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Full Length Research Paper

STK1, a MAP kinase gene from *Setosphaeria turcica*, confers preferable tolerance to sodium salt stress

Po Li[1,2#], Shouqin Gu[1#], Shen Shen[1#], Jingao Dong[1]*, Min Wu[1], Meijuan Wang[1], Yang Yang[1], Changzhi Zhang[1], Yongshan Fan[1] and Jianmin Han[1]

[1]Mycotoxin and Molecular Plant Pathology Lab., Agricultural University of Hebei, Baoding 071000, Hebei Province, China
[2]Plant Protection Institute, Hebei Academy of Agriculture and Forestry Sciences, Baoding 071000, Hebei Province, China

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***Setosphaeria turcica*, a serious fungal plant pathogen that causes northern corn leaf blight, is a filamentous, heterothallic ascomycete. *STK1* gene, cloned from *S. turcica*, encodes a mitogen-activated protein kinase (MAPK) that is homologous to Hog1 in *Saccharomyces cerevisiae* and other MAP kinases related to osmoregulation in filamentous fungi. Its encoded protein, Stk1, contains all the conserved domains that characterize Hog1-homologues. *STK1* expression restores the *S. cerevisiae hog1* null mutant phenotype in the complementation test under salt and oxidative stress. The signal transduction pathway may be similar between *S. turcica* and *S. cerevisiae*, in which *STK1* plays a key function in resistance to environmental stress. Transformants with *STK1* are more tolerant of 1 M NaCl than the wild-type strains based on the growth rate test and determination of intracellular glycerol concentration.**

Key words: Mitogen-activated protein kinase (MAPK), *Setosphaeria turcica.*

INTRODUCTION

Diverse mechanisms have been employed in eukaryotic cells to determine the stress signal and activate protein expression to resists the toxic effects of the stress and promote survival and eventual cell growth under the new condition (Gasch, 2003; Nagiec and Dohlman, 2012). In yeast *Saccharomyces cerevisiae*, the pathway that responds to these changes is the high osmolarity glycerol (HOG) signaling pathway. The HOG-MAPK (mitogen-activated protein kinase) pathway in budding yeast plays an important and somewhat specialized role in adapting to hyperosmotic stress (Parmar, et al., 2011; Van Wuytswinkel et al., 2000). Similar to other organisms that synthesize and/or accumulate small-molecular-weight compounds to adapt to hyperosmotic stress, the level of intracellular glycerol increases in budding yeast when cultured with various salt solutions (Nevoigt and Stahl,1997; Torres-Quiroz et al., 2010). Therefore, the HOG pathway is considered responsible for the osmoregulation of *S. cerevisiae*, in which the *HOG1* gene plays a key role.

HOG-MAPK plays an important role and has been identified in many filamentous fungi (Smith, et al. 2010; Xu 2000). In *Neurospora crassa*, the *HOG1*-homologous *os-2* gene replacement mutant is sensitive to high osmolarity and resistant to phenylpyrrole fungicides, which stimulate intracellular glycerol accumulation in wild-type (WT) strains but not in *os-2*Δ mutants (Lew and Nasserifar, 2009; Zhang et al., 2002). In *Botrytis cinerea*, the Hog1 homologue BcSak1 is phosphorylated under osmotic stress, as well as exposure to specific fungicides and oxidative stress. In contrast to most other fungal systems, Δ*bcsak1* mutants are significantly impaired in conidiation and sclerotial development, and are unable to penetrate unwounded plant tissue (Segmuller et al., 2007). Thus, hog1 homologues play an important role in osmoregulation and are related to vegetative growth and pathogenesis in pathogenic fungi.

*Corresponding author. E-mail: shmdjg@hebau.edu.cn. Tel: +86-312-7528266. Fax: +86-312-7528266.

Po Li, Shouqin Gu and Shen Shen are the co-first authors.

Considering the well conserved sequences of MAPKs in different species, the expression of MAPKs from other organisms in yeast often play complementary roles in yeast wherein homologous MAPKs is invalid. For example, the *EhHOG* of *Eurotium herbariorum*, isolated from Dead Sea water, and the *OSM1* in *Magnaporthe grisea* functionallycomplement the osmotic sensitivity of *S. cerevisiae hog1*Δ mutant (Dixon et al., 1999; Jin et al., 2005). Slt2 in yeast can be functionally replaced with mammalian ERK5 (Truman et al., 2006). However, no information is available for the HOG pathway or the molecular mechanism of stress tolerance in *Setosphaeria turcica*. *S. turcica*, a fungal plant pathogen that causes Northern Corn Leaf Blight (NCLB), is a filamentous, heterothallic ascomycete (Fan et al., 2004). This pathogen which first identified in Italy in 1876, is a serious threat to maize production. This disease most happened in the low temperature and humid regions in worldwide. In China, it was found in North China Spring Corn Area and the mountainous areas with lower temperature and higher elevations. It could cause serious economic losses at the epidemic year with production drop of 30 to 50%. So our group has been working on molecular mechanism of growth, development especially on pathogenicity of this fungus. In our previous research, we cloned a MAP kinase gene of *S. turcica*, which was designated STK1 gene (*S. turcica* kinase1 gene).

