Osmoregulation and Fungicide Resistance: the *Neurospora crassa* os-2 Gene Encodes a HOG1 Mitogen-Activated Protein Kinase Homologue†

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*Neurospora crassa* osmosensitive (os) mutants are sensitive to high osmolarity and therefore are unable to grow on medium containing 4% NaCl. We found that os-2 and os-5 mutants were resistant to the phenylpyrrole fungicides fludioxonil and fenpiclonil. To understand the relationship between osmoregulation and fungicide resistance, we cloned the os-2 gene by using sib selection. os-2 encodes a putative mitogen-activated protein (MAP) kinase homologous to HOG1 and can complement the osmosensitive phenotype of a *Saccharomyces cerevisiae* hog1 mutant. We sequenced three os-2 alleles and found that all of them were null with either frameshift or nonsense point mutations. An os-2 gene replacement mutant also was generated and was sensitive to high osmolarity and resistant to phenylpyrrole fungicides. Conversely, os-2 mutants transformed with the wild-type os-2 gene could grow on media containing 4% NaCl and were sensitive to phenylpyrrole fungicides. Fludioxonil stimulated intracellular glycerol accumulation in wild-type strains but not in os-2 mutants. Fludioxonil also caused wild-type conidia and hyphal cells to swell and burst. These results suggest that the hyperosmotic stress response pathway of *N. crassa* is the target of phenylpyrrole fungicides and that fungicidal effects may result from a hyperactive os-2 MAP kinase pathway.

Wild-type *Neurospora crassa* strains can grow on media with different osmotic strengths. The members of one class of *N. crassa* mutants, known as os (osmosensitive) mutants, however, are sensitive to hyperosmotic pressure and are unable to grow on media supplemented with 4% NaCl (wt/vol) or 1 M sorbitol (25). Several os mutants, including os-1, os-2, os-3, os-4, os-5, os-6, and cut mutants, and the sorbose-resistant sor (T9) mutant have been described (25). Most os mutants have aberrant colony morphology on regular Vogel’s medium N and form sticky, close-cropped aerial hyphae. The aggregated hyphae are intensely pigmented and have a tendency to rupture and bleed (25). In addition, os mutants have reduced conidiation and altered cell wall compositions (10, 16).

Only one of the *N. crassa* os genes, the os-1 gene (also designated the nik-I gene), has been identified (1, 30). os-1 encodes a putative two-component histidine kinase that is homologous to the osmosensing Sln1p histidine kinase of *Saccharomyces cerevisiae*. The osmoregulation mediated by the yeast Sln1p, known as the high-osmolarity glycerol response (HOG), is well characterized (for a review, see reference 11). The major component of the HOG pathway is a mitogen-activated protein (MAP) kinase module consisting of one MAP kinase (Hog1p), one MAP kinase kinase (Pbs2p), and three MAP kinase kinase kinases (Ste11p, Ssk2p, and Ssk22p). The HOG1 MAP kinase kinase can be activated by either of two branches of upstream osmosensing pathways that converge at Pbs2p. One branch involves a two-component histidine kinase phosphorelay system comprised of Sln1p (24), Ypd1p (27), and Ssk1p (17). The other upstream osmosensing branch involves a putative membrane protein, Sho1p, that activates Pbs2p via Ste11p (17, 28). Hyperosmotic stresses activate the HOG pathway, which in turn increases the transcription of key enzymes involved in glycerol synthesis, including glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol-3-phosphatase (*HOR2*). Hyperosmotic stresses also reduce the permeability of the plasma membrane to glycerol, primarily by inhibiting the activity of the Fps1p glycerol transporter. The resulting cytosolic glycerol accumulation leads to increased internal osmolarity and restores the osmotic gradient between the cells and their environment. Yeast mutants defective in HOG1, PBS2, or *GPD1* are osmosensitive (12).

