Identification of OS-2 MAP kinase-dependent genes induced in response to osmotic stress, antifungal agent fludioxonil, and heat shock in *Neurospora crassa*

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Abstract

Two-component signal transduction comprising of OS-1 (histidine kinase), OS-4 (MAPKK kinase), OS-5 (MAPK kinase), and OS-2 (MAP kinase) plays an important role in osmotic regulation in *Neurospora crassa*. To identify the genes regulated downstream of OS-2 MAP kinase, quantitative real-time RT-PCR analysis was conducted in selected genes based on Hog1 MAP kinase regulated genes in yeast. In response to osmotic stress and fludioxonil, expression of six genes that for glycerol synthesis (*gcy-1*, *gcy-3*, and *dak-1*), gluconeogenesis (*fbp-1* and *pck-1*), and catalase (*ctt-1*) was activated in the wild-type strain, but not in the *os-2* mutant. A heat shock treatment also induced their expression in the same way. Consisting with the gene expression, the enzyme activity of glycerol dehydrogenase, but not glycerol-3-phosphate dehydrogenase, was increased in response to osmotic stress and fludioxonil in the wild-type strain. OS-2 was phosphorylated by the OS-1 cascade in response to relatively low osmotic stress and fludioxonil. However, OS-2 phosphorylation by heat shock and a higher osmotic stress was found in the *os-1* mutant normally but not in the *os-4* and *os-5* mutants. These results suggested that non-OS-1 signaling activates OS-2 in an OS-4-dependent manner in such conditions.

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Keywords: *Neurospora crassa*; Catalase; Glycerol dehydrogenase; Osmotic stress; MAP kinase; Histidine kinase; Gluconeogenesis; Heat shock

1. Introduction

In filamentous fungi, histidine kinase signal transduction pathways play an important role in osmotic response and sensitivity to some fungicides. The *Neurospora crassa os-1* (also known as *nik-1*) gene encodes an osmosensor-like histidine kinase (Alex et al., 1996; Schumacher et al., 1997) and the *os-4*, *os-5*, and *os-2* genes encode homologs of the Ssk2/Ssk22 MAPKK kinase (Fujimura et al., 2003), Pbs2 MAPK kinase (Fujimura et al., 2003), and Hog1 MAP kinase (Zhang et al., 2002) in the *Saccharomyces cerevisiae* HOG pathway (Maeda et al., 1994), respectively. The osmoregulation mediated by the yeast Sln1, known as the high-osmolality glycerol response (HOG), has been well characterized (O’Rourke et al., 2002; Hohmann, 2002). Hyperosmotic stresses activate the HOG pathway, which in turn increases the transcription of many genes such as glycerol-3-phosphate dehydrogenase (*GPD1*), glycerol-3-phosphatase (*GPP2*), catalase (*CTT1*), and the heat shock protein *HSP12* (Akhtar et al., 1997).

Signal transduction downstream of *S. cerevisiae* Sln1 and *N. crassa* OS-1 appear to be similar because the *N. crassa os-2* gene functionally complements osmotically
sensitive hop1 mutants of *S. cerevisiae* (Zhang et al., 2002). However, there are several differences in the two-component pathway between *N. crassa* and *S. cerevisiae*. The disruption of the SLN1 gene of *S. cerevisiae*, which has only one histidine kinase gene, is lethal due to the overactivation of the HOG pathway. In contrast, filamentous fungi have several histidine kinase genes, including SLN1-like genes, in their genome. *N. crassa* has 11 putative histidine kinase genes (Borkovich et al., 2004). The SLN1 homolog *tesB* gene of *Aspergillus nidulans*, which functionally complements the *sln1* mutant of *S. cerevisiae*, is not essential and the *AtesB* strain did not exhibit a detectable phenotype on standard or stress media (Furukawa et al., 2002). In *N. crassa*, the mutation of the *os-1* histidine kinase gene confers sensitivity to osmotic stress, but this gene is not essential for growth under normal hypotonic conditions. The predicted osmosensor protein encoded by *os-1* differs from *Sln1* in its cytoplasmic location and the addition of six 90-amino acid repeat regions between the sensor and the kinase domains (Alex et al., 1996; Schumacher et al., 1997).

These differences in the histidine kinase pathway between yeast and filamentous fungi indicate functional diversity; however, little is known about the types of metabolic pathways that are regulated downstream of histidine kinase signaling in filamentous fungi. Therefore, in this study, we focused on the genes of *N. crassa* that encode putative catalase and the enzymes involved in glycerol synthesis and gluconeogenesis; these genes are orthologs of the Hop1-regulating genes of *S. cerevisiae*. The mRNA transcripts produced in response to osmotic stress and fluoroxy- nil were measured using quantitative real-time PCR and were compared between the wild-type strain and the os-2 mutant. Our finding revealed that the genes induced by osmotic stress in an OS-2-dependent fashion were also upregulated by heat shock. Furthermore, we examined the phosphorylation of OS-2 MAP kinase in the wild-type strain and os mutants. We found that OS-4 MAPK kinase and OS-5 MAPK kinase are essential for OS-2 phosphorylation, and that heat shock also activated OS-2 MAP kinase but independent of OS-1 histidine kinase.

