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-1* 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 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*

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gene encodes a putative MAP kinase highly homologous to yeast Hog1p. We found that null mutations in *os-2* confer resistance to phenylpyrroles 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 phenylpyrrole fungicides may exert their fungicidal effects by interfering with the *os-2* MAP kinase pathway.

MATERIALS AND METHODS

Strains and media. The *os-2* cDNA clone W08A8, 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-OR23-1A and 74-OR8-1a and *os* mutants AR2 (*os-1*), ALS10 (*os-2*), UCLA80 (*os-2*), UCLA93 (*os-2*), NM201o (*os-4*), P5341 (*os-5*), and 14-3 (*os-6*) were cultured on Vogel's medium N agar plates supplemented with 2% sucrose (VMS medium [6]) at 30°C. *S. cerevisiae* strains YPH102 (*MATa ura3 leu2 his3 ade2 lys2 trp1*) and JBY10 (*MATa ura3 leu2 his3 ade2 lys2 trp1 hog1Δ::TRP1*) were provided by Michael Gustin 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 modification. 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% L-sorbose, 0.05% fructose, 0.05% glucose, 1.5% agar, and an appropriate selection drug (VMSFG agar). The plates were dried with their lids ajar in a biosafety hood for at least 7 h. For sib selection, 228 cosmid 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 3 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 agar 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). Pool 8 contained transformants resistant to 4% NaCl, and DNAs from all 24 cosmid clones in this pool were used to transform UCLA80 (*os-2*) individually.

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 following primer pairs were used for PCR amplification and sequencing of different regions of the *os-2* alleles: HogN14 (5'-AGAGGTATCCGAATTCCCGT-3') and HogC13 (5'-GTGAGGATTGCTGG AAGACG-3'); HogN15 (5'-CACTGAATCAAACCTCGCGT-3') and HogC1 (5'-TGGTTGGTGAGCTGGTCTTT-3'); HogN2 (5'-CTACCTTTGAGATCA CCTCG-3') and HogC3 (5'-TGCCAAGTGAGCATGATCTC-3'); HogN4 (5'-TGCGACTTGAAGATTGCGA-3') and HogC2 (5'-CTTCCATGTGTCGAC CGGGAG-3'); and HogN18 (5'-GAGTACCTTGCTCCTTACCA-3') and HogC10 (5'-ATCCTAACCCCAAACATCGC-3').

Construction of an *os-2* gene replacement vector and mutant screening. The *os-2* gene replacement vector pYZN16 was constructed with plasmid pBC KS(+) (Stratagene, La Jolla, Calif.) by ligating a 1.8-kb *SalI-EcoRI* *os-2* left flanking fragment, the 1.4-kb hygromycin phosphotransferase gene (*hph*), and a 1.7-kb *HpaI-BamHI* *os-2* right flanking fragment. Plasmid pYZN16 was used to transform *N. crassa* wild-type strain 74-OR8-1a. To avoid selecting against *os-2* gene replacement transformants, transformation mixtures were incubated at 30°C in regeneration solution (Vogel's medium N supplemented with 2% L-sorbose, 0.05% fructose, 0.05% glucose, and 1 M sorbitol) for 12 h to regenerate cell walls and then plated onto VMSFG agar without any osmotic stabilizer. Hygromycin-resistant transformants were screened for *os-2* gene replacement by performing PCR with primers HogN20 (5'-CCCAATTTCTAGCAAGG-3') and Hph855R (5'-GCTGATCTGACCAGTTGC-3') and with primers HogC18 (5'-AGTCTT CTGTGCGCTGTGT-3') and Hph856F (5'-GTCGATGCGACGCAATCGT-3'). These two 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 HogN20 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 cDNA clone W08A8 containing the entire *os-2* open reading frame was inserted into pYES2 (Invitrogen, Carlsbad, Calif.). The resulting plasmid, pYZN11, was used to transform strains YPH102 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, YPRG (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, 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 YZN61 (IC₅₀, 0.5 µM), and 100 µM for *os-2* mutant UCLA80 (IC₅₀, >100 µM) and *Δos-2* isolate YZN70 (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 phenylpyrrole fungicides.** *N. crassa os* mutants and wild-type strains were tested to determine their abilities to grow on VMS medium containing 4% NaCl or phenylpyrrole 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