Fludioxonil and fenpiclonil are phenylpyrrole fungicides derived from the antibiotic pyrrolnitrin (9, 21). They are broad-spectrum fungicides used to control a variety of important plant-pathogenic fungi. Previous studies with *Fusarium sulphureum* Schlecht indicated that fenpiclonil inhibited the transport of monosaccharides and caused intracellular accumulation of polyols, such as glycerol and mannitol (13, 14). Wild-type *N. crassa* strains treated with fenpiclonil and fludioxonil at concentrations that inhibited fungal growth by 50% accumulated high concentrations of intracellular glycerol (26). Some *Ustilago maydis* and *F. sulphureum* mutants resistant to phenylpyrrole fungicides also were sensitive to high osmotic pressure (13, 14). These studies suggested that there is a relationship between osmoregulation and phenylpyrrole fungicide resistance.

To determine the relationship between osmoregulation and resistance to phenylpyrrole fungicides in filamentous fungi, in this study we cloned the *N. crassa* os-2 gene because os-2 mutants are resistant to phenylpyrrole fungicides. The os-2...
gene encodes a putative MAP kinase highly homologous to yeast Hog1p. We found that null mutations in os-2 confer resistance to phenylpyrinol but result in sensitivity to high osmotic stresses in N. crassa. In germinating conidia or germ tubes treated with fludioxonil, the fungicide stimulates glycerol accumulation and causes fungal cells to swell and burst. Our data suggest that os-2 is critical for osmoregulation in N. crassa and that phenylpyrinol fungicides may exert their fungicidal effects by interfering with the os-2 MAP kinase pathway.

MATERIALS AND METHODS

Strains and media. The os-2 DNA clone W08AS, the ordered pmOcosX N. crassa cosmid library (22), and all N. crassa strains were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City). Wild-type strains 74-OR23A-1 and 74-OR8-1a and os mutants AR2 (os-1), ALS10 (os-2), UCLA80 (os-3), UCLA93 (os-4), NM201o (os-5) were cultured on Vogel's medium N supplemented with 2% sucrose (VMS medium [6] at 30°C. S. cerevisiae strains YHP102 (MATα ura3-2 leu2-3 his3-1 ade2-1 trp1 his3 dpy30 trpl) and JBY10 (MATα ura3-2 leu2-3 his3 dpy30 trpl hoga12:TRP1) were provided by Michael Gustafson of Rice University (Houston, Tex.) and were cultured on YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C.

N. crassa transformation and sib selection. N. crassa was transformed as previously described by Vollmer and Yanofsky (33), with the following modifications. Instead of using regenerating top agar, we added 5 ml of 1 M sorbitol to the transformation mixture and then poured the mixture onto transformation bottom agar which contained Vogel's medium N supplemented with 2% sucrose, 0.05% fructose, 0.05% glucose, 1.5% agar, and in appropriate concentration of drug (VMSFG agar). The plates were dried with their lids ajar in a biosafety hood for at least 7 h. For sib selection, 228 cosmids clones specific for linkage group IV were propagated individually in 5 ml of Luria-Bertani broth overnight at 37°C. The cultures were combined into 11 pools, and cosmid DNAs were isolated from individual pools. Approximately 5 µg of DNA from each pool was used to transform UCLA80 (os-2). Transformation mixtures were poured onto 20 ml of solidified VMSFG agar containing 100 µg of hygromycin B per ml. After the plates were dry, they were overlaid with 20 ml of VMS medium containing 8% NaCl. We selected for transformants that could grow on VMS medium containing 4% NaCl and 50 µg of hygromycin B per ml since os-2 mutations are recessive and os-2 mutants are sensitive to hypertonic growth conditions (25).

Molecular techniques and sequence analysis. Standard protocols were used for routine molecular manipulations (29). DNA sequences were analyzed with DNASIS V.2.5 for Windows (Hitachi Software Engineering Co., Ltd.). Searches for homologues in DNA and protein sequence databases were performed with the BLAST programs (3). The three primer pairs were used for PCR amplification and sequencing of different regions of the os-2 alleles: HogN14 (5'-GCTGATCTGACCAGTTGC-3'), HogC18 (5'-GCTGATCTGACCAGTTGC-3'), and HogN18 (5'-GCTGATCTGACCAGTTGC-3'). These three primer pairs amplify PCR products only in transformants in which there is homologous recombination between pYZN16 and the native os-2 gene, because the sequences of both HogpN20 and HogC18 are outside the flanking sequences used to construct the gene replacement vector. Putative os-2 gene replacement isolates were purified by isolation of single conidia and were analyzed further by Southern blot hybridization.