2. Materials and methods

2.1. Fungal strains and culture

*Neurospora crassa* strains, C1-T10–37A and C1-T10–19a, are wild-type strains that are closely related to the standard Oak Ridge wild-type strains (Tamaru and Inoue, 1989). The osmotic sensitive mutants, *os-1* (alleles: NM233(t)), *os-2* (ALS10), *os-4* (Y256M223), and *os-5* (NM216o), were obtained from the Fungal Genetic Stock Center (Kansas City, MO, USA). The *os-1*, *os-3*, and *os-2* mutants produce truncated and most likely nonfunctional proteins lacking kinase catalytic domain; The *os-1* histidine kinase (1298 amino acids), the OS-5 MAPKK kinase (637 amino acids), and the OS-2 MAP kinase (358 amino acids) are truncated at 307, 308, and 96 residues in the *NM233* (*Ochiai et al., 2001), in the *NM216* (Fujimura et al., 2003), and in the ALS10 (Zhang et al., 2002), respectively. We identified that the *os-4* mutant (Y256M223) has a single point mutation at codon 651 (AAA–TAA) of 1367 of the relevant gene, resulting production of an immature OS-4 MAPKK kinase protein. A putative kinase domain was found to locate on the C-terminal region of the OS-4 (amino acid 1055–1324) by SMART domain search program (http://smart.embl-heidelberg.de/), suggesting the *os-4* mutant totally lost the MAPKK kinase function.

The strains were grown on agar-solidified Vogel’s medium N (VM) and 1.2% (w/v) sucrose at 25 °C (Vogel, 1964). For conidial production, the strains were cultured on medium A (4% malt extract, 1% casamino acids, 5% malt extract, 1% glycerol, and 1.5% agar) at 25 °C.

2.2. Quantitative real-time RT-PCR

For isolation of total RNA, conidia (3.3 × 10⁶/ml) were germinated in liquid VM medium for 4 h at 25 °C and then transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy- nil (1 μg/ml), 0.7 M NaCl, or 1 M sorbitol for 30 min. For heat shock experiments, germinating conidia at 25 °C were transferred to VM medium containing fluoroxy-ni...
to 45°C for 30 min. Following this, the samples were transferred into pre-chilled centrifugation tubes and centrifuged (2500 rpm) for 10 min at 4°C, and then frozen in liquid nitrogen. Total RNA was isolated using a FastRNA Pro Red kit (Qbiogene, Inc., Illkirch, France). In order to remove contaminating genomic DNA, the RNA samples were treated with 10 U of DNase I (Takara, Tokyo, Japan) per 50 µl of RNA at 37°C for 1 h. Each RNA sample (total RNA, 50 ng) was subjected to one-step PCR amplification. Real-time PCR was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany) with SYBR Green detection. The QuantiTect SYBR Green RT-PCR kit was purchased from Qiagen (Qiagen, Hilden, Germany). The amplification conditions consisted of the following three consecutive phases: (i) a reverse transcription step (50°C for 20 min), (ii) an initial denaturation step to activate the HotStarTaq DNA polymerase (95°C for 15 min), and (iii) an amplification step consisting of 50 cycles (94°C for 15 s, 65°C for 20 s, and 72°C for 20 s). The primers shown in Table 1 were used to quantify the expression of the target genes. We designed each primer based on the genome information (www.broad.mit.edu). The PCR product was verified for its length by gel electrophoresis, and for specificity by the melting curve analysis (LightCycler Software 3.5). PCR efficiency was calculated according to equation $E=10^{-1/slope}$, where maximal efficiency $E=2$ means that every single template is replicated in each cycle.

All assays displayed efficiencies in the range 1.80–1.97 (data not shown). Mean crossing point (CP) values (threshold cycle) were calculated from CP data obtained from three biologically independent RNA samples, used for calculations of expression ratios with primer-specific efficiencies, and the significance of differential expression was verified by means of REST (Pfaffl, 2001; Pfaffl et al., 2002). The CP values for an amplification of the β-tubulin *Bml* gene with primer pair Bt2a/Bt2b were used as a reference for normalization.

### 2.3. Enzyme assays of glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase

For enzyme assays of glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase, the conidia (3 × 10^7/ml) were pre-incubated in 100 ml of VM liquid medium in a 500-ml Sakaguchi flask and incubated on a reciprocating shaker (150 rpm) for 24 h at 25°C, and the resultant mycelial suspension was incubated in the VM liquid medium with 1 µg/ml fludioxonil or with 0.7 M NaCl for 4 h at 25°C. The treated mycelia, thus grown, were harvested by filtration and frozen in liquid nitrogen. Approximately 100 mg of mycelia (wet weight) was mixed with 1 ml of extraction buffer [20 mM Tris–HCl, pH 7.5; 2 mM EDTA; complete protease inhibitor cocktail set (Roche), 1 tablet per 50 ml] and 0.4 g of glass beads (φ = 0.5 mm) and then shaken with a bead beater (FastPrep Instruction, Qbiogene) for 20 s. The resulting homogenate was centrifuged at 10,000g for 20 min at 4°C and the supernatant was used to analyze both glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase activity at 25°C. Protein concentrations in the supernatant were determined with the Bio-Rad Protein Assay kit (Bio-Rad, Munich, Germany) according to the

