

Group III Histidine Kinase Is a Positive Regulator of Hog1-Type Mitogen-Activated Protein Kinase in Filamentous Fungi

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We previously reported that the group III histidine kinase Dic1p in the maize pathogen *Cochliobolus heterostrophus* is involved in resistance to dicarboximide and phenylpyrrole fungicides and in osmotic adaptation. In addition, exposure to the phenylpyrrole fungicide fludioxonil led to improper activation of Hog1-type mitogen-activated protein kinases (MAPKs) in some phytopathogenic fungi, including *C. heterostrophus*. Here we report, for the first time, the relationship between the group III histidine kinase and Hog1-related MAPK: group III histidine kinase is a positive regulator of Hog1-related MAPK in filamentous fungi. The phosphorylation pattern of *C. heterostrophus* BmHog1p (Hog1-type MAPK) was analyzed in wild-type and *dic1*-deficient strains by Western blotting. In the wild-type strain, phosphorylated BmHog1p was detected after exposure to both iprodione and fludioxonil at a concentration of 1 μ g/ml. In the *dic1*-deficient strains, phosphorylated BmHog1p was not detected after exposure to 10 μ g/ml of the fungicides. In response to osmotic stress (0.4 M KCl), a trace of phosphorylated BmHog1p was found in the *dic1*-deficient strains, whereas the band representing active BmHog1p was clearly detected in the wild-type strain. Similar results were obtained for *Neurospora crassa* Os-2p MAPK phosphorylation in the mutant of the group III histidine kinase gene *os-1*. These results indicate that group III histidine kinase positively regulates the activation of Hog1-type MAPKs in filamentous fungi. Notably, the Hog1-type MAPKs were activated at high fungicide (100 μ g/ml) and osmotic stress (0.8 M KCl) levels in the histidine kinase mutants of both fungi, suggesting that another signaling pathway activates Hog1-type MAPKs in these conditions.

All living cells must appropriately sense and respond to various environmental stimuli. In eukaryotic cells, the mitogen-activated protein (MAP) kinase cascades are vital to the intracellular transmission of a variety of extracellular signals and to the regulation of growth and differentiation processes (7, 36). The MAP kinase cascades generally consist of three protein kinases that act in series: MAP kinase (MAPK), MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). In mammalian cells, a variety of receptor types, such as G-protein-coupled receptors, receptor tyrosine kinases, and integral membrane proteins, are known to perceive the extracellular signals and then promote the activation of MAPK cascades (36). The activated MAPK phosphorylates substrates, which include transcriptional activators, resulting in altered patterns of gene expression and protein activity.

Cellular signaling pathways that are mediated by MAPK cascades have also been characterized in many fungi (9, 21, 22, 23, 33, 42, 44, 47). In *Neurospora crassa*, for example, three MAPK modules have been identified by genome sequence analysis. *os-2* mutants of *N. crassa*, which contain a null mutation in the MAPK gene that is homologous to *Saccharomyces cerevisiae* HOG1, were found to be unable to adapt to high osmolarity (47). Also, a Mak-2p MAPK of *N. crassa* that is related to the *S. cerevisiae* Fus3p/Kss1p was found to be re-

quired for hyphal fusion events (33). As another example, *Colletotrichum lagenarium*, which causes cucumber anthracnose, was discovered to have a Maf1p MAPK that is related to *S. cerevisiae* Mpk1p (Slr2p) and a Cmk1p MAPK that is related to *S. cerevisiae* Fus3p/Kss1p, which regulate diverse aspects of fungal pathogenesis (22, 42). Despite extensive studies of the functions of individual MAPKs in filamentous fungi, many unsolved problems remain concerning the signaling mechanisms of MAPK cascades. The upstream activators acting on the cascade and the mechanisms of dephosphorylation of MAPK modules are still unclear.

In contrast to eukaryotes, prokaryotic organisms commonly employ a His-Asp phosphorelay signaling strategy known as the two-component regulatory system (3, 34, 41). The prototypical system is composed of a histidine protein kinase and a response regulator protein. Extracellular signals are sensed by a histidine kinase that transfers a phosphoryl group to a response regulator, which results in the activation of a downstream effector that elicits the specific response. Although some eukaryotes, especially fungi, also use two-component signaling systems, the two proteins (a histidine kinase and a response regulator) are typically fused (1, 6, 32).