Yeast complementation assay. The 1.6-kb SacI-KpnI fragment of DNA clone W08AS containing the entire os-2 open reading frame was inserted into pYES2 (Invitrogen, Carlsbad, Calif.). The resulting plasmid, pYZ11, was used to transform strains YHP102 and JBY10 with a yeast alkali-cation transformation kit (Bio 101, Inc., La Jolla, Calif.), and the resulting Ura+ transformants were transferred onto YPR (1% yeast extract, 2% peptone, 2% raffinose) agar. After overnight incubation at 30°C, these transformants were inoculated onto YPR agar, YPGR (1% yeast extract, 2% peptone, 1% raffinose, 1% galactose) agar, and YPD (1% yeast extract, 2% peptone, 2% dextrose) agar in the absence or presence of 4% NaCl. Yeast growth was examined after 2 days of incubation at 30°C.

Cytological and biochemical analyses of fludioxonil effects in N. crassa. Fludioxonil concentrations that resulted in a 50% reduction in growth (IC50) in different N. crassa strains were determined as previously described (26). To study the effect of fludioxonil on conidial germination, N. crassa conidia from wild-type strain 74-OR8-1a and mutant UCLA80 (os-2) were collected from 1-week-old cultures and resuspended at a concentration of 1 × 106 conidia/ml in VMS medium. Fludioxonil was added to each conidial suspension to a final concentration of 0.1, 0.4, or 81 µM (81 µM was equivalent to 20 ppm of fludioxonil). Since conidial germination was completely eliminated in 74-OR8-1a by treatment with 4 µM fludioxonil, the concentration of fludioxonil was decreased to 0.1 µM in order to observe reduced conidial germination and limited germ tube growth in the wild-type N. crassa strain. Samples were taken at time zero and after 2, 6, 8, and 24 h of fungicide treatment at 30°C and examined for conidial germination and swelling. To study the effect of fludioxonil on hyphal growth, conidial suspensions of wild-type strain 74-OR8-1a and mutant UCLA80 (os-2) containing 1 × 106 conidia/ml were incubated at 30°C for 6 h before fludioxonil was added at final concentrations of 0.1, 4, and 81 µM. Samples were taken at time zero and after 2, 6, 8, and 24 h of fungicide treatment at 30°C and examined for hyphal swelling and bursting. All samples were examined with an Eclipse E600 microscope (Nikon, Melville, N.Y.), and photographs were taken with Kodak Tmax 100 films (Eastman Kodak, Rochester, N.Y.). To study the effect of fludioxonil on the intracellular level of glycerol, N. crassa mycelia were treated with fludioxonil at concentrations of 0.1 µM for wild-type strain 74-OR8-1a (IC50, 0.1 µM), 0.5 µM for Os-2+ transformant YZ105 (IC50, 0.5 µM), and 100 µM for os-2 mutant UCLA80 (IC50 > 100 µM) and Δos-2 isolate YZ120 (IC50 > 100 µM). Glycerol concentrations were determined as described previously (26).

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of os-2 is AF297032.

RESULTS

N. crassa os-2 and os-5 mutants are resistant to phenylpyrinol fungicides. N. crassa os mutants and wild-type strains were tested to determine their abilities to grow on VMS medium containing 4% NaCl or phenylpyrinol fungicides, including fludioxonil and fenpiclonil (Table 1). As previously reported (25), none of the os mutants could grow on VMS medium containing 4% NaCl (Table 1). Also, os-1, os-4, and os-6 mutants did not grow on VMS medium containing fludioxonil or fenpiclonil. The os-6 mutant tested was isolated following fungal transformation (19), and the os-6 gene may be allelic to os-1, to which it is linked. We found that os-2 and os-5 mutants were able to grow on VMS medium containing fludioxonil or fenpiclonil (Table 1), and both os-2 and os-5 mutants were also resistant to vinclozolin, as previously reported (10, 11). On the other hand, os-1 mutants were resistant to vinclozolin but were sensitive to fludioxonil and fenpiclonil (Table 1).