### Table 1

<table>
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<th>Genes</th>
<th>Accession Nos.</th>
<th>Primer Position</th>
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<td>GD-D03</td>
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<td>GD-U03</td>
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<td>Bt2a</td>
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<td></td>
<td></td>
<td>Bt2b</td>
<td>304</td>
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suppressor’s instructions, by using bovine serum albumin (BSA) as a standard. For the enzyme assay, the protein concentration in the reaction mixture was adjusted to 0.5 mg/ml. The activity of glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase were determined in the previously described spectrophotometric assays (Seidl et al., 2004). Glycerol-3-phosphate dehydrogenase activities were assayed in 20 mM Tris–HCl buffer, pH 7.5, containing 1 mM MgCl₂, 0.1 mM NADH, 0.67 mM dihydroxyacetone-phosphate (DHAP), and 1 mM dithiothreitol (DTT). Glycerol dehydrogenase activities were determined in 100 mM glycine buffer, pH 9.6, containing 1 mM NAD⁺ and 100 mM glycerol. Activities are expressed as units (U), i.e., one unit (1 U) corresponds to the conversion of 1 μmol of substrate per minute, and are represented as specific activities (U/mg protein).

2.4. Phosphorylation of OS-2 MAP kinase assay

The conidia (3 × 10⁵/ml) were inoculated in VM liquid medium for 24 h at 25 °C, and then the culture was challenged with various stress for 10 min. The resultant mycelia were collected by vacuum filtration and frozen in liquid nitrogen. Approximately 100 mg of mycelia was suspended in an ice cold extraction buffer [50 mM Tris–HCl, pH 7.5; 1% sodium deoxycholate; 1% Triton X-100; 0.1% sodium dodecyl sulphate (SDS); 50 mM NaF; 5 mM sodium pyrophosphate; 0.1 mM sodium vanadate; complete protease inhibitor cocktail set (Roche), 1 tablet per 50 mL], and homogenized with a bead beater (FastPrep Instruction, Qiogene) for 20 s. Samples containing equal amounts of protein (25 μg/lane) were separated on 10% SDS/polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (ADVANTEC, Tokyo, Japan). The Hog1 antibody and phospho-p38 MAP kinase (Thr180/Tyr182) 28B10 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology, Inc. (Danvers, MA), respectively.

Antibody binding was visualized with a secondary antibody conjugated with horseradish peroxidase and diaminobenzidine (DAB) (Sigma, St. Louis, MO) reaction in the presence of hydrogen peroxide. The size standards used were biotin-labeled protein standards (Bio-Rad).

3. Results

3.1. Comparison of the basal level of expression of target genes between the wild-type strain and the os-2 mutant

The hog1 mutant of S. cerevisiae is functionally complemented by the N. crassa os-2 gene. Therefore, to determine the target genes regulated by OS-2, we searched the N. crassa genome sequence (www.broad.mit.edu) and selected the homolog genes regulated by Hog1, including their related genes (Table 2). Although most of them encode hypothetical proteins as per the N. crassa genome information, the putative proteins encoded by gpd-1, gpp-1, dak-1, ctt-1, fbp-1, and pck-1 show highest homology to Gpd1 (AAT27375; identity 42%), Gpp2 (P40106; 33%), Dak1 (NP_013641; 38%), Ctt1 (P06115; 38%), Fbp1 (CAA68723; 57%), and Pck (AAA76693; 67%) of S. cerevisiae, respectively. Three putative glycerol dehydrogenase genes gcy-1, gcy-2, and gcy-3 of N. crassa showed 40, 43, and 44% identity to Gcy1 (CAA99318) of S. cerevisiae, respectively. Three heat shock protein genes hsp-30, hsp-70, and hsp-88, were also selected for analysis of the induction of gene expression. No PCR products were produced by any of the primer sets when the RNA sample without the reverse transcriptase reaction was used as a template. The expression levels of these target genes were analyzed using quantitative RT-PCR and the crossing point (CP) of the target genes regulated by OS-2, we searched the N. crassa genome sequence (www.broad.mit.edu) and selected the homolog genes regulated by Hog1, including their related genes (Table 2). Although most of them encode hypothetical proteins as per the N. crassa genome information, the putative proteins encoded by gpd-1, gpp-1, dak-1, ctt-1, fbp-1, and pck-1 show highest homology to Gpd1 (AAT27375; identity 42%), Gpp2 (P40106; 33%), Dak1 (NP_013641; 38%), Ctt1 (P06115; 38%), Fbp1 (CAA68723; 57%), and Pck (AAA76693; 67%) of S. cerevisiae, respectively. Three putative glycerol dehydrogenase genes gcy-1, gcy-2, and gcy-3 of N. crassa showed 40, 43, and 44% identity to Gcy1 (CAA99318) of S. cerevisiae, respectively. Three heat shock protein genes hsp-30, hsp-70, and hsp-88, were also selected for analysis of the induction of gene expression. No PCR products were produced by any of the primer sets when the RNA sample without the reverse transcriptase reaction was used as a template. The expression levels of these target genes were analyzed using quantitative RT-PCR and the crossing point (CP) of the untreated control in the wild-type strain were compared with those in the os-2 mutant. The intensity of PCR amplification is dependent on the primer set used (Table 1) in the real-time PCR quantification; therefore, the CP values of one gene cannot be compared with that of another gene.

