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 *hog1*^{F315S} 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 *hog1*^{F315S} 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 *hog1*^{F315S} 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.

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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.*,

Abbreviations: CsA, cyclosporin A; DAPI, 4',6-diamidino-2-phenylindole; EST, expressed sequence tag; MAPK, mitogen activated protein kinase.

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; Motoyama *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 & Penttilä, 1998), which makes this genus a unique source to identify polymer-degrading enzymes with industrial value (Penttilä, 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). Yeasts 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 0.5% (w/v) ammonium sulphate (Penttilä *et al.*, 1987). When indicated, 10 µg cyclosporin A ml⁻¹ (Calbiochem), 1.2 M sorbitol, 1 M NaCl, 10 mM H₂O₂, 40 mM CuSO₄ 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. Drop assays were performed using 3 µl drops containing 2 × 10⁷ spores ml⁻¹ and tenfold serial dilutions. *T. harzianum* was transformed using the PEG-based protocol (Penttilä *et al.*, 1987). For antifungal ability tests, fungal strains were previously grown for 7 days on PDA plates. Plugs 6 mm in diameter were inoculated on fresh PDA plates and incubated at 25 °C for 7 days to observe overgrowth.

Mycelia for both Northern and array experiments and the preparation of intracellular extracts were obtained in two steps as follows. One hundred millilitres of TSB medium (Tryptic Soy Broth, Merck) was inoculated with a spore suspension (final concentration: 10⁶ spores ml⁻¹) and incubated for 16 h. Mycelia were filtered, and used to

reinoculate fresh MM as described for each experiment. Unless otherwise 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'-CTCGTAAGGATGGAGGATCGCAGCGGG-3' and commercial adaptor primer were used, and the band obtained was re-amplified using the nested specific primer 5'-GGATCGCAGCG-GGAGGATACGAGTAAA-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::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^{F315S} (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'-GGAAGCTTCATCACGAGTCAAATCGATCC-3' and 3'-GGGCGGCCGCTCCGCTGTCCATTTCATCA-5', and cloned in pES86 using *Hind*III and *Not*I enzymes (sites underlined). Using the resulting pES86-*hog1* as a template, pES86-*hog1*^{F315S} was obtained using the QuickChange Mutagenesis kit (Stratagene). Primers used in mutagenesis were 5'-GCCGATGAGAAGTCTGACTGGAGCTTTA-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 pLMRS3 (Mach *et al.*, 1994). The genomic clone of *hog1* was amplified using primers 5'-GGTCTAGAGCCATCACGAGTCAAATCGATC-3' and 5'-GGATGCATAGGTCCGCTGTTCATTTCATTC-3', which introduced *Xba*I and *Nsi*I sites, respectively (underlined). Using the resulting pLMRS3-*hog1* as a template, pLMRS3-*hog1*^{F315S} was obtained using the QuickChange Mutagenesis kit (Stratagene) with primer 5'-GCCGATGAGAAGTCTGACTGGAGCTTTA-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 *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'-GGAAGCTTACTAGTAGATCTTTGGCACCACGTTC-3' and 5'-GGGAATTCGGATCCGCTGTAGAAGGCATGTTAG-3', which introduced *Hind*III and *Spe*I sites and *Eco*RI and *Bam*HI sites, respectively (underlined and italicized). The fragment in direct orientation was cloned using *Spe*I and *Bam*HI sites, whereas an inverted repeat was introduced using *Eco*RI and *Hind*III 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 radiation (125 mJ) 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 composed of RNAs obtained by *in vitro* transcription of some plant-specific genes. RNA was isolated as described under 'DNA and RNA hybridizations'. For macroarray hybridization, the probe was prepared 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 × 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 × 12 cm membrane. The radioactive probe was added appropriately diluted to obtain 5 × 10⁵ 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 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 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, tosyllysine 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'-GGTCGACCTGTGTC-CCTTCATTACTTC-3' and 5' TCACTAGTTTGTGTGGGGAATTGC-TCC-3', which added *Sall* and *SpeI* sites, respectively (underlined). These enzymes were used to clone the fragment into pZEGA1 vector (Zeilinger *et al.*, 2003) previously cut with *XhoI* and *XbaI*. The resultant vector pHog1-GFP was used to transform *T. harzianum* using a PEG-based protocol (Penttilä *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× 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 (10⁶ 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-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/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 *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 osmolarity 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*^{F315S}) showed the same phenotype. As expected, *hog1Δ* yeasts transformed with pES86-*HOG1*^{F318S} did not grow in YNB+0.4 M NaCl, whereas strains transformed with pES86-*hog1*^{F315S} grew copiously even at higher salt concentrations (Fig. 1). Hence, in contrast to the yeast allele *HOG1*^{F318S} the *T. harzianum* *hog1*^{F315S} 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*^{F315S} 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*^{F315S} 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