Isolation of the os-2 gene. To clone the os-2 gene by sib selection, we used 228 linkage group IV specific clones from the N. crassa pmOcosX cosmid library to transform os-2 mutant UCLA80 because os-2 was mapped to the right arm of linkage group IV. Only one pool containing 24 cosmids (pool 8) gave rise to transformants that could grow on VMS medium.
containing 4% NaCl and 50 μg of hygromycin B per ml. After transforming os-2 mutant UCLA80 with individual clones from this pool, we found that cosmids G18G04 was the only clone that complemented os-2. This clone also complemented the other two os-2 mutants, ALS10 and UCLA93. Homokaryotic G18G04 transformants had wild-type morphology and grew on VMS medium containing 4% NaCl but were sensitive to flu-dioxonil and fenpiclonil (data not shown). In addition, cosmids G18G04 was mapped to the os-2 locus on linkage group IVR (Table 1) grown on VMS medium containing 150 μg of hygromycin B per ml (+Hyg), 4% NaCl (+NaCl), or 81 μM flu-dioxonil (+Fludioxonil) after 3 days of incubation at 30°C. The strains examined were wild-type (WT) strain 74-OR8-1a, os-2 mutant UCLA80, a transformant of UCLA80 transformed with the wild-type OS-2 gene (YZN1), and an os-2 gene replacement mutant (YZN70).

**TABLE 1. Osmotic sensitivity and fungicide resistance of *N. crassa* os-2 mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth in the presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4% (wt/vol) NaCl</td>
</tr>
<tr>
<td>74-OR23-1A (wild type)</td>
<td>+</td>
</tr>
<tr>
<td>74-OR8-1a (wild type)</td>
<td>+</td>
</tr>
<tr>
<td>AR2 (os-1)</td>
<td>−</td>
</tr>
<tr>
<td>ALS10 (os-2)</td>
<td>−</td>
</tr>
<tr>
<td>UCLA80 (os-2)</td>
<td>−</td>
</tr>
<tr>
<td>UCLA93 (os-2)</td>
<td>−</td>
</tr>
<tr>
<td>NM201o (os-4)</td>
<td>−</td>
</tr>
<tr>
<td>P5341 (os-5)</td>
<td>−</td>
</tr>
<tr>
<td>14-3 (os-6)</td>
<td>−</td>
</tr>
</tbody>
</table>

* All tests were done on VMS agar supplemented with 4% (wt/vol) NaCl, 81 μM fludioxonil, or 5 μM fenpiclonil. Fungal growth was scored after 2 days of incubation at 30°C. +, growth; −, no growth.

Since the Hog1p MAP kinase plays a key role in the yeast hyperosmotic response, we were interested in examining the function of a homologous *N. crassa* MAP kinase. A putative yeast HOG1 homologue in the *N. crassa* EST database generated at the University of New Mexico had been identified previously by a sequence similarity analysis (20). We acquired this cDNA clone, clone W08A8, from the Fungal Genetic Stock Center and used it as a probe to screen the *N. crassa* os-2 mutant UCLA80. Homokaryotic G18G04 transformants had wild-type morphology and grew on VMS medium containing 4% NaCl but were sensitive to flu-dioxonil and fenpiclonil (data not shown). In addition, cosmids G18G04 was mapped to the os-2 locus on linkage group IVR (Table 1) grown on VMS medium containing 150 μg of hygromycin B per ml (+Hyg), 4% NaCl (+NaCl), or 81 μM flu-dioxonil (+Fludioxonil) after 3 days of incubation at 30°C. The strains examined were wild-type (WT) strain 74-OR8-1a, os-2 mutant UCLA80, a transformant of UCLA80 transformed with the wild-type OS-2 gene (YZN1), and an os-2 gene replacement mutant (YZN70).

**Complementation of yeast hog1 with *N. crassa* os-2.** We cloned the entire os-2 open reading frame from cDNA clone W08A8 into the yeast expression vector pYES2 under GAL1 promoter control. The resulting construct, pYZN11, was transformed into *S. cerevisiae* Δhog1 strain JBY10 and isogenic HOG1 wild-type strain YPH102 (4). URA* transformants were examined to determine their abilities to grow on both YPD and YPRG agar plates in the presence or absence of 4% NaCl (Fig. 2). Transformants of JBY10 containing plasmid pYZN11 grew on media containing 4% NaCl, whereas JBY10 itself did not (Fig. 2). Thus, the *N. crassa* os-2 gene complemented the osmo-sensitive phenotype of yeast hog1 mutants. Interestingly, even though os-2 expression is under GAL1 promoter control, pYZN11 transformants of JBY10 grew on media containing 4% NaCl and either glucose (repressing conditions) or galactose (inducing conditions) as the carbon source (Fig. 2). These results were confirmed in three independent experiments, suggesting that the low level of expression of *N. crassa* os-2 under repressing conditions was sufficient to complement the hog1 defect in the yeast for growth on hypertonic medium.