### Table 2

Mean crossing point of target genes of untreated control in the wild-type strain and the os-2 mutant

<table>
<thead>
<tr>
<th>Genes</th>
<th>NCU Nos.</th>
<th>Putative gene product</th>
<th>Crossing point ± SD</th>
<th>os-2 Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>gpd-1</td>
<td>00742.2</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>21.1 (±0.86)</td>
<td>22.3 (±0.57)</td>
</tr>
<tr>
<td>gpp-1</td>
<td>03068.2</td>
<td>Glycerol-3-phosphatase</td>
<td>23.9 (±1.81)</td>
<td>22.1 (±1.18)</td>
</tr>
<tr>
<td>gcy-1</td>
<td>04923.2</td>
<td>Glycerol dehydrogenase</td>
<td>25.8 (±0.47)</td>
<td>24.1 (±0.38)</td>
</tr>
<tr>
<td>gcy-2</td>
<td>01906.1</td>
<td>Glycerol dehydrogenase</td>
<td>24.0 (±0.43)</td>
<td>25.2 (±0.83)</td>
</tr>
<tr>
<td>gcy-3</td>
<td>04510.1</td>
<td>Glycerol dehydrogenase</td>
<td>20.5 (±0.37)</td>
<td>22.0 (±0.64)</td>
</tr>
<tr>
<td>dak-1</td>
<td>03779.1</td>
<td>Dihydroxyacetone kinase</td>
<td>26.9 (±1.23)</td>
<td>26.7 (±1.73)</td>
</tr>
<tr>
<td>ctt-1</td>
<td>05169.2</td>
<td>Catalase</td>
<td>26.6 (±0.81)</td>
<td>26.2 (±0.92)</td>
</tr>
<tr>
<td>fbp-1</td>
<td>04797.2</td>
<td>Fructose-1,6-bisphosphatase</td>
<td>26.7 (±0.10)</td>
<td>25.5 (±0.34)</td>
</tr>
<tr>
<td>pck-1</td>
<td>09873.2</td>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>25.8 (±0.15)</td>
<td>22.8 (±1.73)</td>
</tr>
<tr>
<td>hsp-30</td>
<td>09364.1</td>
<td>Heat shock protein 30</td>
<td>23.2 (±0.23)</td>
<td>22.6 (±0.59)</td>
</tr>
<tr>
<td>hsp-70</td>
<td>09602.1</td>
<td>Heat shock protein 70</td>
<td>20.4 (±0.16)</td>
<td>20.5 (±0.18)</td>
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<tr>
<td>hsp-88</td>
<td>05269.1</td>
<td>Heat shock protein 88</td>
<td>18.0 (±0.49)</td>
<td>18.7 (±1.22)</td>
</tr>
<tr>
<td>Bnl</td>
<td>04054.1</td>
<td>β-Tubulin</td>
<td>16.1 (±1.89)</td>
<td>17.6 (±0.33)</td>
</tr>
</tbody>
</table>

- a The average crossing points (CP) in quantitative real-time RT-PCR analysis in three replicated experiments. RNA samples (total RNA 50 ng) of untreated control in the wild-type and the os-2 mutant were subject to one-step PCR amplification. SD means standard deviation.
- b Gene locus number in Neurospora genome project.
However, the CP value of each target gene of the wild-type strain was very similar to that of the corresponding target genes of the os-2 mutant; no significant differences were found between the wild-type strain and os-2 mutant (Table 2). These results indicate that lack of OS-2 MAP kinase did not affect the basal levels of mRNA at least of these genes, including the β-tubulin (Bml) gene.

3.2. Identification of gene responding to osmotic stress and fludioxonil in an OS-2-dependent manner

In order to identify genes that are regulated in response to osmotic stress and fludioxonil, the germinating conidia were treated with fludioxonil (1 μg/ml) and exposed to osmotic stress (0.7 M NaCl and 1 M sorbitol) for 30 min. The mRNA levels of the genes in the resultant samples were assessed using quantitative real-time RT-PCR. The relative expression level of each gene was calculated using software tool REST® (Pfaffl et al., 2002) based on the PCR efficiency and the mean crossing point difference (ΔCP) between the treated sample versus the control group. As a reference gene, we used the Bml (β-tubulin) gene, and the CP for 50 ng of total RNA isolated from the wild-type strain was 16.1 (±1.89), 15.8 (±0.25), 15.1 (±0.09), and 15.5 (±0.31) in the untreated control, fludioxonil-treated sample, NaCl-treated sample, and sorbitol-treated sample, respectively. In the os-2 mutant, the CPs were 17.6 (±0.33), 17.5 (±0.26), 16.7 (±0.38), and 16.8 (±0.26) in the untreated control, fludioxonil-treated sample, NaCl-treated sample, and sorbitol-treated sample, respectively.