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

Strain	Growth in the presence of ^a :		
	4% (wt/vol) NaCl	81 μ M fludioxonil	5 μ M fenpiclonil
74-OR23-1A (wild type)	+	—	—
74-OR8-1a (wild type)	+	—	—
AR2 (<i>os-1</i>)	—	—	—
ALS10 (<i>os-2</i>)	—	+	+
UCLA80 (<i>os-2</i>)	—	+	+
UCLA93 (<i>os-2</i>)	—	+	+
NM201o (<i>os-4</i>)	—	—	—
P5341 (<i>os-5</i>)	—	+	+
14-3 (<i>os-6</i>)	—	—	—

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

containing 4% NaCl and 50 μ g of hygromycin B per ml. After transforming *os-2* mutant UCLA80 with individual clones from this pool, we found that cosmid 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 fludioxonil and fenpiclonil (data not shown). In addition, cosmid G18G04 was mapped to the *os-2* locus on linkage group IVR by restriction fragment length polymorphism mapping (data not shown).

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* pMOcosX cosmid library. Interestingly, cosmid G18G04, the cosmid clone isolated by sib selection for complementing *os-2* mutants, was identified as a positive clone with a strong hybridization signal. This led us to speculate that the *N. crassa os-2* gene probably encodes a MAP kinase homologous to the yeast Hog1p.

We cloned and sequenced the 3.8- and 2.2-kb *Sal*I fragments that hybridized to cDNA clone W08A8 from cosmid G18G04. Sequence analysis of the genomic and cDNA clones revealed an open reading frame that encoded a protein that was 83% identical and 90% similar to *S. cerevisiae* Hog1p and 85% identical and 93% similar to *Schizosaccharomyces pombe* Sty1p. A *Bsr*GI-*Sac*I fragment from the two *Sal*I fragments containing the gene was then cloned into pCB1004 (5). The resulting plasmid, pYZN12, was used to transform *os-2* mutant UCLA80. Hygromycin-resistant transformants generated from all three *os-2* mutants (Table 1) grew on VMS medium containing 4% NaCl (Fig. 1 and data not shown). These transformants, including YZN61, also became sensitive to fludioxonil upon purification for homokaryons (Fig. 1). Thus, we concluded that the *os-2* gene encodes a MAP kinase homologous to yeast Hog1p.

FIG. 1. Growth of *N. crassa os-2* mutants. Fungal growth was examined on VMS medium supplemented with 150 μ g of hygromycin B per ml (+Hyg), 4% NaCl (+NaCl), or 81 μ M fludioxonil (+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 *GAL*I promoter control. The resulting construct, pYZN11, was transformed into *S. cerevisiae* $\Delta hog1$ 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 osmosensitive phenotype of yeast *hog1* mutants. Interestingly, even though *os-2* expression is under *GAL*I 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 fludioxonil 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

FIG. 2. Complementation of *S. cerevisiae* $\Delta hog1$ mutant by the *N. crassa os-2* gene. The same yeast strains, including YPH102 (*HOG1*), JBY10 ($\Delta hog1$), 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 YPG plates on the right were supplemented with 4% NaCl. The photographs were taken after 3 days of incubation at 30°C.

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 fludioxonil 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 fludioxonil on *N. crassa* conidial and hyphal growth. We observed that fludioxonil dramatically inhibited conidial germination even at a concentration as low as 0.1 μ M 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 μ M fludioxonil after 24 h. In conidia treated with higher concentrations of fludioxonil (4 and 81 μ M), 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 μ M (data not shown). However, conidia from *os-2* mutants germinated and grew in VMS medium containing fludioxonil at concentrations up to 100 μ M (the highest concentration tested).

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

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.

FIG. 4. Effect of fludioxonil treatment on *N. crassa* conidial germination and hyphal growth. (A) Untreated wild-type *N. crassa* conidia. (B and D) Ungerminated and germinated conidia, respectively, incubated in VMS medium containing 81 μ M fludioxonil for 24 h at 30°C. (C) Germ tubes of *os-2* mutant UCLA80 incubated in VMS medium containing 81 μ M fludioxonil for 24 h at 30°C. Scale bars = 20 μ m.

added, while it only slightly inhibited hyphal growth at a concentration of 0.1 μ M. 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 μ M 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, $\Delta os-2$ 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 μ M 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 μ g 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 homolo-

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 μ M for the wild type, 0.5 μ M for *os-2*⁺ rescued transformant YZN61, and 100 μ M for *os-2* mutant UCLA80 and Δ *os-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.

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 hyperosmolarity (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 osmolarity (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 osmoregulatory 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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