**Null mutations in os-2 result in osmotic sensitivity and flu-dioxonil resistance.** We used five primer pairs, the HogN14-HogC13, HogN15-HogC1, HogN2-HogC3, HogN4-HogC2, and HogN18-HogC10 primer pairs, to PCR amplify different regions of the DNA sequences from the os-2 mutants (Table 1) and then sequenced the PCR products directly by using these primers. All mutations identified by the sequence analysis were verified by repeating the PCR and performing sequencing analysis at least once to rule out PCR and sequencing errors. The ALS10 allele had a 7-bp duplication at Thr97 that led to a frameshift (Fig. 3). The UCLA80 and UCLA93 alleles had nonsense point mutations (from TGG to TGA) at Trp188 and...
Trp198, respectively (Fig. 3). The mutations in all three os-2 alleles were located in the well-conserved protein kinase domains. Proteins encoded by these os-2 alleles were predicted to be truncated and most likely nonfunctional as MAP kinases. Our data indicated that a functional os-2 protein is essential for the response to hyperosmotic stress and for conferring fungicide sensitivity.

To further confirm that null mutations in os-2 were responsible for osmotic sensitivity and resistance to fungicides, we constructed an os-2 gene replacement vector (pYZN16) and transformed it into N. crassa wild-type strain 74-OR8-1a. After more than 100 hygromycin-resistant transformants were screened by performing PCR with the HogN20-Hph855R and HogC18-Hph856F primer pairs, one os-2 gene replacement mutant was identified. YZN17 is a homokaryotic isolate that was derived from this transformant after two rounds of single-conidium isolation. Deletion of the native os-2 gene in YZN17 was confirmed by Southern blot analysis using the PCR product amplified with HogN2 and HogC3 as the probe (data not shown). Like the os-2 null mutants, YZN70 produced short, cropped aerial hyphae and fewer conidia on VMS medium (data not shown). It was also resistant to flufoxonil and was unable to grow on VMS medium containing 4% NaCl (Fig. 1). These data confirmed that the N. crassa os-2 gene is essential for hyperosmoregulation and suggested that phenylpyrrole fungicides interfere with normal functions of the os-2 MAP kinase pathway.

**Effect of flufoxonil on N. crassa conidial and hyphal growth.**

We observed that flufoxonil dramatically inhibited conidial germination even at a concentration as low as 0.1 μM in wild-type N. crassa cultures. Untreated conidia germinated after 5 h of incubation (Fig. 4A) and formed hyphae within 24 h, but only short germ tubes were observed in conidia treated with 0.1 μM flufoxonil after 24 h. In conidia treated with higher concentrations of flufoxonil (4 and 81 μM), no germination was observed after 24 h of incubation. Instead, most conidia became swollen, and approximately 80% of the swollen conidia ruptured and released cytoplasmic contents (Fig. 4B). Fludioxonil also caused Botrytis cinerea conidia to burst after 24 h of treatment with a concentration of 4 μM (data not shown). However, conidia from os-2 mutants germinated and grew in VMS medium containing flufoxonil at concentrations up to 100 μM (the highest concentration tested).

We also examined the effect of flufoxonil on N. crassa hyphal growth. Conidia were grown in VMS medium at 30°C for 6 h before flufoxonil was added. At concentrations of 4 and 81 μM, flufoxonil inhibited germ tube elongation in the wild-type strain almost immediately after the fungicide was applied.

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**FIG. 2.** Complementation of S. cerevisiae Δhog1 mutant by the N. crassa os-2 gene. The same yeast strains, including YPH102 (HOG1), JBY10 (Δhog1), and YPH102 and JBY10 transformed with the os-2 cDNA construct pYZN11, were used to inoculate YPRG medium (top photograph) and YPD medium (bottom photograph). The YPRG and YPD plates on the right were supplemented with 4% NaCl. The photographs were taken after 3 days of incubation at 30°C.

