ThHog1 controls the hyperosmotic stress response in *Trichoderma harzianum*

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*Trichoderma harzianum* is a widespread mycoparasitic fungus, able to successfully colonize a wide range of substrates under different environmental conditions. Transcript profiling revealed a subset of genes induced in *T. harzianum* under hyperosmotic shock. The *hog1* gene, a homologue of the MAPK *HOG1* gene that controls the hyperosmotic stress response in *Saccharomyces cerevisiae*, was characterized. *T. harzianum hog1* complemented the *hog1Δ* mutation in *S. cerevisiae*, but showed different features to yeast alleles: improved osmoresistance by expression of the *hog1* allele and a lack of lethality when the *hog1F315S* allele was overexpressed. ThHog1 protein was phosphorylated in *T. harzianum* under different stress conditions such as hyperosmotic or oxidative stress, among others. By using a ThHog1-GFP fusion, the protein was shown to be localized in nuclei under these stress conditions. Two mutant strains of *T. harzianum* were constructed: one carrying the *hog1F315S* allele, and a knockdown *hog1*-silenced strain. The silenced strain was highly sensitive to osmotic stress, and showed intermediate levels of resistance against oxidative stress, indicating that the main role of ThHog1 protein is in the hyperosmotic stress response. Stress cross-resistance experiments showed evidences of a secondary role of ThHog1 in oxidative stress. The strain carrying the *hog1F315S* allele was highly resistant to the calcineurin inhibitor cyclosporin A, which suggests the existence of links between the two pathways. The two mutant strains showed a strongly reduced antagonistic activity against the plant pathogens *Phoma betae* and *Colletotrichum acutatum*, which points to a role of ThHog1 protein in fungus–fungus interactions.

**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 via a MAPK member of the family of stress-activated protein kinases (SAPKs) (Roux & Blenis, 2004). In yeasts, the MAPK (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; Shiue 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., 2002).
T. harzianum (Israel). Yeast strains were performed using 3% w/v ammonium sulphate (Penttila et al., 1987). When indicated, 2% w/v glucose and 0-5% 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 L02T34P026R02431 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’-CTCGTAAGATGGAGATCGCCCATGAGG-3’ and commercial adaptor primer were used, and the band obtained was re-amplified using the nested specific primer 5’-GGATCCATGAGGATGGATGATGAA-3’ and commercial adaptor primer used, and the band obtained was re-amplified using the nested specific primer 5’-GGATCCATGAGGATGGATGATGAA-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 (MATα his3 leu2 ura3 hog1::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 pES86/HOG1 and pES86/HOG1<sup>Δ5188</sup> (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 pES86 vector, the hog1 cDNA was amplified using primers 5’-GGAACCTTATCAGAGATGCAATCTCC-3’ and 3’-GGCG-GCGGCTCCGCTGTCCATTTCCATTCAA-3’, and cloned in pES86 using HindIII and NotI enzymes (sites underlined). Using the resulting pES86-hog1 as a template, pES86 hog1<sup>Δ5188</sup> was obtained using the QuickChange Mutagenesis kit (Stratagene). Primers used in mutagenesis were 5’-GCGGATGAGAAGTCTGACTGGAGCTTTA-3’ and 3’-GCGGATGAGAAGTCTGACTGGAGCTTTA-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 were derived from pLMRS3 (Mach et al., 1994). The genomic clone of hog1 was amplified using primers 5’-GGTCTAGAGCCCATCAGCTTCAAATGCAGTC-3’ and 5’-GGATGATAGGTGGTTCCTATTGCGCATT-3’, which introduced XbaI and NsiI sites, respectively (underlined). Using the resulting pLMRS3-hog1 as a template, pLMRS3-hog1<sup>F315S</sup> was obtained using the QuickChange Mutagenesis kit (Stratagene) with primer 5’-GCGGATGAGAAGTCTGACTGGAGCTTTA-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 (tss1), an intron sequence and the ceb2 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’-GGAACCTTATCAGAGATGCAATCTCC-3’ and 3’-GGGATCCGAGAATCTCAGACTGGAGCTTTA-3’, respectively (underlined and italicized). The fragment in direct orientation was cloned using SpeI and BamHI 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 starva-
tion, 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 microtitre of the PCR pro-
ducts 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
light (125 mJ) in a Vilber-Lourmat cross-linker. Membranes were stored at room temperature until use. A set of filters was pre-
pared containing all unique sequences found at the time of the analysis (M. Rey & F. Gonzalez, 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*
genomes), negative (library vector, PCR buffer) and charge controls composed of RNAs obtained by *in vitro* transcription of some specific genes. RNA was isolated as described under ‘DNA and RNA hybridizations’. For macroarray hybridization, the probe was pre-
pared by reverse transcription using 12.5 μg total RNA, 7.5 μCi
(227 kBq) [α-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 scintil-
lation counter.