Both fludioxonil and osmotic stress induce glycerol accumulation in the wild-type strain (Pillonel and Meyer, 1997). The genes GPD1 and GPP2, which encode glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively, for glycerol synthesis, respond in a Hog1-dependent manner in S. cerevisiae (Akhtar et al., 1997). Therefore, we first estimated the mRNA levels of the corresponding genes in N. crassa named gpd-1 and gpp-1. However, the mRNA levels of both these genes did not increase by the treatment of 1 μg/ml of fludioxonil, 0.7 M NaCl, or 1 M sorbitol (Fig. 1a). In contrast, a significant increase was detected in the mRNA levels of the gcy-1 and gcy-3 genes, which encode putative glycerol dehydrogenases. Both osmotic stress and fludioxonil increased gcy-1 mRNA levels by more than 50-fold in the wild-type strain. Although another putative glycerol dehydrogenase gene gcy-2 did not respond to the fungicide and osmotic stress, the upregulation of gcy-3 in response to fludioxonil and osmotic stress was also detected in the wild-type strain. In the os-2 mutant, gcy-1 expression was only slightly activated by fludioxonil and osmotic stress, and increased expression of the gcy-3 genes was not observed (Fig. 1b); this suggests that these genes were not regulated in an OS-2-dependent manner.

![Fig. 1](https://example.com/image1.png)

**Fig. 1.** Relative mRNA levels of target genes in response to fludioxonil and osmotic stress in the wild-type strain and os-2 mutant. Relative quantification of mRNA of the selected genes in response to fludioxonil (1 μg/ml, white boxes), 0.7 M NaCl (gray boxes), and 1 M sorbitol (black boxes) using real-time RT-PCR. The expression levels of the six target genes, namely, gpd-1, gpp-1, gcy-1, gcy-2, gcy-3, and dak-1, which encode putative enzymes for the glycerol synthesis pathway in the wild-type strain (a) and in the os-2 mutant (b), are shown. The expression levels of the other six target genes cct-1, fbp-1, pck-1, hsp-30, hsp-70, and hsp-88 in the wild-type strain (c) and in the os-2 mutant (d) are shown. Conidia at 3.3 × 10^6 cells/ml were germinated in the VM liquid medium for 4 h at 25 °C and treated with 1 μg/ml of fludioxonil, 0.7 M NaCl, and 1 M sorbitol for 30 min at 25 °C, and RNA was then extracted. Total RNA (50 ng) isolated from the wild-type strain and the os-2 mutant was analyzed by real-time PCR (LightCycler; Roche). The relative expression of each target gene was calculated using software tool REST® (Pfaffl et al., 2002) based on the mean crossing point difference between the treated versus the control group and the PCR efficiency. The target gene expression was normalized via the β-tubulin gene as a reference. Three independent experiments were carried out and the standard deviations are indicated as error bars.
two genes were upregulated in an OS-2-dependent manner. In the wild-type strain, the mRNA level of \textit{dak-1}, which encodes a putative dihydroxyacetone kinase, also increased in parallel with that of the \textit{gcy-3} gene in response to fludioxonil and osmotic stress, but did not change significantly in the \textit{os-2} mutant. These results indicate that the upregulated genes for glycerol synthesis in \textit{N. crassa} are different from the Hog1-dependent genes in \textit{S. cerevisiae}.

In the same way, we verified the quantitative mRNA expression of one catalase gene \textit{ctt-1}; two gluconeogenesis related genes \textit{fbp-1} and \textit{pck-1}; and three heat shock protein genes \textit{hsp-30}, \textit{hsp-70}, and \textit{hsp-88} (Fig. 1c and d). In the wild-type strain, the \textit{ctt-1}, \textit{fbp-1}, and \textit{pck-1} mRNAs were accumulated in response to fludioxonil and osmotic stress. The expression of these genes did not increase in the \textit{os-2} mutant, although \textit{pck-1} mRNA levels increased slightly in response to fludioxonil. On the other hand, heat shock protein gene expressions were not affected by fludioxonil and osmotic stress in both the wild-type strain and the \textit{os-2} mutant. The OS-2-dependent upregulation of these genes was also observed when the mycelia were treated with fludioxonil (1 μg/ml) and 1 M sorbitol (data not shown).

3.3. Effect of heat shock stress on expression of the genes upregulated in response to osmotic stress and fludioxonil

Although heat shock protein genes were not activated by osmotic stress (Fig. 1c), all three genes \textit{hsp-30}, \textit{hsp-70}, and \textit{hsp-88}, displayed high mRNA expression levels in response to heat shock in the wild-type strain (Fig. 2a). Similar levels of activation were observed in the \textit{os-2} mutant (Fig. 2b). These results indicate that the three heat shock protein genes are activated by heat shock treatment but their regulation is essentially independent of OS-2 MAP kinase. However, the six genes \textit{gcy-1}, \textit{gcy-3}, \textit{dak-1}, \textit{ctt-1}, \textit{fbp-1}, and \textit{pck-1}, whose expressions were upregulated in an OS-2-dependent manner in response to osmotic and fludioxonil, were also activated by heat shock in the wild-type strain, while the levels of \textit{gpd-1} and \textit{gpp-1} were not altered in response to heat shock stress (Fig. 2a). Interestingly, the heat shock-induced increased expression of the mRNA of these six genes, which was observed in the wild-type strain, was almost absent in the \textit{os-2} mutant (Fig. 2b); this was also observed under high osmotic conditions (and fludioxonil treatment) (Fig. 1). In contrast, the CP values for β-tubulin expression during heat shock was 18.5 (±0.75), of which untreated control was 17.6 (±0.33), in the wild-type, and 18.8 (±0.47), of which untreated control was 17.7 (±0.24), in the \textit{os-2} mutant, respectively. This consistency between the upregulation of genes in response to osmotic stress and heat shock stress suggests that OS-2 may be activated by not only osmotic stress but also heat shock.

