A MAPK gene from Dead Sea fungus confers stress tolerance to lithium salt and freezing–thawing: Prospects for saline agriculture

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The Dead Sea is one of the most saline lakes on earth (≈ 340 g/liter salinity) and is ≈ 10 times saltier than the oceans. *Eurotium herbariorum*, a common fungal species, was isolated from its water. EhHOG gene, encoding a mitogen-activated protein kinase (MAPK) that plays an essential role in the osmoregulatory pathway in yeast and many other eukaryotes, was isolated from *E. herbariorum*. The deduced amino acid sequences of EhHOG indicated high similarity with homologous genes from *Aspergillus nidulans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* and contained a TGY motif for phosphorylation by MAPK kinase. When EhHOG was expressed in *S. cerevisiae* *hog1* Δ mutant, the growth and aberrant morphology of *hog1* Δ mutant was restored under high osmotic stress condition. Moreover, intracellular glycerol content in the transformant increased to a much higher level than that in the mutant during salt-stress situations. *hog1* Δ mutant complemented by EhHOG outperformed the wild type or had higher genetic fitness under high Li^+ and freezing–thawing conditions. The present study revealed the putative presence of a high-osmolarity glycerol response (HOG) pathway in *E. herbariorum* and the significance of EhHOG in osmotic regulation, heat stress, freeze stress, and oxidative stress. The Dead Sea is becoming increasingly more saline while the fungi living in it evolutionarily adapt to its high-saline environment, particularly with the extraordinarily high Li^+ concentration. The Dead Sea is potentially an excellent model for studies of evolution under extreme environments and is an important gene pool for future agricultural genetic engineering prospects.

Eurotium herbariorum | HOG | salinity | genetic resources | crop improvement

The Dead Sea is one of the most saline lakes on earth (salinity ≈ 340 g/liter). The studies on the biology of the Dead Sea revealed a variety of microorganisms including red halophilic Archaea, unicellular green algae (*Dunaliella parva* Lerche), different types of bacteria, and possibly even protozoa (1). Recently, filamentous fungi were isolated from surface water to 300 m down in the Dead Sea (2). Although the isolated fungi did not grow in undiluted Dead Sea samples, they showed a remarkable salt tolerance and, in many cases, even had a requirement for high salt concentrations, making them halophilic. *Eurotium herbariorum* is the most common species isolated from the Dead Sea water, from the surface to a depth of 300 m in all investigated seasons (3). All of these organisms need to adapt to the extremely high salinity of the Dead Sea brines.

Exposure to high environmental osmolarity leads to dehydration in microorganisms; thus, consequently, cell viability decreases. To cope with this challenge, the cells of both prokaryotic and eukaryotic microorganisms have developed mechanisms to adapt to severe osmotic changes in their environments, often called osmoregulation. To adapt to salt stress, microorganisms balance high external osmotic pressure by synthesizing and/or accumulating low-molecular-mass compounds, which are compatible with cellular function and do not inhibit enzymes (4). Increased synthesis and/or accumulation of glycerol and other

compatible solutes, mainly polyols, have been the major feature of fungi osmoregulation (5).

Mitogen-activated protein kinase (MAPK) is a key component of the evolutionarily conserved signal transduction cascades consisting of MAPK/extracellular signal-related kinase (ERK) activated by an MAPK/ERK kinase (MEK), which in turn is activated by an MEK kinase (6). Eukaryotic organisms use different MAPK cascades to regulate various aspects of cellular function (7, 8). MAPKs that specifically transmit environmental stress signals are also known as stress-activated protein kinases (SAPKs). This pathway is called the high-osmolarity glycerol (HOG) response pathway in *Saccharomyces cerevisiae* (9). Members of this MAPK subfamily include Hog1 in *S. cerevisiae*, Spc1 (also called Sty1) in *Schizosaccharomyces pombe*, SakA in *Aspergillus nidulans*, and p38/JNK in the mammals. Indeed, *S. cerevisiae* *hog1* mutants are sensitive to high osmolarity, whereas *spc1* mutations in *S. pombe* result in sensitivity to high osmolarity, heat shock, and oxidative stress. Activation of the HOG pathway increases the transcription of some proteins, including enzymes involved in glycerol synthesis (10). As a result, a high accumulation of glycerol inside the cell occurs and leads to increased internal osmolarity and restores the osmotic gradient between the cells and their environment (11). Therefore, HOG1 gene holds a key position in osmo-adaptation of the yeast *S. cerevisiae*.