Table 1. ESTs induced after 2 h growth in MM containing 1·2 M sorbitol

Induction level*	EST			Similarity detected by BLAST analysis of ESTs†				
	Sequence code	bp	Acc. no.	Acc. no.	Organism	Putative function	Score‡	E-value§
> 100	L06T34P029R02710	685	AJ898191	emblCAD21180·11	<i>Neurospora crassa</i>	Serine/threonine protein kinase NRC-2	415	e-115
> 100	L06T34P031R02920	655	AJ898372	gb AAS76670·11	<i>Trichophyton rubrum</i>	Leucine aminopeptidase 2	114	2e-24
> 100	L91T34P045R04188	613	AJ919183	emblCAF05963·11	<i>Neurospora crassa</i>	Related to transcription initiation factor	115	9e-25
> 100	L06T34P031R02954	675	AJ898399	gb AAQ04633·11	<i>Paracoccidioides brasiliensis</i>	Cell cycle regulator protein Clf1	297	2e-79
> 100	L06T34P029R02707	659	AJ898188	gb AAL08510·11	<i>Ophiostoma piliferum</i>	Subtilase	291	1e-77
> 100	L06T34P030R02792	700	AJ898267	emblCAA39364·11	<i>Hypocrea jecorina</i>	Orotate phosphoribosyltransferase	239	5e-62
> 100	L06T34P030R02794	656	AJ898269	gb EAL86518·11	<i>Aspergillus fumigatus</i>	Chromatin remodelling complex subunit (Arp9)	110	2e-23
> 100	L03T34P023R02176	695	AJ896946	gb EAL86188·11	<i>Aspergillus fumigatus</i>	Monosaccharide transporter	207	3e-52
> 100	L06T34P029R02713	546	AJ898194	gb EAL89460·11	<i>Aspergillus fumigatus</i>	ATP-dependent RNA helicase, putative	261	7e-69
> 100	L06T34P031R02902	734	AJ898356	gb EAL89621·11	<i>Aspergillus fumigatus</i>	Oxidoreductase, zinc-binding dehydrogenase family	132	8e-30
> 100	L06T34P029R02703	369	AJ898184	gb EAA77489·11	<i>Gibberella zeae</i>	Hypothetical protein FG07472.1	48	8e-005
> 100	L02T34P026R02420	487	AJ894575	gb EAL91198·11	<i>Aspergillus fumigatus</i>	Glucose-repressible gene protein-related protein	65	7e-010
> 100	L06T34P030R02818	664	AJ898290	gb EAA77567·11	<i>Gibberella zeae</i>	Hypothetical protein FG07334.1	186	4e-46
> 100	L06T34P029R02729	756	AJ898208	gb AAB58299·11	<i>Hypocrea virens</i>	Carbamoyl phosphate synthetase, small subunit	421	e-117
> 100	L02T34P013R01198	661	AJ893972	gb EAA78739·11	<i>Gibberella zeae</i>	Conserved hypothetical protein	375	e-103
10-20	L06T34P029R02715	655	AJ898196	gb EAL85453·11	<i>Aspergillus fumigatus</i>	K ⁺ /H ⁺ antiporter, putative	94	2e-18
10-20	L03T34P016R01470	730	AJ896746	No hits found				
10-20	L02T34P054R05008	735	AJ894944	emblCAI67598·11	<i>Hypocrea lixii</i>	Glucosylase	411	e-114
10-20	L02T34P013R01182	299	AJ893956	dbj BAA01859·11	<i>Saccharomyces cerevisiae</i>	Cks1 protein	82	5e-15