3.4. Enzyme activity of glycerol-3-phosphate dehydrogenase and glycerol dehydrogenase in response to osmotic stress and fludioxonil

To clarify whether upregulation of the \textit{gcy-1} and \textit{gcy-3} genes reflects quantitative changes in the corresponding enzymes, the activities of NAD-dependent glycerol-3-phosphate dehydrogenase and NADP-dependent glycerol dehydrogenase were assayed (Table 3). Both fludioxonil and osmotic stress did not affect the activity of glycerol-3-phosphate dehydrogenase in both the wild-type strain and the \textit{os-2} mutant. Exposure to fludioxonil (1 μg/ml) for 4 h led to a significant increase in the glycerol dehydrogenase activity in the wild-type strain. In the \textit{os-2} mutant, fludioxonil treatment showed only a slight increase or no change in the levels of these enzymes. However, the levels of \textit{dak-1} and \textit{gcy-3} enzymes were increased in the \textit{os-2} mutant. These results suggest that OS-2 may be activated by not only osmotic stress but also heat shock.

Fig. 2. Relative quantification of mRNA of the target genes in response to heat shock in \textit{N. crassa}. The germinating conidia (for 4 h at 25 °C) were shifted to 45 °C for 30 min, and RNA was then extracted. The mRNA quantification of the 12 target genes was analyzed as shown in Fig. 1. Three independent experiments were carried out and the standard deviations are indicated as error bars.
glycerol dehydrogenase activity. These enzyme activities are well correlated with their gene expressions. However, the presence of 0.7 M NaCl resulted in a significant increase in the glycerol dehydrogenase activity not only in the wild-type strain but also in the os-2 mutant.

### 3.5. Phosphorylation of MAP kinase OS-2 in the os mutants

Phosphorylation of OS-2 MAP kinase was investigated in the wild-type and os mutants after exposure to fluodoxinol (1 μg/ml), NaCl (0.2 M and 0.7 M), KCl (0.2 M and 0.8 M), sorbitol (1 M), and heat shock (45°C) by Western blotting using an antiphospho-p38 antibody (Fig. 3). Both fluodoxinol and osmotic stress stimulated OS-2 phosphorylation in the wild-type strain as described by Irmlera et al. (2005), and OS-2 phosphorylation induced by fluodoxinol and low levels of osmotic stress (0.2 M NaCl and 0.2 M KCl) decreased in the os-1 mutant as described by Yoshimi et al. (2005). We found that clear phosphorylation of OS-2 was induced not only by exposure to fluodoxinol and osmotic stress but also by heat shock in the wild-type strain. The OS-2 protein of the os-1 mutant was significantly phosphorylated in response to heat shock treatment. These results suggest that both fluodoxinol and low levels of osmotic stress phosphorylate OS-2 MAP kinase via OS-1 histidine kinase, however, heat shock stress, as well as high level of osmotic stress, is recognized independent of OS-1 and then transferred to the OS-2 protein.

The os-4 and os-5 mutants as well as the os-2 mutant did not show any phosphorylation in response to fluodoxinol, osmotic stress, and also heat shock stress (Fig. 3), although a detectable level of signal was observed even in the untreated control in the wild-type strain. In the os-1 mutant, OS-2 phosphorylation induced by fluodoxinol and low levels of osmotic stress decreased drastically, but a basal level of phosphorylation persisted. However, OS-2 phosphorylation was absent in the os-4 and os-5 mutants, suggesting that activation of OS-2 was almost completely dependent on the OS-4 MAPKK kinase and OS-5 MAPK kinase.

### 4. Discussion

A two-component histidine kinase pathway plays an important role in osmoregulation in fungi. We verified that six genes (gcy-1, gcy-3, dak-1, ctt-1, fbp-1, and pck-1) were simultaneously upregulated by increased osmolarity, heat elevation and a fungicide chemical, dependent upon the phosphorylation of OS-2 MAP kinase in N. crassa (Fig. 4). Interestingly, when compared to that of wild-type, these stress caused distinct effect on the phosphorylation of OS-2 in the os-1 mutant (Fig. 3): unchanged by the heat treatment and the high osmotic stress (0.7 M NaCl and 0.8 M KCl), considerably lower by lower osmotic stress (0.2 M NaCl and 0.2 M KCl), and no stimulation by fluodoxinol. Recent study also showed that the high osmotic stress (0.7 M NaCl and 0.8 M KCl) activated OS-2 in a N. crassa os-1 mutant (Yoshimi et al., 2005). These observations indicate that OS-1 histidine kinase is not essential for the phosphorylation of OS-2 and the other signaling pathway(s), of which osmotic sensor may have a lower function than