The results of fungal genome sequencing projects (<http://www.broad.mit.edu>) and recent studies (5, 16) have revealed that filamentous ascomycetes, such as the model filamentous fungi *N. crassa* and *Aspergillus nidulans*, and phytopathogenic fungi, such as *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*), *Cochliobolus heterostrophus* (anamorph: *Bipolaris maydis*), *Gibberella moniliformis*, and *Magnaporthe grisea*, possess several genes for hybrid histidine kinases, which are typically classified into eleven groups. Among them, six groups (III, V, VI,

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VIII, IX, and X) contain histidine kinases that are highly conserved in those fungal species, and other groups are more divergent (5). To date, however, there have been few reports regarding the functional analysis of the histidine kinase genes in filamentous fungi except for the group III histidine kinase gene.

The group III histidine kinases, e.g., *N. crassa* Os-1p (also known as Nik1p), *B. fuckeliana* Daf1p (also known as BcOs1p), and *C. heterostrophus* Dic1p (also known as ChNik1p), are characterized by a unique N-terminal region consisting of a HAMP domain repeat (5). The mutation of these histidine kinase genes resulted in resistance to the fungicides phenylpyrrole and dicarboximide and also in increased osmosensitivity (8, 30, 45, 46). In *C. heterostrophus*, null mutants for *dic1* and mutants with a deletion or point mutation in the HAMP domain repeat were highly sensitive to osmotic stress and highly resistant to the previously mentioned fungicides (46). Similar effects resulting from mutations were observed in *N. crassa* *os-1* and *B. fuckeliana* *daf1* (8, 30). A single amino acid change within the kinase domain or the regulator domain of *C. heterostrophus* *dic1* altered the sensitivity to osmotic stress and conferred a moderate resistance to the fungicides. Thus, the group III histidine kinase is considered to be a putative osmosensor (38). However, no membrane-spanning domain is predicted in the group III histidine kinase, so the protein is likely cytoplasmic. The precise function and mechanism of the group III histidine kinase-mediated response to extracellular osmotic stress are still obscure.

In *S. cerevisiae*, osmotic stress is processed by the osmoregulatory system HOG (high-osmolarity glycerol) pathway MAPK cascade that is controlled by a His-Asp phosphorelay signaling system employing the group VI histidine kinase Sln1p (19, 20). *Sln1* is the sole histidine kinase occurring in *S. cerevisiae* and is essential. The group VI histidine kinases are also conserved and are found in several fungal species (5). However, the mutant of the *Sln1* orthologue in *A. nidulans*, *TcsB*, has no significant alterations to its phenotype (15), yielding no obvious clue as to the function of the *Sln1* homologue in filamentous fungi.

Osmotic stress in filamentous fungi also appears to be processed by the Hog1-related MAPK cascade. The *os-2* and *osc1* MAPK gene mutants, which are orthologues of *S. cerevisiae* *HOG1* in *N. crassa* and *C. lagenarium*, respectively, were found to be sensitive to high osmolarity (23, 47). The *Osc1p* MAPK was phosphorylated and accumulated in the nucleus under high osmotic conditions, indicating the activation of the *Osc1p* by high osmotic stress (23). The mutants also showed increased resistance to phenylpyrrole and dicarboximide fungicides similar to that of the group III histidine kinase mutant (23, 47). Furthermore, these fungicides led to the improper activation of Hog1-related MAPKs in *C. lagenarium*, *C. heterostrophus*, and *B. cinerea* (23), which indicates that an osmotic stimulus is mediated by the group III histidine kinase and Hog1-related MAPK pathways in filamentous fungi and that the fungicides interfere with those osmoregulatory systems. However, the histidine kinases have not been proven to regulate the activation of the Hog1-related MAPK pathway, and the mechanism of the fungicide-induced, inappropriate activation of the Hog1-related MAPKs is unknown.