The presence of HOG1 homologous genes has been reported in fungal species (12, 13) and mammals (14), indicating that this pathway is conserved among eukaryotes. However, no information is available for the HOG pathway or the molecular mechanism of stress tolerance in *E. herbariorum*. Here, we cloned the gene EhHOG of the filamentous fungus *E. herbariorum* from the Dead Sea and then transformed it into a wild-type (WT) and *hog1* mutant of the baker's yeast *S. cerevisiae*. The expression of EhHOG functionally complemented *S. cerevisiae* *hog1* mutant, and, remarkably, the complemented *hog1* mutant displayed higher tolerance to several stresses than that of the WT strain of *S. cerevisiae*. Consequently, we propose the genetic resource of the Dead Sea fungi as a potentially promising resource to improve salt tolerance in other organisms in relation to various biological systems, including saline agriculture.

Materials and Methods

Fungus, Yeast Strains, and Culture Conditions. *E. herbariorum*, isolated from the Dead Sea water, was cultured in 1% yeast extract/10% glucose medium (GY) at 25°C (3). *Escherichia coli*

Conflict of interest statement: Carmel-Haifa University Economic Corp. Ltd. has filed for a patent on EhHog in the names of Y.J., S.W., and E.N.

Abbreviations: ERK, extracellular signal-related kinase; HOG, high-osmolarity glycerol; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ229154).

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strain XL/B was used for transformation and plasmid propagation. *E. coli* XL/B was grown in Luria–Bertani (LB) medium at 37°C for plasmid manipulation. The *S. cerevisiae* strains used in this study were WT YSH6.142–3A (*MATa leu2–3/112 ura3–1 trp1–1 his3–11/15 ade2–1 can1–100 GAL SUC2 mal0*) and YSH444 (*MATa hog1Δ::TRP1* mutant) (10). Yeast cells were grown in a rich medium containing 1% yeast extract, 2% peptone, and 2% dextrose (YPD) or in a minimal medium containing 0.67% yeast nitrogen base and 2% (wt/vol) glucose (SD) supplemented with required amino acids on a rotary shaker at 30°C (15). Agar (1.5%) was added to these media when required.

DNA and RNA Analysis. Mycelia of *E. herbariorum* cultured in GY medium for 2 weeks and harvested by filtration were then soaked in fresh GY medium with 2 M NaCl for various periods of time. Total RNA was prepared by using the Total RNA Isolation Kit (Beit Haemek, Ashrat, Israel). DNA from *E. herbariorum* was isolated and purified by modifying the Raeder and Broda method (16). Genomic DNA of yeast was isolated according to basic protocol (17). For Southern blot analysis, 10 µg of high-purity genomic DNA was digested with enzymes EcoRI, HindIII, and BamHI (New England Biolabs) and then transferred to a Hybond N nylon membrane (Amersham Pharmacia). Southern hybridizations (60°C) were carried out as described by Sambrook *et al.* (18). For the probe, an EhHOG-containing DNA fragment (~800 bp) was obtained by PCR amplification with oligonucleotides 5'-AAGAAGATTATGAAGCCTTTCAGC-3' and 5'-CATAATTTTCCATGTGTGCGACCGG-3' as the primers and was labeled with [³²P]dCTP by a random primer-labeling kit (Beit Haemek). For Northern blot analysis, the method of hybridization (68°C) was the same as for Southern blots.