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**Table 3**

<table>
<thead>
<tr>
<th>Condition</th>
<th>GPD (mU/mg)</th>
<th>GLD (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.77 (±0.30)</td>
<td>0.89 (±0.15)</td>
</tr>
<tr>
<td>NaCl (0.7 M)</td>
<td>0.95 (±0.43)</td>
<td>1.29 (±0.39)</td>
</tr>
</tbody>
</table>
| Samples were taken after 4 h of replacement in the corresponding media. The results are averages from three independent experiments.
OS-1, was activated in these conditions and the signal transferred to OS-4 MAPKK kinase in the filamentous fungus. It is known that *N. crassa* has eleven histidine kinase genes on its genome (www.broad.mit.edu). Although their histidine kinase functions remain unclear, with the exception of OS-1, possessing several sensor histidine kinases allow *N. crassa* to detect multiple abiotic stress and transmit the signal to activate OS-2 MAP kinase (Fig. 4). Only a few Hog1 homologous have shown to be activated by multiple stresses. *A. nidulans* SakA was activated by osmotic and oxidative stress (Kawasaki et al., 2002), and *Schizosaccharomyces pombe* Spc1/Sty1 was activated in response to heat and oxidative stress as well as osmotic stress (Degols et al., 1996). Hog1-type MAP kinase might be activated by multiple stress factors in filamentous fungi. On the other hand, the budding yeast *S. cerevisiae* has only one histidine kinase gene **SLN1**, and the HOG pathway plays a role mainly in osmotic adaptation. Although a report suspected that Hog1 is also activated by heat stress (Winkler et al., 2002), Hog1 is considered to be specifically activated by increased extracellular osmolarity.

The phosphorylation of OS-2 was totally diminished in the *os-4* MAPKK Kinase and os-5 mutants even if a basal level of it was found in the untreated cells of the wild-type and the *os-1* mutant strains (Fig. 3). This result indicates that the phosphorylation of OS-2 is solely dependent on the upstream OS-4 MAPKK kinase and OS-5 MAPK kinase in *N. crassa*. Meanwhile, *S. cerevisiae* Hog1 MAP kinase is phosphorylated by the two upstream osmosensor proteins Snl1 (histidine kinase) and Sho1 (non-histidine kinase). The Sho1 signal activates Hog1 via Pbs2 MAP kinase and independent of Snl1 (Maeda et al., 1995). Therefore, the signal from Sho1 is not transferred to a MAPKK kinase in the yeast. Recently Furukawa et al. (2005) revealed that activation of the *A. nidulans* HOG (AnHOG) pathway depended solely on the two-component signaling system, and MAPK kinase activation mechanisms in the AnHOG pathway differed from those in the yeast HOG pathway. These data suggest that not only MAPK kinase but also MAPKK kinase could be essential for the activation of the Hog1-type MAP kinase in filamentous fungi (Fig. 4).

In response to osmotic pressure, both the filamentous fungus *N. crassa* and the yeast *S. cerevisiae* accumulate glycerol within their cells to increase the internal turgor pressure. Although such a response is commonly accomplished by the stimulation of glycerol production pathway, results described herein indicated that MAP kinase of each organism upregulated different glycerol synthesis genes. In *S. cerevisiae*, the osmotic stress-induced expression of *GPD1* and *GPP2*, which encode glycerol-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphatase (GPP), respectively, is controlled by the MAP kinase Hog1 (Akhhtar et al., 1997), and the *GPD1* gene is essential for growth under osmotic stress (Albertyn et al., 1994). A genome search suggested that *N. crassa* has *gpd-1* for a probable GPD and *gpp-1* for a probable GPP, homolog of *GPD1* and *GPP2*, respectively. However, exposure to high osmolality (0.7 M NaCl or 1 M sorbitol) never influenced the expression of *gpd-1* and *gpp-1* in the filamentous fungus (Fig. 1). The enzyme activity of GPD also remained constant after the osmotic stress treatments (Table 3). In contrast, reflect to an *os-2*-dependent induction of two putative glycerol dehydrogenase (GLD) genes, *gcy-1* and *gcy-3* (Fig. 1a and b), a large increase of cellular GLD activity was observed in response to osmotic stress in *N. crassa* (Table 3). Additionally, *dak-1*, which encodes a putative dihydroxyacetone kinase (DAK), was also upregulated in the same way. We conclude that *N. crassa* employs GLD and DAK for glycerol synthesis in the osmoregulation. In contrast, *S. cerevisiae*, glycerol is synthesized from DHAP.

Fig. 4. Schematic diagram of two-component signal transduction pathway in *N. crassa* and *S. cerevisiae*. The treatments by fludioxonil and low levels of osmotic stress activate the OS-2 MAP kinase OS pathway thought OS-1 histidine kinase. On the other hand, the high osmotic stress and heat shock stress phosphorylated OS-2 MAP kinase in OS-1-independent manner. The activation of OS-2 by these stresses was dependent on OS-4 and OS-5, and the six genes, such as *gcy-1*, *gcy-3*, *dak-1*, *ctt-1*, *fbp-1*, and *pek-1*, were upregulated in *N. crassa*. 