In the present study, we investigate whether *S. turcica STK1* restores the stress sensitivity phenotypes of yeast with a non-functional *HOG1* gene. Even more interesting is the fact that transformants display higher tolerance to several types of salt stress, especially Na^+ stress, than that of the WT strain of *S. cerevisiae*. Consequently, the transformation of *STK1* into crops will likely be able to improve the salt tolerance of transgenic plants, which would take full advantage of saline–alkaline soil, in which Na^+ is the chief element. This idea provides genetic potential for exploration of saline–alkaline soil and development of salt-tolerant agriculture.

MATERIALS AND METHODS

Strains, media and culture condition

S. turcica [anamorph *Exserohilum turcicum* (Pass.)] strain 01-23 (Race 0) was cultured on potato dextrose agar (PDA) or in PD at 25°C. The *S. cerevisiae* strains used in this study were the WT YSH689 and the *hog1*Δ mutant YSH444 (*hog1*::*TRP1* mutant). Yeast cells were cultured in yeast extract peptone dextrose (YPD) medium or in a minimal synthetic drop-out (SD) medium supplemented with appropriate amounts of sterile 10× uracil dropout solution at 30°C.

DNA manipulation and analysis

Basic DNA manipulations were performed according to standard protocols. Phylogenetic tree generation based on protein sequence alignment was performed using DNAStar software (Madison, WI), whereas homology searches were performed with the BLAST program on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

STK1 expression in *S. cerevisiae* and stress-tolerance assay

To express *STK1* in *S. cerevisiae*, the corresponding ORF was cloned into a pVT102U plasmid (Vernet et al., 1987). The plasmid containing *STK1* was transformed into *S. cerevisiae* via the lithium acetate method. Complementation analysis was performed on the strains maintained on YPD medium with indicative stresses. For salt stress, the yeast cells were cultured until the concentration of the *S. cerevisiae* suspension reached on OD_{600} of 1.8 at 30°C. Serially diluted (1/10) cell suspensions were spotted to examine the growth of the different strains on different media in the presence of NaCl, KCl, $CaCl_2$, LiCl, and sorbitol (Raffaello et al., 2012) at 30°C for 2 days. For the growth rate test, positive colonies were grown overnight in YPD or SD medium, adjusted to an OD_{600} of 0.3 with fresh medium, and incubated at 30°C. At various time intervals, the cultures were measured at OD_{600}. For the oxidative shock experiments, the cells were grown in YPD medium at 30°C until the mid-log phase and then aliquots were incubated for 1 h with increasing H_2O_2 concentrations. The cells were then washed, diluted, and plated onto solid YPD medium to determinate their survival rate. Each stress tolerance assay was performed in triplicate.

Glycerol measurement

For the glycerol production assays in yeasts, the yeast strains were cultured in YPD medium at 30°C until the exponential phase (OD_{600} = 0.7). Then, the yeast cells were collected and subsequently resuspended in new YPD medium with or without 0.5 M NaCl, and incubated for 1 h at 30°C. Subsequently, the cultures were boiled for 10 min to lyse the cells to release the intracellular glycerol. The determination of glycerol content was performed following the protocols of the glycerol-F kit (r-Biopharm Roch).

RESULTS

Characterization of *STK1* from *S. turcica*

The deduced *STK1* (AY849317) protein (Stk1) consists of 356 amino acid residues, with protein molecular weight of 40.80 KDa. The Stk1 protein shows high similarity to Hog1 of *S. cerevisiae* and also shares a significantly striking identity to the MAPK of filamentous fungi of stress-activated MAPK subfamily (Figure 1B,1C and Figure 1A).

Comparison of Stk1 with other MAPKs revealed the presence of all characteristic conserved subdomains. A TGY motif (Figure 1A), the characteristic structure of hyperosmolarity-activated MAPKs (Cano and Mahadevan, 1995) and the site for Thr and Tyr phosphorylation, was located in amino acids 171 to 173. Subsequently, a binding site responsible for its stable interaction with MAPKKs, designated common docking (CD) domain, was found. It was located on the C-terminal side of Stk1 and was composed of residues 299 to 312 (Figure 1A) (Tanoue et al., 2000). Two crucial amino acids, Asp-304 and Asp-307, were present in the CD domain, and functioned with Tyr-302 and His-303