**Hybridization and image acquisition.** Prehybridization and hybri-
dization were performed at 65 °C. Membranes were prehydrized 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 10^5 c.p.m. per ml hybrid-
ization 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 subtrac-
tion 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 con-
trols were discarded for further analysis. Each EST was dotted in
duplicate, and each membrane was hybridized three times. Dot and
replica 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 differ-
ence in intensity levels was established to identify genes as differenti-
tially 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 tosylphenyl-
alanine chloromethyl ketone, toslylsine chloromethyl ketone, pep-
statin-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 separ-
ated 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 ampli-
fied from genomic DNA by PCR using primers 5'-GGTGGACCTGTGC-
CCCTCATTACTTC-3' and 5' TCACATTGTGTGTTGGAATTTGC-
TCC-3', which added SalI and SpeI 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 resul-
tant 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. Formal-
dehyde was then added up to a final concentration of 4% (w/v)
and maintained for 10 min at room temperature. Cells were then
centri-
fuged, 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 fluores-
cence 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 [α-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 (10^6 conidia
ml^-1) for 9 h. Cultures were treated for 1 h with the following condi-
tions: untreated control, oxidative stress (0.4 mM H2O2) and
osmotic stress (0-3 M NaCl). Mycelia were collected by centrifuga-
tion, 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

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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-oxodicarboxylate 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(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 HOGL overexpression does not enable pbs2Δ cells to grow on high-salt media (Bell et al., 2001). Taking advantage of this phenotype, a hyperactive *HOG1*F318S allele was identified since it rescued Δpbs2 cells on high osmolality medium (Kojima et al., 2004) and the filamentous fungus *T. harzianum* hog1 gene grew profusely when *hog1*+ plus the *hog1*F318S allele allowed *hog1Δ* cells to grow under osmotic shock.

**T. harzianum** 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 (1-2 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 (Fig. 2). Only extracts obtained from mycelia grown in MM supplemented with glucose and ammonium showed an almost undetectable signal.

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.

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*F318S allele overexpressed, and a knockdown *hog1*-silenced strain, since gene deletion has not been established in *T. harzianum* CECT 2413. The latter strain was obtained by the use of a hairpin construct (with inverted repeats separated by an intron) that provokes gene silencing mediated by small RNAs (siRNA) (Fig. 4a).

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 carrying the *hog1*F318S allele were isolated and checked by Southern blotting. Some of them had integrated one copy of the construct and were chosen for further analysis; only FS12 is shown here. The selection of silenced mutants was performed by isolating small colonies from transformation plates. A strain (SHM1) that showed one integration event and no disruption of the endogenous *hog1* gene was chosen for further characterization.

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 SHM1 showed decreased, although detectable, levels of *hog1* mRNA. When this was quantified, strain SHM1 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 SHM1 was analysed under basal and hyperosmotic shock conditions.

Phenotypic analysis of strains FS12 and SHM1

The phenotypes of wild-type, FS12 and SHM1 strains were studied using a drop assay (Fig. 5). The two mutants grew, developed and conidiated like the wild-type. Strain SHM1 showed reduced resistance to hyperosmotic shock (1-2 M sorbitol) and salt stress (1 M NaCl). Lower effects were
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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: $S$ (bits) = $\hat{\lambda} \times S$ (raw) − $\ln K$/$\ln 2$, where $\hat{\lambda}$ 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 (Rodríguez-Hernández 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 conidiate on the surface of the phytopathogenic fungi. Moreover, a slight clearing hallus was observed in the interaction zone, suggesting a role of ThHog1 in those 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 whether 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 \textit{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 \textit{T. harzianum}.