10-20	L05T34P051R04715	633	AJ897844	gb EAL93520·11	<i>Aspergillus fumigatus</i>	Cytochrome c oxidase assembly protein Cox11	143	3e-33
10-20	L05T34P051R04776	695	AJ897903	emblCAE76503·11	<i>Neurospora crassa</i>	Probable ribosomal protein L35	197	2e-49
5-10	L05T34P051R04763	703	AJ897891	gb EAL90014·11	<i>Aspergillus fumigatus</i>	Cytosolic large ribosomal subunit L11, putative	294	1e-78
5-10	L02T34P013R01227	681	AJ893999	emblCAB99174·11	<i>Neurospora crassa</i>	Related to GTP-binding protein Rab2	275	1e-72
5-10	L02T34P054R05051	675	AJ894980	gb EAL88455·11	<i>Aspergillus fumigatus</i>	Autophagic death protein (Idi-7), putative	232	6e-60
5-10	L03T34P075R07048	599	AJ897783	No hits found				
5-10	L08T34P062R05757	699	AJ899490	gb EAL88981·11	<i>Aspergillus fumigatus</i>	ABC transporter, putative	206	6e-52
5-10	L06T34P031R02926	712	AJ898378	gb EAA69900·11	<i>Gibberella zeae</i>	Hypothetical protein FG02621.1	143	5e-33
5-10	L10T34P073R06887	551	AJ900032	gb EAL89736·11	<i>Aspergillus fumigatus</i>	CSL family zinc-finger-containing protein	107	1e-22
5-10	L02T34P020R01850	693	AJ894410	emblCAE85545·11	<i>Neurospora crassa</i>	Related to translation initiation factor eI	252	6e-66
5-10	L06T34P032R03066	574	AJ898485	gb EAA76320·11	<i>Gibberella zeae</i>	RS7_NEUCR 40S ribosomal protein S7	256	3e-67
5-10	L05T34P051R04760	690	AJ897888	dbj BAB85760·11	<i>Fusarium oxysporum</i>	Putative mitochondrial carrier protein	364	1e-99
5-10	L05T34P051R04747	703	AJ897875	gb AAX07669·11	<i>Magnaporthe grisea</i>	Succinyl-CoA ligase beta-chain-like protein	358	8e-98
2-5	L02T34P055R05095	599	AJ895019	gb EAL89660·11	<i>Aspergillus fumigatus</i>	Fructose-1,6-bisphosphatase	143	3e-33
2-5	L10T34P072R06754	692	AJ899902	gb EAL92207·11	<i>Aspergillus fumigatus</i>	Cation-transporting ATPase, putative	133	4e-30
2-5	L10T34P072R06753	628	AJ899901	ref NP_967878·11	<i>Bdellovibrio bacteriovorus</i>	ThiJ/PfpI family protein	96	1e-18
2-5	L02T34P004R00295	464	AJ893779	emblCAD21258·11	<i>Neurospora crassa</i>	Related to Glc8 protein	112	4e-24
2-5	L05T34P051R04775	611	AJ897902	gb EAL91198·11	<i>Aspergillus fumigatus</i>	Glucose-repressible gene protein-related protein	105	7e-22
2-5	L02T34P013R01183	297	AJ893957	gb EAL92477·11	<i>Aspergillus fumigatus</i>	SNI2 protein	84	1e-15
2-5	L05T34P052R04820	633	AJ897944	gb EAA76043·11	<i>Gibberella zeae</i>	Hypothetical protein FG07070.1	211	2e-53