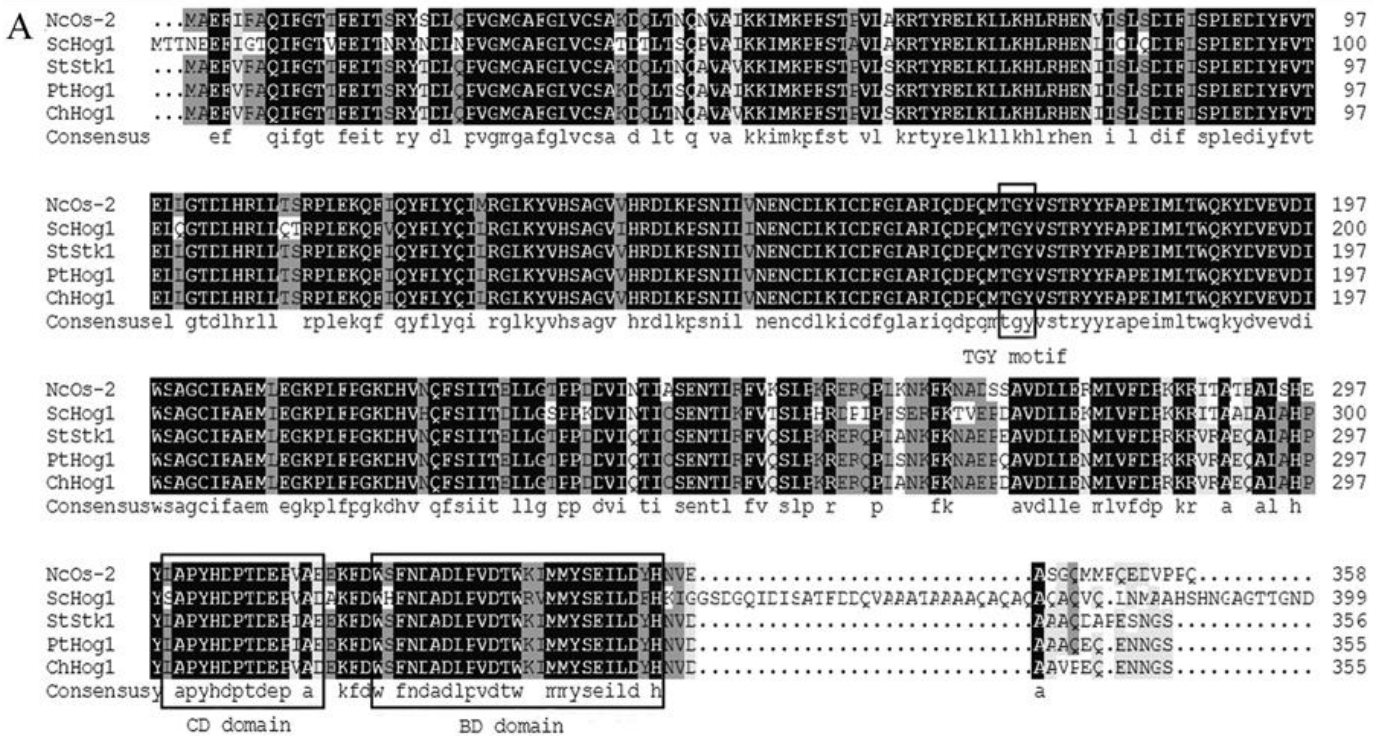


Figure 1A. Comparison between Stk1 and other stress-activated MAP kinases. Sequence alignment of *Setosphaeria turcica* Stk1 (ScStk1) with amino acid sequences of Stk1 homologues from other fungi: Hog1 from *Saccharomyces cerevisiae* (ScHog1, 82% identity, 91% similarity); Os-2 from *Neurospora crassa* (NcOs-2, 92% identity, 96% similarity); Hog1 from *Pyrenophora tritici* (PtHog1, 97% identity, 99% similarity); and Hog1 from *Cochliobolus heterostrophus* (ChHog1, 97% identity, 98% similarity). The TGY motif, CD domain, and BD domain in all MAPKs are underlined.

during interactions with their upstream and downstream effectors (Figure 1A). This docking domain serves as a docking site for efficient enzymatic reactions (Enslen and Davis, 2001). Another MAPKKs docking site, adjacent to the CD domain and designated as the binding domain (BD domain) (Murakami et al., 2008), was present in Stk1 from residues 317 to 341. The BD docking site appears to be sterically blocked in the intact Stk1 molecule. As shown in Figure 1B, the aligned sequences are clustered primarily by the type of MAPK, which forms three major clades: Hog1-homologues, Fus1/Kss1-homologues, and Slt2-homologues. On this tree, Stk1 is classified with the Hog1-homologous MAPKs subgroup, which is characterized by the TGY motif.

Functional complementation of the budding yeast *hog1*Δ mutant by *STK1* during osmotic stress

Upon deletion of the *HOG1* gene in *S. cerevisiae*, the mutant strains became more sensitive to salt compared with the WT strains because *GPD1* activity, which synthesizes glycerol in response to osmotic stress, was low (Albertyn et al., 1994). As shown in Figure 2A, under 1 M KCl, 1 M sorbitol, 0.3 M LiCl, and 0.4 M $CaCl_2$, the growth of the transformant strains with the *STK1* gene approximated the growth of the WT; on 1 M NaCl YPD, the transformant strains grew even faster than the WT strains, but *hog1*Δ mutant strains were more sensitive to salt stress compared with the WT and transformant strains. The results indicate that *STK1* expression is involved in the osmoregulation of the native Hog1 MAPK in yeast.