In this study, we determined a specific signature pattern of Hog1-related MAPK phosphorylation in *C. heterostrophus* wild-type and *dic1*-deficient strains after the cells were exposed to either osmotic stress or dicarboximide and phenylpyrrole fungicides. In addition to *C. heterostrophus*, *N. crassa* Os-2p MAPK activation in wild-type and *os-1* strains was also assessed. The results revealed that the group III histidine kinases Dic1p and Os-1p were positive regulators of Hog1-related MAPK in each fungal species. This indicates that osmotic signal transmission in *C. heterostrophus* and *N. crassa* is regulated differently from that of *S. cerevisiae* because Sln1p, which is the sole histidine kinase in *S. cerevisiae*, negatively regulates the activation of the HOG pathway. Furthermore, we also demonstrated that these fungi possessed another upstream activator of Hog1-related MAPK.

MATERIALS AND METHODS

Strains and growth conditions. The *C. heterostrophus* strain HITO7711 was used as the wild-type strain. The dicarboximide- and fludioxonil-resistant strains E4504, E4509, N9006, and N9010 were obtained in our previous work (45). Strains E4504, N9006, and N9010 had *dic1* null mutations resulting from premature termination due to the presence of the stop codon at repeat 5 of the HAMP domain (E4504), or frameshifts within repeat 1 of the HAMP domain (E4504) or after the H-box of the kinase domain (N9010) (46).

The mutant DHOG101, which has a disrupted Hog1-related MAPK gene, *BmHOG1* (GenBank accession no. AB208438), was generated as described following. The *BmHOG1* was cloned using the degenerate PCR and the inverse PCR methods. The *BmHOG1* of the wild-type strain was disrupted using the targeted insertion method by homologous recombination. The DNA fragment (from the 548th to the 1762nd nucleotide in the open reading frame), which not contained part of 5' and 3' coding region of *BmHOG1*, was amplified by PCR. The amplified fragment was introduced into the plasmid, pCB1004, containing the *hph* gene. Fungal transformation was performed using a method described previously (46). Genomic DNA from the resulting transformants was extracted and Southern analysis was performed to confirm occurring homologous recombination event (data not shown). One of the resulting disruptants, DHOG101, was used as the *BmHOG1*-disrupted mutant.

All *C. heterostrophus* strains were maintained on complete medium agar (CMA) at 25°C. CMA contains (per liter): 1.5 g Ca(NO₃)₂ · 4H₂O, 0.5 g MgSO₄ · 7H₂O, 0.5 g KCl, 0.4 g KH₂PO₄, 30 mg K₂HPO₄, 10 g glucose, 1.0 g tryptone, 1.0 g yeast extract, and 15 g agar. All *N. crassa* strains were obtained from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, KS). The wild-type strain 74-OR8-1a and the *os* mutants NM233t (*os-1*), Y256M209 (*os-1*), UCLA80 (*os-2*), and UCLA93 (*os-2*) were grown at 25°C on agar-solidified Vogel's medium N plus 1.5% (wt/vol) sucrose (43). To prepare the vegetative mycelia for analysis of the phosphorylation of Hog1-related MAPKs, *C. heterostrophus* and *N. crassa* were incubated in liquid complete medium (CM; CMA minus agar) and Vogel's liquid medium N plus sucrose, respectively. The fungicides used in this study were iprodione (Rovral WP, 50% active ingredient; Aventis Crop Sci.), and fludioxonil (Sapphire flowable, 20% active ingredient; Novartis Agro) and were obtained commercially. The fungicides were added to the media from 1000-fold concentrated stock solutions in 70% ethanol (iprodione) or dimethyl sulfoxide (fludioxonil).

Southern blot and RT-PCR analysis. The total DNA of *C. heterostrophus* was isolated using the method of Nakada et al. (29). DNA digestion and gel electrophoresis were performed according to standard methods (37). The gene fragments amplified by PCR using the primer sets ProbeA (5'-TTTGAGCAG GTGATTGAGAGTTTG-3' as forward and 5'-TGCATGAATGTGCTCGTCA CAAT-3' as reverse) and ProbeB (5'-ACCACAGTTTACCTCCAATT CA-3' as forward and 5'-ATACCGTTTCATTTCTCAGTGAG-3' as reverse) were used as DNA probes. DNA probes were labeled using the AlkPhos Direct labeling kit (Amersham Biosciences) according to the manufacturer's instructions. Hybridization and detection were carried out according to the manufacturer's instructions with the AlkPhos Direct detection system (Amersham Biosciences).