**FIG. 3.** Map of the os-2 gene and three os-2 mutant alleles. The 5.98-kb genomic DNA fragment containing the os-2 gene and relevant restriction enzyme sites is shown. The thick line represents the transcribed region, and the potential transcription start site is indicated by an arrow. Introns are indicated by vertical bars. Mutations in the os-2 alleles are indicated by numbers on the map and are described below the map.

**os-2 alleles:**

1. ALS10: cttgtt → cttgcttctgtt
2. UCLA80: tgg (Trp188) → tga (stop)
3. UCLA93: tgg (Trp198) → tga (stop)

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N. CEREA os-2 GENE ENCODES HOG1 MAP KINASE HOMOLOGUE
added, while it only slightly inhibited hyphal growth at a concentration of 0.1 μM. Interestingly, 24 h after fludioxonil addition, the wild-type strain exhibited a different extent of hyphal swelling (Fig. 4D). Approximately 10 and 40% of wild-type hyphae treated with 4 and 81 μM fludioxonil, respectively, ruptured (Fig. 4D). Fludioxonil at the same concentrations did not inhibit hyphal growth or result in hyphal swelling and rupture in cultures of os-2 mutants (Fig. 4C).

**Effect of fludioxonil on intracellular glycerol accumulation.** It was reported previously that phenylpyrrole fungicide treatment stimulates glycerol accumulation in wild-type N. crassa strains (26). We measured the glycerol contents in os-2 mutant UCLA80, Δos-2 gene replacement strain YZN70, Os-2" rescued transformant YZN61, and wild-type strain 74-OR8-1a in the presence of 100, 100, 0.5, and 0.1 μM fludioxonil, respectively (Fig. 5). os-2 mutant UCLA80 and YZN70 accumulated little or no intracellular glycerol in response to fludioxonil treatment, whereas, like the wild type, YZN61 accumulated approximately 50 μg of glycerol per mg (dry weight) of mycelia when it was treated with fludioxonil. This response is similar to the hyperosmotic response in yeast mediated by activation of the Hog1p MAP kinase cascade. Fludioxonil treatment appears to activate the hyperosmotic response in N. crassa, and this activation is mediated by the os-2 MAP kinase.

**DISCUSSION**

Previous work showed that some N. crassa osmosensitive mutants, including os-1, os-2, os-4, and os-5 mutants, were resistant to dicarboximide fungicides, such as vinclozolin (10, 11). Even though dicarboximide fungicides have been on the market for a long time, their mode of action has not been clearly defined. In U. maydis, mutation in a Ser/Thr protein kinase homologous to the catalytic subunit of protein kinase A was reported to be responsible for vinclozolin resistance (23). In this study, we found that N. crassa os-2 and os-5 mutants were resistant to both phenylpyrrole fungicides and dicarboximide fungicides (Table 1). During preparation of this paper, os-2 and os-5 mutants were reported to be resistant to phenylpyrrole fungicides by another research group (7, 8). Assuming that these osmotic loci are involved in the same osmoregulation pathway (see below), these data indicate that dicarboximide and phenylpyrrole fungicides may interfere with different steps of the hyperosmoregulatory response in N. crassa.

To further understand the relationship between fungal osmoregulation and fungicide resistance, we cloned the N. crassa os-2 gene. os-2 encodes a MAP kinase homologous to yeast HOG1, which regulates responses, including glycerol accumulation, in yeast cells grown under hypertonic conditions (Fig. 1). Null mutations in os-2 resulted in osmosensitivity and fungicide resistance, and all os-2-associated phenotypes could be restored by reintroducing the wild-type os-2 gene (Fig. 1 and 3). The finding that the os-2 gene replacement transformants were sensitive to 4% NaCl and resistant to fludioxonil confirmed that a functional os-2 gene is essential for osmoregulation and for the fungicidal effect of phenylpyrrole compounds in N. crassa.