WT yeast is generally oval shape, as shown in Figure 2B, the *S. cerevisiae hog1*Δ mutant has an abnormal cell morphology under osmotic stress because of large multinucleated cells with multiple elongated buds (Degols et al., 1996). However, the transformants that contain the *STK1* gene have a normal cell shape, similar to the WT strains. The result indicates that the aberrant cell morphology of the *hog1*Δ mutant is suppressed by *STK1* expression.

Better tolerance to Na^+ than that of *HOG1* of WT by *STK1*

From Figure 2A, under 1 M NaCl, to our interest, the growth of transformants containing *STK1* is better than WT, which contains *HOG1*. To confirm this phenomenon, the growth rates of these yeast strains we measured by determining their OD at 600 nm. In YPD without any salt,

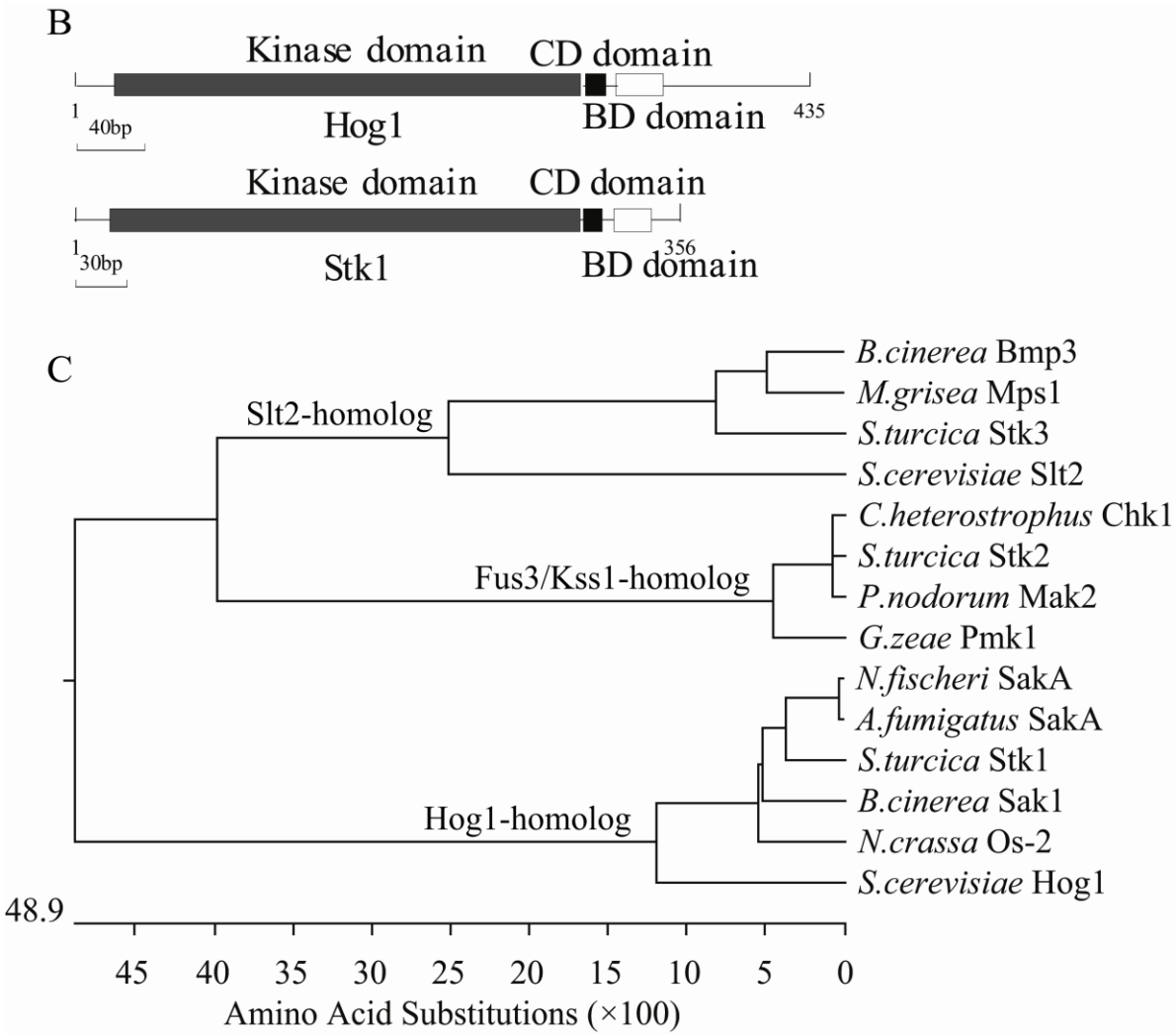


Figure 1B and 1C. Conserved domains of Stk1 and phylogenetic analysis of the Stk1 MAPK homologues based on amino acid sequence alignments. (B) Schematic diagram of the Stk1 MAPK. The locations of the kinase catalytic domain (gray rectangle), the CD domain (black rectangle), and BD domain (white rectangle) are shown. (C) Sequence alignment and phylogenetic tree construction were done using MegAlign, a program of Lasergene (DNAStar).