Table 1. cont.

Induction level*	EST			Similarity detected by BLAST analysis of ESTs†				
	Sequence code	bp	Acc. no.	Acc. no.	Organism	Putative function	Score‡	E-value§
2–5	L03T34P022R02080	360	AJ896869	emblCAC28672·11	<i>Neurospora crassa</i>	Glucose-repressible protein Grg-1	85	8e–16
2–5	L91T34P045R04199	650	AJ919191	No hits found				
2–5	L06T34P032R03046	423	AJ898470	gbLEAA68083·11	<i>Gibberella zeae</i>	RL44_PICJA 60S ribosomal protein L44 (L41)	221	7e–57
2–5	L06T34P030R02847	601	AJ898315	gbLEAA72805·11	<i>Gibberella zeae</i>	Hypothetical protein FG04424.1	213	4e–54
2–5	L03T34P014R01335	467	AJ896711	gbLEAA76695·11	<i>Gibberella zeae</i>	Hypothetical protein FG09376.1	70	3e–11
2–5	L91T34P046R04274	677	AJ919246	emblCAE85513·11		Related to DNA mismatch repair protein PMS1	183	3e–45
2–5	L10T34P076R07157	667	AJ900107	gbLEAL93821·11	<i>Aspergillus fumigatus</i>	Small oligopeptide transporter, OPT family	155	1e–36
2–5	L10T34P076R07101	667	AJ900056	emblCAC28847·21	<i>Neurospora crassa</i>	Related to HFB30	307	2e–82
2–5	L02T34P020R01920	679	AJ894457	No hits found				
2–5	L10T34P077R07256	608	AJ900194	emblCAD70936·11	<i>Neurospora crassa</i>	Related to cleft lip and palate transmembrane	186	3e–46
2–5	L06T34P029R02700	536	AJ898182	gbLEAL91000·11	<i>Aspergillus fumigatus</i>	Fatty acid elongase (Gig30), putative	84	3e–15
2–5	L10T34P072R06724	704	AJ899872	gbLEAL90295·11	<i>Aspergillus fumigatus</i>	Mitochondrial 2-oxodicarboxylate carrier protein	299	4e–80
2–5	L03T34P014R01254	665	AJ896635	gbLEAL86695·11	<i>Aspergillus fumigatus</i>	Ser/Thr protein phosphatase family	227	2e–58
2–5	L06T34P031R02959	691	AJ898404	gbLEAA78762·11	<i>Gibberella zeae</i>	Hypothetical protein FG11590.1	266	3e–70
2–5	L05T34P051R04764	681	AJ897892	gbLEAL85583·11	<i>Aspergillus fumigatus</i>	Elongation factor 1-gamma 2	233	3e–60
2–5	L02T34P003R00211	685	AJ893718	gbLEAA70102·11	<i>Gibberella zeae</i>	Hypothetical protein FG10259.1	90	6e–17
2–5	L03T34P016R01468	673	AJ896744	No hits found				
2–5	L10T34P076R07132	515	AJ900085	No hits found				
2–5	L02T34P001R00060	407	AJ893596	gbLEAL92964·11	<i>Aspergillus fumigatus</i>	Glutaryl-CoA dehydrogenase	181	8e–45
2–5	L91T34P046R04287	666	AJ919256	gbLEAA73743·11	<i>Gibberella zeae</i>	Hypothetical protein FG06252.1	153	5e–36
2–5	L03T34P059R05469	554	AJ897207	gbLEAA74083·11	<i>Gibberella zeae</i>	Hypothetical protein FG05206.1	48	2e–04
2–5	L03T34P023R02114	666	AJ896900	gbLAAD16095·11	<i>Aspergillus oryzae</i>	Phosphatidylglycerol/phosphatidylinositol transfer protein	149	5e–35
2–5	L02T34P050R04607	681	AJ894750	gbLEAA48690·11	<i>Gibberella zeae</i>	Hypothetical protein MG00348.4	51	3e–05
2–5	L08T34P063R05842	612	AJ899574	No hits found				
2–5	L02T34P004R00319	697	AJ893796	emblCAE09055·11	<i>Gibberella zeae</i>	Cytochrome P450 oxidoreductase	327	2e–88
2–5	L02T34P054R05069	699	AJ894995	gbLEAA74999·11	<i>Gibberella zeae</i>	Hypothetical protein FG10742.1	268	1e–70
2–5	L03T34P060R05584	693	AJ897313	emblCAC27396·11	<i>Podospora anserina</i>	Alternative oxidase	323	3e–87
2–5	L91T34P045R04191	671	AJ919185	gbLAO32939·11	<i>Neurospora crassa</i>	TIM50	345	8e–94
2–5	L02T34P057R05358	710	AJ895274	gbLEAL88570·11	<i>Aspergillus fumigatus</i>	Actin-binding protein, putative	333	2e–90
2–5	L03T34P022R02092	669	AJ896880	gbLEAA77790·11	<i>Gibberella zeae</i>	CT1B_Fusso cutinase transcription factor 1 B	341	1e–92
2–5	L10T34P072R06790	635	AJ899937	gbLEAA77138·11	<i>Gibberella zeae</i>	Hypothetical protein FG09581.1	230	2e–59
2–5	L10T34P076R07178	578	AJ900122	gbLAAX55622·11	<i>Hypocrea lixii</i>	Small heat-shock protein	141	1e–32
2–5	L03T34P016R01467	566	AJ896743	gbLAAB28355·11	<i>Neurospora crassa</i>	NAD ⁺ -specific glutamate dehydrogenase; NAD	180	2e–44
2–5	L03T34P022R02091	352	AJ896879	gbLEAA47045·11	<i>Magnaporthe grisea</i>	Hypothetical protein MG10856.4	75	6e–013