**DISCUSSION**

In this paper we describe the stress response of \textit{T. harzianum}. We have characterized the \textit{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 \textit{T. harzianum}. Overexpression of \textit{T. harzianum hog1} and \textit{hog1}\textsuperscript{F315S} alleles in \textit{Δhog1} yeasts resulted in enhanced osmotic resistance, contrasting with yeast \textit{HOG1} and \textit{HOG1}\textsuperscript{F318S} gene overexpression, which caused lethality under osmotic shock. The presence (in yeast) or absence (in \textit{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 \textit{hog1} silenced mutants under oxidative or heavy metal stress. This situation is similar to that of baker’s yeast. \textit{S. cerevisiae hog1Δ}

![Fig. 3. Localization of the Hog1-GFP fusion protein. Twenty-four-hour germlings of the \textit{T. harzianum} strain carrying the Hog1-GFP fusion were grown for 1 h under different conditions: MM (minimal medium with glucose and ammonium sulphate, as control); MM + 1M sorbitol; MM + 10mM H2O2. Cells were then fixed with formaldehyde and the nuclei stained with DAPI (see Methods). Fluorescence of DAPI and the GFP fusion (HOG-GFP) was examined. The Nomarski technique was used as a control.](image-url)
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 _sty1_/spc1 inactivates the response against different stresses (Degols & Russell, 1997; Degols et al., 1996; Shiozaki & Russell, 1997).

Although the silencing of _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 _T. harzianum_ to control oxidative stress is provided by cross resistance experiments. The wild-type, silenced mutant SHM1 and strain FS12 showed a similar 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 ThHog1F315S protein (in strain FS12), but also activated by another kinase(s) when ThHog1 is absent (in strain SHM).

**Fig. 4.** Mutant strains of _T. harzianum_. (a) Constructs used to transform _T. harzianum_ CECT 2413: pLMRS3 : _hog1<sup>F315S</sup>_, carrying the pyruvate kinase promoter from _T. reesei_ controlling the expression of _hog1<sup>F315S</sup>_ allele; pSILHOG, containing inverted repeats of the 5′ end of the coding sequence of _hog1_ (black bar in pLMRS3 : _hog1_ construct) separated by an intron. The strong _tss1_ promoter was used to express the hairpin construct to provoke _hog1_ silencing. (b) Northern blot analysis. Wild-type (wt), the strain carrying pLMRS3 : _hog1<sup>F315S</sup>_ (FS12) and the silenced strain (SHM1) were grown for 16 h in TSB, then washed and inoculated in minimal medium (MM), and MM supplemented with 1·2 M sorbitol (Sor), and grown for 1 h. RNA was blotted onto nylon membranes and hybridized with _hog1_ probe and with 18S rRNA as loading control. (c) Western blot analysis of intracellular proteins extracted as indicated in (b). Proteins were blotted and membranes analysed with specific antibodies against the phosphorylated TGY domain of p38 MAPK. The arrow indicates the signal revealed with anti-p38 antibodies. Coomassie blue (CB) staining was used as loading control.
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 Ca^{2+} pathways acting over common processes (Maeta et al., 2005), since only overexpression of hog1F315S can restore, at least in

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**Fig. 5.** Phenotypic analysis of hog1 mutant strains. (a) Three microlitres of conidial suspensions (2 × 10^7 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 CuSO_4 (MM+Cu); MM plus 10 mM H_2O_2 (MM+H_2O_2) 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 H_2O_2, ■). Cells were then washed and treated with increasing H_2O_2 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.

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 Ca^{2+} pathways acting over common processes (Maeta et al., 2005), since only overexpression of hog1F315S can restore, at least in
part, the normal growth inhibited by CsA. Moreover, the silenced strain was highly affected by CsA.

Hog1 kinases have been naturally 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 Tuzdinsky, 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*. THog1 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 THog1 for some parasitic interactions. The phenotype observed for strain FS12 can be explained if the *hog1* F351S 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.

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