We think that N. crassa may have an osmotic response signal transduction pathway similar to that of yeast since os-2 encodes a MAP kinase homologous to yeast HOG1 and os-1 encodes an SLN1-like osmosensing histidine kinase (1, 30). In addition, we have found that os-5 encodes a MAP kinase kinase homol-
gous to *S. cerevisiae* PBS2 (J.-R. Xu and S. Lam, unpublished data). It is possible that other components of the osmoregulation pathway are encoded by other osmotic loci in *N. crassa*. However, it should be noted that *S. cerevisiae* is resistant to both phenylpyrrole and dicarboximide fungicides. Also, deletion of *SLN1* is lethal in yeast (18), while *N. crassa* os-1 deletion mutants are viable (1). These results indicate that there are differences between the *N. crassa* and yeast osmoregulation pathways, even though many of the components may be conserved.

Fludioxonil treatment of *N. crassa* wild-type strains causes the conidia and germ tubes to swell and rupture (Fig. 4) and also causes intracellular glycerol to accumulate in the wild-type strain but not in os-2 mutants (Fig. 5) (6). These observations led us to hypothesize that phenylpyrrole fungicides kill fungal cells by overactivating the os-2 osmoregulation pathway. In yeast, hyperactivation of the *HOG1* MAP kinase pathway by deletion of its upstream negative regulators *SLN1* and *YPD1* is lethal (16, 27). The mechanism of the hyperactive lethal effect in yeast (12) and *N. crassa* is not clear yet, but there are at least two mechanisms that could explain the lethal effect of an overactivated os-2 osmoregulation pathway. One possibility is that intracellular glycerol accumulation caused by fungicide treatment results in extremely high internal turgor pressure and causes fungal cells to swell and rupture. Alternatively, the *N. crassa* os-2 signal transduction pathway may be involved in other cellular processes, such as cell wall and/or cytoskeleton structure or cell cycle progression. In *S. cerevisiae*, the HOG pathway has been implicated in regulation of cell wall modification (15) and cytoskeleton reorganization (4) in response to hyperosmotic stresses. The *STY1* (*HOG1* homologue) MAP kinase pathway of *Schizosaccharomyces pombe* regulates responses to multiple stresses in addition to hyperosmolality (11, 33) and has roles in cell cycle control and sexual reproduction (31, 32). In *Candida albicans*, the MAP kinase homologous to *HOG1* is involved in cytokinesis, cell wall metabolism, and dimorphism (2, 34). The *N. crassa* os-1 gene is involved in hyphal development and regulation of cell wall assembly in response to changes in external osmolality (1). We noticed in this study that os-2 mutants produced fewer aerial hyphae and conidia on Vogel’s medium than the wild-type strains produced. Based on these observations, we think that the osmo-regulatory MAP kinase pathway in *N. crassa* may also have roles in cell wall metabolism and the establishment and elongation of polarized hyphal tip growth. Thus, overactivation of the os-2 pathway by fungicide treatment may lead to a weakened cell wall, abnormal cytokinesis, or aberrant cell cycle control.

Our data are not sufficient to determine whether Os-2 MAP kinase is the direct target of phenylpyrrole fungicides. An intracellular cAMP- and calcium-independent protein kinase isolated from *N. crassa* binds fenpiclonil in vitro (26). However, the identity of this protein kinase and its relationship with the os-2 pathway are unknown. Further characterization of the os-2 signal transduction pathway in *N. crassa* may help us identify the direct target(s) of phenylpyrrole fungicides. Finally, if our hypothesis that Os-2 is the direct target of phenylpyrrole fungicides is confirmed, this will be the first example of a fungicide that works not by inhibiting an intracellular function or pathway but by overstimulating it.

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**FIG. 5.** Intracellular glycerol contents of os-2 mutants treated with fludioxonil. All cultures were grown in liquid VMS medium. Fludioxonil was added to final concentrations of 0.1 μM for the wild type, 0.5 μM for os-2+ rescued transformant YZN61, and 100 μM for os-2 mutant UCLA80 and Δos-2 strain YZN70. Glycerol contents are expressed in micrograms of glycerol per milligram (dry weight [dw]) of mycelia. Symbols: ○, YZN61; □, wild-type strain 74-OR8-1a; △, os-2 mutant UCLA80; □, YZN70. The symbols indicate means based on three replicates, and the error bars indicate standard errors of the means.
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