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*Saccharomyces cerevisiae*  
*Neurospora crassa*

Osmotic stress  
Sho1  
Sln1  
Pbs2  
Hog1  
GPD1, GPP1  
CTT1, HSP12  
Osmotic stress (low)  
Fludioxonil  
Osmotic stress (high)  
Heat shock  
SHO-1  
10 other histidine kinases  
OS-4  
OS-5  
OS-2  
gcy-1, gcy-3, dak-1  
ctt-1, fbp-1, pck-1  
MAP Kinase  
MAP Kinase Cascade  
via glycerol-3-phosphate by a sequential action of GPD1 and GPP2 and its dissimilation is mediated by an NADP-dependent GLD and a putative DAK (Norbeck and Blomberg, 1997). Formation of glycerol in A. nidulans under osmotic stress conditions is also mainly dependent on GLD activity and not on GPD activity (Füllinger et al., 2001; Han and Prade, 2002; de Vries et al., 2003). A similar result has also been reported in Trichoderma atroviride (Seidl et al., 2004). These data suggest that, in filamentous fungi, DHAP may be first converted to dihydroxyacetone and only then to glycerol by a GLD. Curiously in the N. crassa os-2 mutant, GLD activity increased in response to osmotic stress without induction of the gcy genes (Table 3). This mutant and the other os mutants were known to accumulate glycerol, even though considerably lower than that of the wild-type strain, under high osmolarity conditions (Fujimura et al., 2000a,b). Although the reason behind this contradiction between the gene induction and the enzyme activity remains to be elucidated, an increased enzyme activity could be attributed to the expression of other os-2-independent genes involved in the glycerol synthesis or the activation of the posttranscriptional regulatory system by osmotic stress. In contrast, fludioxonil did not affect the gene expression of gcy genes and GLD activity in the os-2 mutant, suggesting that stimulation of glycerol synthesis by the fungicide occurs only via the activation of OS-2 MAP kinase.

In addition to the glycerol synthesis genes, two genes fbp-1 and pck-1, which involve gluconeogenesis, were activated by an increased osmolarity and the fludioxonil treatment in an OS-2-dependent manner (Fig. 1c and d). The expression of S. pombe fbp1, which encodes a key gluconeogenesis enzyme fructose-1,6-bisphosphatase, is regulated by the cAMP-PKA signaling pathway and MAP kinase Spc1/Sty1 pathway (Stettler et al., 1996). The Spc1/Sty1 pathway mutants fail to activate the transcription of fbp1, whereas fbp1 is constitutively expressed in mutants defective in adenylate cyclase (git2) or cAMP-dependent protein kinase (pka1). In Neurospora, the expression of a homologous gene fbp-1 was induced by osmotic stress and fludioxonil in the wild-type strain, but not in the os-2 mutant (Fig. 1c and d). The expression of another putative gluconeogenic gene pck-1 encoding phosphoenolpyruvate carboxykinase was similarly increased, suggesting that the gluconeogenic pathway was activated by the osmotic stress. On the other hand, the induction of the ctt-1 gene, which encodes a putative cytoplasmic catalase, by several stress indicates that the OS-mediated signal affects the expression of genes whose products play a more general role in protection from stress-induced damage, similar to the HOG pathway of S. cerevisiae (Rep et al., 1999). Meanwhile, the HSP12 gene encoding a heat shock protein has been reported to be strongly regulated downstream of the HOG pathway in the yeast (Rep et al., 1999). We could not find any HSP12 homologous genes in the N. crassa genome; therefore, we monitored mRNA levels of three heat shock protein genes hsp-30, hsp-70, and hsp-88, which encode α-crystallin-related protein, chaperone, and chaperone-related protein, respectively (Plesofsky-Vig and Brambl, 1998). In N. crassa, however, these heat shock protein genes did not respond to osmotic stress or fludioxonil, and OS-2 did not involve in their expression. The transcription regulation of the OS-dependent genes predicts the motifs for binding of specific transcriptional factors. In the promoter region (–1 kb from start codon) of these OS-dependent genes, putative stress response elements (STREs: CCCCT and AGGGG) were found in the gcy-I (at positions –487 and –322 bp), dak-I (–770, –735, –411, and –395 bp), fbp-1 (–948, –820, –472, and –452 bp) and, pck-1 (–829, –495 bp) genes. These elements might be recognized by S. cerevisiae Msn2/42-like transcriptional factors (Rep et al., 2000) and involved in upregulation of these genes in N. crassa, although further studies are necessary for the direct evidence.

In this study, we identified the OS-2-dependent genes in N. crassa based on the evidence derived from the yeast HOG pathway. The function and regulation of the two-component histidine kinase pathway in N. crassa are essentially similar to those of the HOG pathway in S. cerevisiae. However, several important differences appear to exist in these organisms. Although further studies on the actual functions of OS-2-dependent genes and analysis of their promoters and transcriptional regulators are necessary, our result provides useful information to elucidate the function of the histidine kinase signaling pathway and its regulatory mechanism in filamentous fungi.

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