Isolation of EhHOG Gene from *E. herbariorum*. To isolate the HOG gene from *E. herbariorum*, PCR was performed by using genomic DNA as the template and sequences 5'-ATGGCGGAATTCGTGCGTGCCACGATT-3' and 5'-GGCCGCGAATGCTGCTGGCCATCCCC-3' as the primers for 35 cycles each consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The amplified fragments were subcloned into pGEM-T easy (Promega) and sequenced. To obtain full-length HOG cDNA, RT-PCR was performed. For RT-PCR, two HOG-specific primers (5'-CAAAGCTTATGGCGGAATTCGTGCGTGCCACGATT-3' and 5'-GGCCGCGAATGCTGCTGGCCATCCCC-3') were synthesized. First-strand cDNA synthesis was performed in a 20-µl reaction mixture according to cDNA Synthesis system (Promega). A 1-µl sample of first-strand cDNA products was then used as template DNA in PCR for *E. herbariorum* HOG cDNA. PCR amplification was performed with 50-µl reaction that contained 5 µl of 10× buffer, 0.2 mM dNTPs (2 µl of 5 mM dNTPs), 1 µl each of 10 µM HOG-specific primer, and 2 units of Taq polymerase (Biotools, Madrid) for 30 cycles each consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The amplified fragments were subcloned into pGEM-T Easy and sequenced.

Phylogenetic Analysis. Phylogenetic trees based on the amino acid sequences alignment of 36 MAPKs were constructed by using the neighbor-joining method (19) implemented in MAGA2.1 (20). Reliability of the tree topology was evaluated by bootstrap analysis of 1,000 replicates. The resulting estimated amino acid distances were corrected for multiple amino acid substitutions per single site by Poisson correction.

Function Expression of EhHOG in *S. cerevisiae*. For expression of EhHOG in *S. cerevisiae*, 1.1-kb full-length EhHOG cDNA was inserted into HindIII/NotI sites under transcriptional control of

the promoter for alcohol dehydrogenase (ADH) in plasmid pADNS resulting in plasmid pADNS–EhHOG. Plasmid pADNS harboring the *S. cerevisiae* HOG1 coding sequences was obtained by subcloning HindIII–NotI fragments, which were obtained by amplification from *S. cerevisiae* genomic DNA through primers 5'-ACAAAGCTTATGACCACTAACGAGGAATT-3' and 5'-CTGGCGGCCGCTTACTGTTGGAATCATTAG-3'. Transformation of yeast cells with pADNS–EhHOG was performed by the lithium acetate/single-stranded carrier DNA/poly(ethylene glycol) methods (21).

Growth Assays. The *EhHOG* gene was expressed in *S. cerevisiae* WT strain YSH6.142–3A and mutant YSH444 (*MATa hog1Δ::TRP1*), which lacks *HOG1*. Cultures of strains transformed with pADNS or pADNS–EhHOG were grown in SD–LEU medium and YPD medium in the presence or absence of various concentrations of NaCl, LiCl, and sorbitol. For the growth test, cells were grown at 30°C for 36 h; serial dilution, 1/10, was made at each step. From each dilution, 10 µl was spotted on different media and incubated at 30°C for 3–5 days. At various time intervals, aliquots of the cultures were measured by optical density at 600 nm. For heat stress, cells were grown to OD₆₀₀ 1.8–2. Serial dilutions of 10 times were made. Ten microliters of each dilution was spotted on solid YPD plates. Cells on YPD plates were incubated at 42°C for 1 day and allowed to recover at 28°C for 36 and 69 h; for 2-day heat stress, cells were recovered at 28°C for 48 h. Freeze-stress tolerance of cells was determined by measuring viability after the number of freeze–thaw cycles (–30°C and room temperature). Percent survival was expressed relative to the initial viability before freezing. For oxidative stress, cells were grown to OD₆₀₀ 1.0–1.5 in SD medium at 30°C and then incubated for 1.5 h with increasing concentrations of H₂O₂ in SD. Percent survival was expressed relative to the initial viability before oxidative stress. Two independent transformants were included, and three independent experiments were performed.

Assays of Intercellular Glycerol Content in Various *S. cerevisiae* Strains. Cells were cultured at 30°C in SD media and harvested at the early exponential phase (OD₆₀₀ = 0.5–0.8). Subsequently, cells were resuspended in a new media with or without 0.4 M NaCl. After incubation for 1 h at 30°C, cells were harvested and prepared as described by André *et al.* (22). The glycerol content was determined according to the application manual of the glycerol-F kit (Roche Molecular Biochemicals).