*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: $S' \text{ (bits)} = [\lambda \times S \text{ (raw)} - \ln K] / \ln 2$, where λ 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.

Fig. 1. Complementation analysis of the *hog1* gene in budding yeast. Yeasts were transformed with: empty vector pES86 (vector); pES86:*HOG1* vector, carrying the *HOG1* gene from *S. cerevisiae* (*HOG1*); pES86:*HOG1*^{F318S} vector, carrying the *HOG1*^{F318S} allele from *S. cerevisiae* (*HOG1* F318S); pES86:*hog1* vector, carrying the *hog1* gene from *T. harzianum* (*hog1*); and pES86:*hog1*^{F315S} vector, carrying the *hog1*^{F315S} allele from *T. harzianum* (*hog1* F315S). Where indicated, YNB plates were supplemented with 0.4 M or 0.8 M NaCl.

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

Fig. 2. Western blot analysis of phosphorylation state. Protein extracts obtained as described in Methods were separated by SDS-12% PAGE and blotted onto membranes. Antibodies specific for the phosphorylation state of p38-related MAPK were used. An identical gel was stained with Coomassie blue (CB) as loading control. Conidia were grown for 16 h in TSB medium. Germlings were filtered and inoculated into different fresh minimal media: MM supplemented with glucose and ammonium sulphate (MM); MM supplemented with 1% (w/v) colloidal chitin instead of glucose as carbon source (Chi); with 0.002% ammonium sulphate (N); without phosphate source (P); with 0.1% glucose (C); or MM supplemented with 1.2 M sorbitol (Sor); 1 M NaCl (NaCl); 10 mM H₂O₂ (H₂O₂); 40 mM CuSO₄ (Cu) or at pH 10 (pH). Incubation was continued for 1 h before protein extraction.

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 germings were treated for 1 h with 0.3 mM H₂O₂ or 0.4 mM NaCl. The germings were then washed and grown for 1 h in media with increasing lethal concentrations of H₂O₂. Finally, the germings were diluted and plated. The untreated wild-type cells showed an extreme sensitivity to H₂O₂, whereas germings pre-treated with NaCl showed higher resistance, which was increased in germings pre-treated with H₂O₂ (Fig. 7a). The same

Fig. 3. Localization of the Hog1-GFP fusion protein. Twenty-four-hour germlings of the *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 + 1 M sorbitol; MM + 10 mM 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.

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*. Over-expression of *T. harzianum* *hog1* and *hog1*^{F315S} alleles in Δ *hog1* yeasts resulted in enhanced osmotic resistance, contrasting with yeast *HOG1* and *HOG1*^{F318S} gene over-expression, 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* *hog1* Δ

Fig. 4. Mutant strains of *T. harzianum*. (a) Constructs used to transform *T. harzianum* CECT 2413: pLMRS3:*hog1*^{F315S}, carrying the pyruvate kinase promoter from *T. reesei* controlling the expression of *hog1*^{F315S} 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*^{F315S} (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.

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 *styl1/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 ThHog1^{F315S} protein (in strain FS12), but also activated by another kinase(s) when ThHog1 is absent (in strain SHM).

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, $\blacklozenge$), or pretreated for 1 h with osmotic stress (0.3 M NaCl, $\blacktriangle$) or low-dose oxidative stress (0.4 mM H_2O_2 , $\blacksquare$). 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 *hog1*^{F315S} 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 *hog1*^{F315S} 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 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*^{F315S} 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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