the difference was minor between these strains. In 1 M NaCl YPD, the growth rate of WT is faster than the *hog1*Δ mutant because the mutant lost the capacity for osmoregulation. Most interestingly, the growth rate of the mutant transformed with pVT102-*STK1* was not only better than the mutant but also better than the WT strains (Figure 3A), although the osmoregulatory HOG-MAPK pathway was not interrupted in the WT strains.

When yeast strains were exposed to hyperosmotic stress, the accumulation of intracellular glycerol was the major feature of *S. cerevisiae* osmoregulation. The glycerol content in the *S. cerevisiae hog1*Δ mutant increased by a much lower level than in the WT strain and the transformant (containing *STK1*) when it was stressed with 0.5 M NaCl. Compared with the WT, the transformant (containing *STK1*) exhibited a higher glycerol content (Figure 3B), which is in accordance with the growth rate under 1 M NaCl stress.

Effects of *STK1* in yeast *hog1*Δ mutant against oxidative stresses

HOG1 is reportedly involved in responses to oxidative stress in *S. cerevisiae* (Winkler et al., 2002) and *S. pombe* (Degols et al., 1996). Under oxidative stress, the *HOG1* null strain was more sensitive to H_2O_2 than the WT strain, whereas the transformants containing *STK1* showed a similar survival rate to that of the WT strain (Figure 4). This indicates that *STK1* has a similar function to *HOG1* in *S. cerevisiae* in resisting oxidative stress aside from salt stress.

