test the influence of the Ca²⁺-calmodulin-activated pathway upon osmotic challenge, the stress sensitivity of the mutants was analysed using the potent inhibitor of the calcium-calmodulin-activated protein phosphatase calcineurin, cyclosporin A (CsA) (Dumont, 2000). In yeast, the calcineurin inhibitor FK506 increases osmotic sensitivity by inducing amino acid starvation (Rodriguez-Hernandez et al., 2003). However, when using CsA in T. harzianum, no changes in stress resistance were observed (Fig. 5b). Strain FS12 showed a slightly enhanced resistance to MM + CsA when compared to the wild-type in MM, whereas strain SHM1 grew poorly, suggesting connections between the two signalling pathways.

T. harzianum is able to develop parasitic interactions with fungal hosts as a part of its antagonistic abilities. During all these biotic interactions, T. harzianum has to confront different metabolites, cell wall polymers and intracellular environments. To study whether the ThHog1 protein is involved in these processes, the wild-type, FS12 and SHM1 strains were assayed for their ability to antagonize plant-pathogenic fungi such as Botrytis cinerea, Rhizoctonia solani, C. acutatum, P. betae and Sclerotium sclerotiorum. No differences were observed except with C. acutatum and P. betae (Fig. 6). In both situations, T. harzianum wild-type strain grew over the other fungus, and sporulated profusely. However, both strains FS12 and SHM1 did not overgrow, and consequently did not condiate on the surface of the phytopathogenic fungi. Moreover, a slight clearing halluc was observed in the interaction zone, suggesting a role of ThHog1 in these fungus–fungus interactions.

**Cross-resistance mediated by Hog1**

The existence of a general stress response in S. cerevisiae, Schiz. pombe and C. albicans has been proposed to explain the cross-resistance phenomenon (Chen et al., 2003; Lewis et al., 1995; Smith et al., 2004). The exposure to a stress agent (e.g. oxidative) at low concentrations (non-lethal) can protect against further stronger related and non-related stress conditions (e.g. salt stress). In order to study wheth this mechanism is also present in T. harzianum we studied the protective effect of H₂O₂ and NaCl against strong oxidative stress. Nine-hour germlings were treated for 1 h with 0-3 mM H₂O₂ or 0-4 mM NaCl. The germlings were then washed and grown for 1 h in media with increasing lethal concentrations of H₂O₂. Finally, the germlings were diluted and plated. The untreated wild-type cells showed an extreme sensitivity to H₂O₂, whereas germlings pre-treated with NaCl showed higher resistance, which was increased in germlings pre-treated with H₂O₂ (Fig. 7a). The same
phenomenon was also observed in strain SHM1 (Fig. 7b). The resistance in non pre-treated FS12 cells was much higher than that observed in the wild-type (Fig. 7c), and the same was observed for the salt pre-treated cells. Hence, a stress cross-protection partially dependent on ThHog1 is also present in *T. harzianum*, which suggests the existence of a general stress response in this fungus. Taken together, these findings also raise the possibility that the cross-resistance mediated by oxidative stress would not be fully dependent upon ThHog1 in *T. harzianum*.

**DISCUSSION**

In this paper we describe the stress response of *T. harzianum*. We have characterized the *hog1* gene, which codes for a protein highly similar to the yeast MAPK Hog1. Some different characteristics related to Hog1 MAPK function are observed between budding yeast and *T. harzianum*. Overexpression of *T. harzianum hog1* and *hog1F315S* alleles in Δ*hog1* yeasts resulted in enhanced osmotic resistance, contrasting with yeast *HOG1* and *HOG1F318S* gene overexpression, which caused lethality under osmotic shock. The presence (in yeast) or absence (in *T. harzianum*) of the long carboxyl-terminal domain as well as other subtle divergences detected may be responsible for the functional differences observed.

ThHog1 seems to be mainly involved in the hyperosmotic shock response since only minor effects are observed in *hog1* silenced mutants under oxidative or heavy metal stress. This situation is similar to that of baker’s yeast. *S. cerevisiae hog1Δ*
mutants are affected in the hyperosmotic shock response (Brewster et al., 1993; Gasch et al., 2000; Hohmann, 2002), and effects related to oxidative stress resistance have only recently been described (Bilsland et al., 2004). This indicates the existence of alternative and complementary mechanisms to counteract oxidative stress (Lev et al., 2005). On the other hand, in Schiz. pombe, deletion of \textit{sty1/spc1} inactivates the response against different stresses (Degols & Russell, 1997; Degols et al., 1996; Shiozaki & Russell, 1997).