Results and Discussion

Isolation and Characterization of the *EhHOG* from *E. herbariorum*. Successful amplification of *HOG1* homolog in *E. herbariorum* was achieved with primers derived from conserved regions of *HOG1* from various fungal species available, with flanking regions obtained by chromosome walking (23). Sequence analysis revealed a long ORF interrupted by eight introns, whose positions were confirmed by sequencing cDNA and genomic DNA clones (see Fig. 8, which is published as supporting information on the PNAS web site). This ORF predicted 366-amino acid residues with protein masses of 41.62 kDa, which were highly similar to kinase from the stress-activated MAPK family. Therefore, the corresponding gene was named *EhHOG* (for *E. herbariorum* HOG). The EhHOG protein shows the highest identity to SakA (81% identity, 86% similarity) from *A. nidulans* and also shares a high homology to other SAPKs such as Spc1 (78% identity, 87% similarity) in *S. pombe*, HOG1 (66% identity, 72% similarity) in *S. cerevisiae*, and Mpkc (60% identity, 74% similarity) in *A. nidulans* (Fig. 1). A TGY motif, characteristic of hyperosmolarity-activated MAPKs (24), was found at amino acids 171–173 (Fig. 1). Similar motifs have been found in other yeast species (13) and also in MAPKs from higher

organisms that could survive in the Dead Sea, such as Archaea (50), *Dunaliella* (2), and filamentous fungi (2).

Our results demonstrate that *EhHOG* could perform similarly to other *HOGs* under a range of stress environments. More importantly, yeast *hog1Δ* mutant, complemented with *EhHOG*, displayed higher tolerance to some types of salts and freezing as well. Evidently, we can attribute the superior tolerance to specific sequences of *EhHOG* in contrast to other *HOGs*. These specific sequences are most likely the products of strong selection pressure from the hypersalinity progressively increasing in the Dead Sea for the last 70,000 years of the Lisan lake duration, particularly during the last 15,000 years.

In the present study, *EhHOG* functioned in a distantly related yeast species without any obvious shortcomings. It is interesting that despite considerable differences between yeast and *E. herbariorum*, we could achieve the functional expression of *EhHOG*, a *MAPK* homolog playing a central role in *HOG* signal transduction, in the yeast *S. cerevisiae*. Because Hog is well conserved from yeast to animals, there might be ample opportunity to introduce *EhHOG* into other eukaryotic genomes to improve stress tolerance. Moreover, other genes from *E. herbariorum*, as well as other organisms residing in the same environment, could prove useful for further genetic engineering efforts. It has been speculated that there is no *HOG* lineage in plants, because no plant *HOG* homolog has been found yet (29). Nevertheless, the *MAPK* homolog from *Pisum sativum* could complement the *HOG* mutant (51). It will be of great interest to see the interaction of *EhHOG* with a plant genome if stable integration can be achieved and thereby reinforce the exploitation of genetic resources of the Dead Sea fungi for crop

improvement. Our preliminary results suggest that *EhHOG* might possess the potential to play a similar role, as shown here with a yeast *hog1* mutant, in conferring better stress tolerance in transgenic *Arabidopsis* (data not shown).

Prospects. In conclusion, isolation and characterization of *EhHOG* show insight into the evolutionary strategies and genetic mechanism of high halotolerance in the Dead Sea fungus *E. herbariorum*. *EhHOG* also can enhance the resistance against multiple stress tolerances (salt, H_2O_2 , low and high temperature), which constitutes a clear advantage in agriculture. These results undoubtedly open theoretical routes for the study of Dead Sea biodiversity of filamentous fungi and other organisms (e.g., Archaea, the alga *Dunaliella*) in terms of stress genomics, proteomics, and phenomics. In addition, genetic resources in the Dead Sea also could be potentially harnessed for engineering transgenic organisms with enhanced stress tolerance, thereby advancing saline agriculture. This application is desperately relevant in view of the increasing desertification and salinization of our planet and the need for crop improvement and higher food production to overcome world hunger.

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