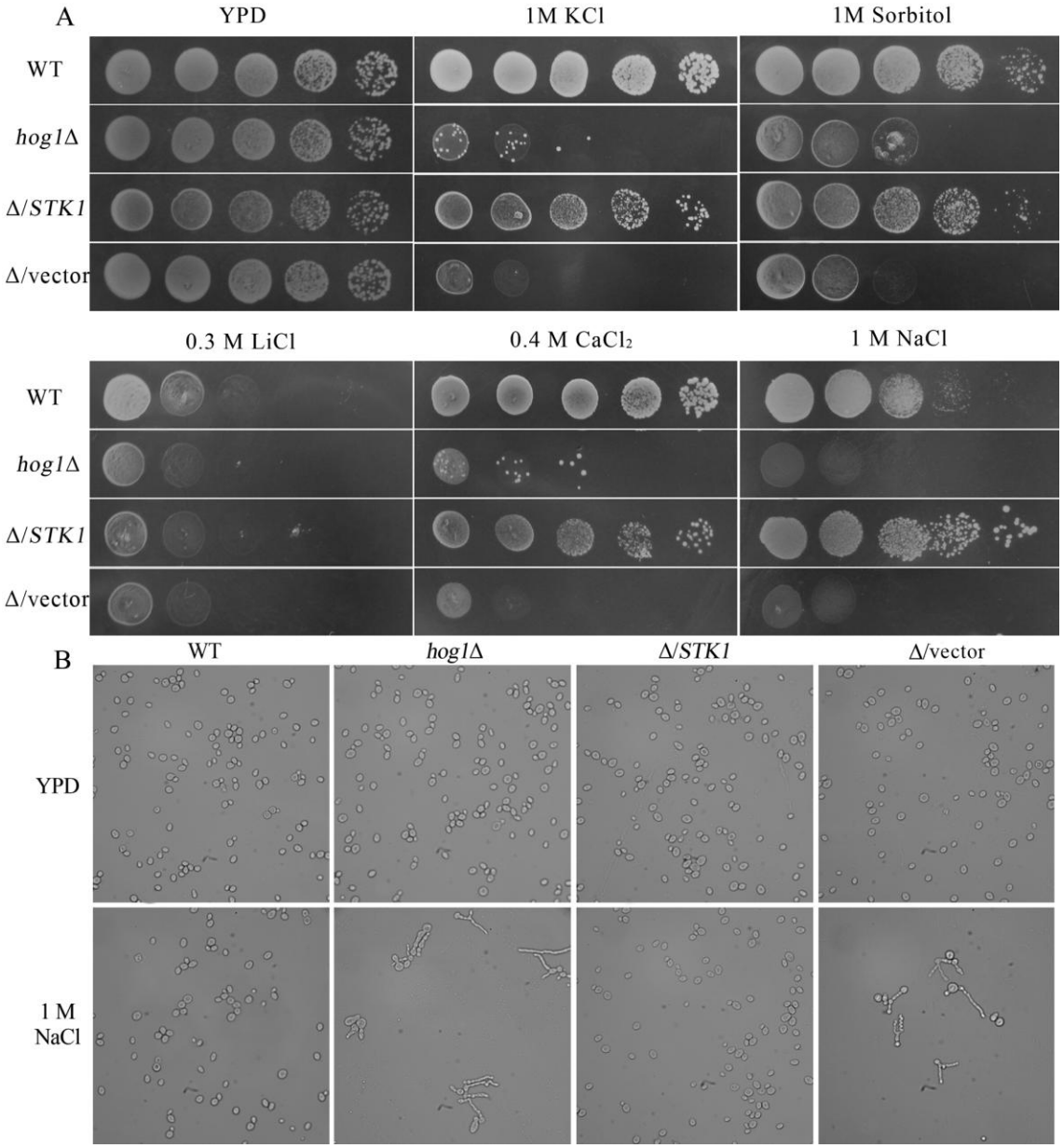


Figure 2. *STK1* complements the salt-sensitive yeast *HOG1* null mutant phenotype. (A) Serial tenfold dilutions of cultures were spotted onto YPD plates without salt (control) or containing KCl, NaCl, sorbitol, LiCl, and $CaCl_2$ at the indicated concentrations: WT (wild-type), *hog1*Δ (*HOG1* null mutant), Δ/*STK1* (*hog1*Δ transformed with pVT102U-*STK1*), and Δ/vector (*hog1*Δ transformed with empty vector pVT102U). The *hog1*Δ displays an osmotic sensitive phenotype, and the *STK1* gene expression restored the salt tolerance of the *hog1*Δ mutant. (B) Cell morphology of the budding yeast strains in YPD or 1M NaCl stress. The *hog1*Δ mutant showed abnormal cell morphology under osmotic stress with multiple elongated buds; *STK1* expression suppressed the mutant phenotype.

DISCUSSIONS

A MAPK encoded by *STK1* in *S. turcica* homologous to Hog1 in *S. cerevisiae*

Given the importance of MAPK in cellular signaling pathway, the activity and specificity of MAPK must be tightly regulated to ensure proper integration of diverse biological stimuli and generation of appropriate cellular responses (Dhanasekaran et al., 2007; Murakami et al., 2008; Takekawa et al., 2005).

MAPKs are all activated in response to the dual Thr/Tyrphosphorylation of a Thr-X-Tyr motif (X may be Glu, Pro, or Gly, which varies in different MAPKs). In