Although the silencing of \textit{hog1} provoked only minor effects over oxidative stress resistance, a role for ThHog1 in this cellular process cannot be discarded. Preliminary evidence of the existence of other factors that may be interacting with ThHog1 in \textit{T. harzianum} to control oxidative stress is provided by cross resistance experiments. The wild-type, silenced mutant SHM1 and strain FS12 showed a similar \textit{H2O2}-induced cross resistance. This suggested a different system to ThHog1 to respond to oxidative damage. Oxidative stress provoked only a barely detectable effect on the viability of strain FS12 when compared to wild-type. This phenotype could be explained if those factor(s) involved in oxidative stress resistance were partially dependent on ThHog1. Candidates are Rck1 and Rck2 kinases or similar proteins (Bilsland et al., 2004), which could be highly phosphorylated by ThHog1\textsuperscript{F315S} protein (in strain FS12), but also activated by another kinase(s) when ThHog1 is absent (in strain SHM).
We also found preliminary evidence of cross-talk between the calcineurin and HOG pathways. CsA, a potent inhibitor of the protein phosphatase calcineurin, did not increase sensitivity to osmotic stress as has been described in yeast for another calcineurin inhibitor, FK506 (Rodriguez-Hernandez et al., 2003). Moreover, strains carrying the hog1F315S allele were less affected by toxicity of CsA. Toxicity could not be relieved by an excess of tryptophan (data not shown). Therefore, this may be a different phenomenon from that described in yeasts and mammals using FK506 (Rodriguez-Hernandez et al., 2003; Sanchez-Perez et al., 2004). This result raises the possibility of the HOG and Ca²⁺ pathways acting over common processes (Maeta et al., 2005), since only overexpression of hog1F315S can restore, at least in

Fig. 5. Phenotypic analysis of hog1 mutant strains. (a) Three microlitres of conidial suspensions (2 × 10⁷ conidia ml⁻¹) of wild-type (wt), FS12 and SHM1 strains and tenfold serial dilutions were dropped onto plates with: minimal medium (MM); MM plus 1-2 M sorbitol (MM+Sorb); MM plus 1 M NaCl (MM+NaCl); MM plus 40 mM CuSO₄ (MM+Cu); MM plus 10 mM H₂O₂ (MM+H₂O₂) and MM incubated at 37°C. (b) CsA plates contained 10 µg cyclosporin A ml⁻¹.

Fig. 6. Mycoparasitic analysis of hog1 mutant strains. Seven-day-old mycelium plugs were inoculated onto PDA plates from wild-type (wt), FS12 and SHM1 strains. The phytopathogenic fungi used were Phoma betae and Colletotrichum acutatum. Plates were incubated for 1 week at 25°C and then photographed.

Fig. 7. Stress cross-protection in T. harzianum. Nine-hour germlings of wild-type (a), SHM1 (b) and FS12 (c) strains were either unstressed (control, ○), or pretreated for 1 h with osmotic stress (0-3 M NaCl, ▲) or low-dose oxidative stress (0-4 mM H₂O₂, ■). Cells were then washed and treated with increasing H₂O₂ concentrations (10, 25 and 50 mM) for 1 h, diluted and plated onto PDA medium to determine remaining viability. The experiment was repeated three times with similar results.
part, the normal growth inhibited by CsA. Moreover, the silenced strain was highly affected by CsA.

Hog1 kinases have been mainly described as being involved in stress control, but there is no evidence supporting other roles. In *M. grisea*, mutation in *osm1* (homologue to *hog1*) did not impair pathogenic ability (Dixon et al., 1999). On the contrary, *hog1* null mutation caused a drastic fall in development in *B. cinerea* pathogenesis (Professor Paul Tudzinsky, personal communication). The role of other MAP kinases in the antagonistic activity of *Trichoderma* species over fungi has been studied. The same MAPK has been cloned and characterized in two different strains of *Trichoderma virens* (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003). Interestingly, the loss-of-function mutant obtained covered three different phenotypes: unaffected interaction with *R. solani*, reduced parasitism over *Sclerotium rolfsii* and enhanced antagonism against a different strain of *R. solani*. This strong variability may be due to the wide variety of mechanisms used by *Trichoderma* to antagonize other fungi (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003). This was also observed in this study since changes in mycoparasitic ability were detected with *P. betae* and *C. acutatum* but not with *B. cinerea, R. solani* or *S. sclerotiorum*. ThHog1 protein could be involved in neutralizing stress agents produced by these parasitized fungi, such as reactive oxygen species. The result obtained with the silenced strain points to the requirement of intact ThHog1 for some parasitic interactions. The phenotype observed for strain FS12 can be explained if the *hog1*<sup>F315S</sup> allele disturbs other signalling pathways required for mycoparasitism. Calcineurin has been claimed as a key regulator of pathogenicity (Fox et al., 2001; Kraus & Heitman, 2003; Lengeler et al., 2000). Moreover, the calcineurin and HOG pathways show antagonistic regulation over some developmental processes in yeasts (Shitamukai et al., 2004). If active calcineurin is involved in fungus–fungus interactions, we cannot rule out the possibility of impaired antagonistic abilities of strains carrying a hyperactive allele of the *hog1* gene.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