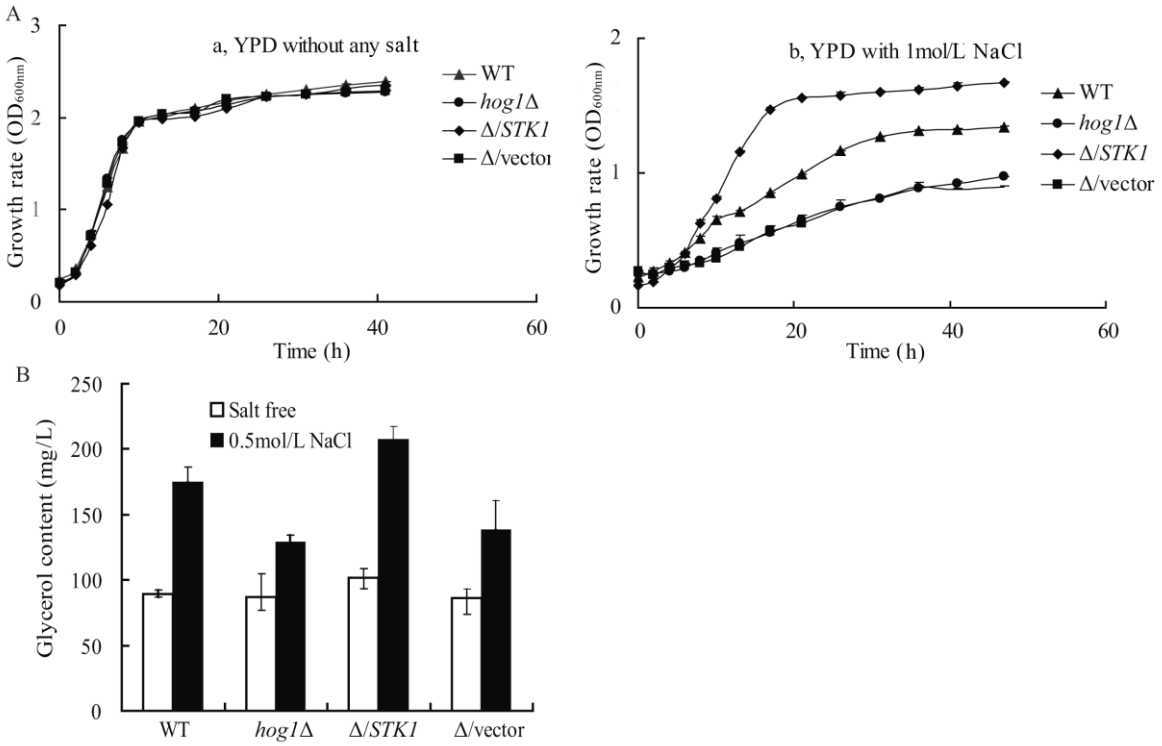


Figure 3. Tolerance of yeast strains to 1 M NaCl. (A) Growth rate of *S. cerevisiae* in liquid YPD without any salt (control, a) and with 1 M NaCl (b). Each curve represents one yeast strain, as shown in the diagram. The results were obtained from triplicate experiments using the same strains. (B) Effect of *STK1* expression on glycerol production in response to salt treatment. The data are representative of triplicate experiments and the standard errors of means are indicated.

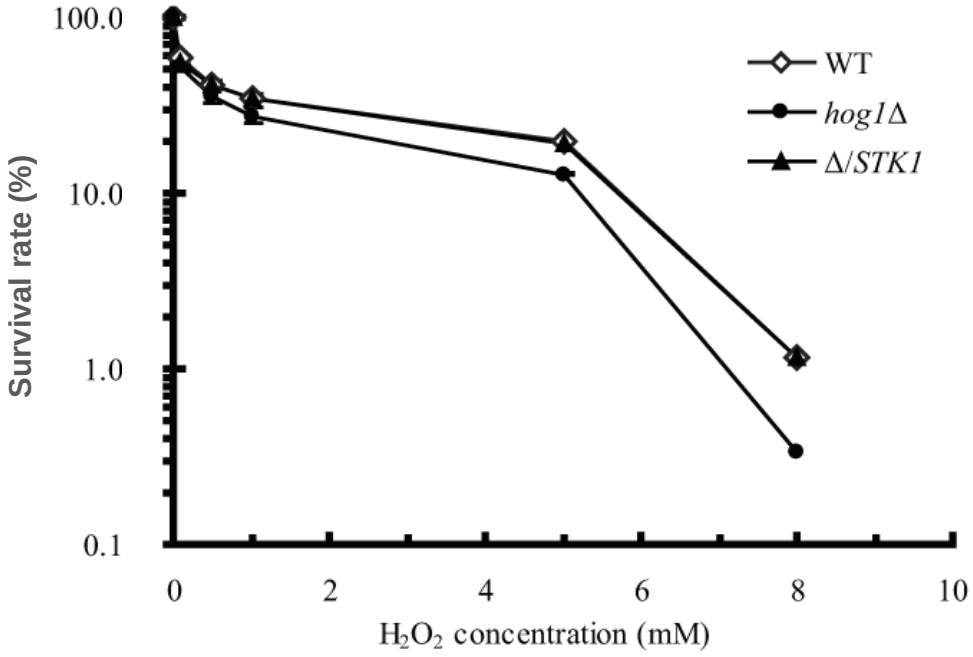
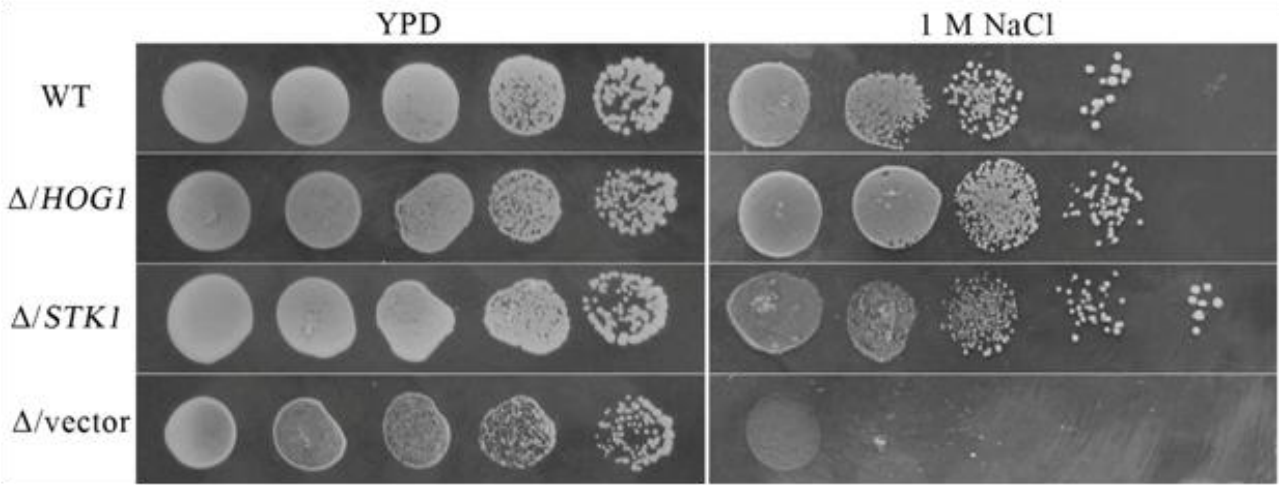


Figure 4. The effects of *STK1* in yeast strains under oxidative stress. The cells were grown up to an OD_{600} of 1.0 at 30 °C, and then the cultures were exposed to increasing H_2O_2 concentrations for 1.5 h. Percent survival is expressed relative to the initial viability before oxidative exposure. The results were obtained from triplicate experiments.



Supplementary 1. Growth of transformants carrying *HOG1* of *S. cerevisiae* and *STK1* in YPD and 1 M NaCl. Serial tenfold dilutions of the cultures were spotted onto YPD plates with and without 1 M NaCl and incubated on YPD at 30 °C: WT (Wild-type), Δ/*HOG1* (*hog1*Δ transformed with pVT102U-*HOG1*), Δ/*STK1* (*HOG1* null mutant transformed with pVT102U-*STK1*), and Δ/EV (*HOG1* null mutant transformed with empty vector pVT102U).

Hog1-homologous MAPKs, TGY is characteristic of MAPKs and its phosphorylation is catalyzed by the appropriate MAPKK. The common docking (CD) domain is utilized commonly for docking interactions with MAPKKs and transcriptional factors. The BD docking site appears to be sterically blocked in the intact MAPK Stk1 through the CD domain, it might induce a conformational change in Stk1, thereby exposing the BD domain to interact with Pbs2 (a MAPK kinase, data unpublished). Consequently, the association of activators with Stk1 via the CD domain might be reinforced by the additional interaction with the BD domain. Subsequently, the TGY motif is phosphorylated and Stk1 becomes functional, thereby transmitting the signal to its substrate. In the present study, only three MAPK homologues, Fus3/Kss1-homologues, Hog1-homologues, and Slt2-homologues have been identified in fungal pathogens. In the current sequence alignment, Stk1 showed a significant identification to the MAPK related to stress responses. It was also tightly clustered with the Hog1-homologue. Thus, Stk1 might potentially function in *S. turcica* similar Hog1 in *S. cerevisiae*.

Stress tolerance of Stk1 in *S. cerevisiae hog1*Δ mutant

STK1 encodes an MAPK that has very high identity with osmoregulatory MAPKs from plant pathogenic fungi, including Hog1 from *S. cerevisiae*. The deduced MAPK Stk1 has all the conserved domains. Therefore, Stk1 is likely homologous to Hog1 and it may have a similar role in stress tolerance if it was transformed into the *S. cerevisiae hog1*Δ mutant.

As shown in the functional complementation data, the *S. turcica STK1* gene decreased the sensitivity to salt and oxidation of the yeast *HOG1* null mutant, and restored its abnormal morphology. The complementation analyses clearly demonstrates that the MAPK encoded by *STK1* functions in the HOG pathway. Accordingly, a similar signal transduction pathway involves Stk1 in *S. turcica*.

STK1 confers preferable tolerance to sodium salt

Furthermore, on 1 M NaCl YPD plate, the transformant growth rate is not only higher than *HOG1* null mutant strains but also higher than WT strains. In addition, the accumulation of intracellular glycerol in the transformant is higher than in the *HOG1* null mutant and the WT, which is accordance with the growth rate experiment. When the *hog1*Δ mutant was transformed with pVT102U-Sc*HOG1*, the transformant did not show to sodium salt tolerance superior to that of the WT, which eliminates the possibility that complementation of *STK1* was derived from the ADH promoter in the expression construct or the copy number of *STK1* (Supplementary 1). These results suggest that *STK1* has similar and preferable function to *HOG1* of *S. cerevisiae* in regulating the *GPD1* gene, which is involved in glycerol biosynthesis in *S. cerevisiae*, and that *STK1* confers preferable tolerance to sodium salt stress.

Interestingly, despite considerable differences between yeast and *S. turcica*, functional expression of *STK1*, a MAPK homologue playing a central role in HOG signal

molecule. Both the CD and BD docking sites are required for the optimal activation of MAPK by MAPK kinase, and in the absence
of both sites, MAPK cannot be activated by MAPKK (Murakami et al., 2008). When MAPK kinase binds to

transduction in the yeast *S. cerevisiae*, was achieved. Considering the Hog1-homologue is well conserved from yeast to plants, *STK1* could potentially be introduced into other eukaryotic genomes to improve their stress tolerance, especially in plants, if stable integration can be achieved. Furthermore, *STK1* confers better tolerance to sodium salt in transgenic *Arabidopsis* (Our unpublished data).

Consequently, whether *STK1* improves the salt tolerance of transgenic plants when it is transformed into crops would take full advantage of saline–alkaline soil, in which Na^+ is a chief element and has an area of up to 954 million hm^2 worldwide according to United Nations Educational, Scientific and Cultural Organization (UNESCO) and Food and Agriculture Organization (FAO) incomplete statistics. This idea will provide genetic potential for the exploration of saline–alkaline soil and the development of salt-tolerant agriculture.

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