RNA isolation and first-strand cDNA synthesis were performed according to the previously described method (46). Three primer sets, PS-A (5'-AAGGAA ATGGACCCTGAAATCACTCTCT-3' as forward and 5'-GTTTACAGTGAG TTCATTCCACATG-3' as reverse), PS-B (5'-AACCATATTCAACGCTCT

CAAGTC-3' as forward and 5'-CGAGATGCCATTTTCCAATGCAGAT-3' as reverse), and GPD (5'-TACGTCGTCGAGTCTACCGGTGTCT-3' as forward and 5'-GTCACTGGAAACAATGTCATCCTCAGT-3' as reverse), were used to amplify the fragments of cDNA from wild-type and *dic1*-mutant strains of *C. heterostrophus*.

Western blot analysis. The phosphorylation of Hog1-related MAPKs in *C. heterostrophus* and *N. crassa* was examined using a Western blot analysis with an anti-dually phosphorylated p38 antibody (Cell Signaling Technology, Beverly, MA). Total protein samples were isolated as described below. Mycelia of each strain were incubated for 2 days in CM or liquid Vogel's medium at 25°C and were filtered through 4-layer gauze. The mycelia were then ground with a mortar and pestle to a fine powder under liquid nitrogen. Ice-chilled buffer containing protease and phosphatase inhibitors [50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1 mM sodium vanadate, protease inhibitor cocktail set (Roche, 1 tablet per 50 ml)] was added to the powder, and the suspensions were sonicated for 20 s to promote solubilization. After centrifuging the suspensions at 8,000 × g for 3 min, the resulting supernatants were separated on 10% polyacrylamide gels and blotted onto nitrocellulose membranes. The concentration of total proteins was calculated using a BCA protein assay reagent (Pierce), and 50 µg of proteins was loaded into each well. Anti-Hog1p antibody was purchased from Santa Cruz Biotechnology (C-terminal anti-Hog1), and antibody binding was visualized using an ECL Plus Western blotting detection reagent (Amersham Biosciences) after the binding of a horseradish peroxidase-conjugated secondary antibody.

RESULTS

Histidine kinase gene *dic1* was not expressed in the E4509 strain of *C. heterostrophus*. To better understand the relationships between the group III histidine kinase Dic1p and Hog1-related MAPK, it was necessary to assess MAPK activation in the cells that did not produce the Dic1 protein. Therefore, we first attempted to find a mutant with a completely defective *dic1* gene. We had previously isolated several *dic1* mutant strains by chemical mutageneses (45). Among these, the E4509 strain was likely to have a deletion in the *dic1* locus, since no fragment was amplified by PCR using the primer sets Dic1F and Dic1R designed for the *DIC1* coding region (46). But it was not clear whether this was a null mutant. To determine if the *Dic1* gene in the E4509 strain was null, Southern blot and RT-PCR analyses were performed.

For the Southern blot analyses, DNA fragments containing parts of 5'-flanking region and *Dic1* open reading frame (probe A: 2,506 bp in the upstream sequence and 542 bp in the *dic1* ORF, probe B: 516 bp in the upstream sequence and 1,675 bp in the *DIC1* ORF) were constructed by PCR (Fig. 1A). Genomic DNA was isolated from the wild-type HITO7711 and the *dic1*-deficient strain E4509. The isolated genomic DNA was digested with EcoRV and HindIII, and hybridized with probe A. The results showed that HITO7711 contained the 8.0-kb EcoRV and 4.4-kb HindIII fragments (Fig. 1B left, lanes 1 and 3), but these fragments were not found in E4509. In contrast, the E4509 strain contained other truncated fragments (Fig. 1B left, lanes 2 and 4). In addition, no band was detected in the same membrane, using probe B in E4509 (Fig. 1B right, lanes 2 and 4). These gel blot analyses indicate that at least 2,191 bp fragment lying across the 5' flanking region and *Dic1* open reading frame is completely deleted in the E4509 strain.

For RT-PCR analyses, two primer sets, PS-A and PS-B, were designed for the 5' and 3' ends of the *DIC1* coding region, respectively. As shown in Fig. 1C, no band was detected in the E4509 strain, but the HITO7711 strain contained frag-

ments that were consistent with the expected length. These data indicate that the histidine kinase gene *dic1* was not expressed in the E4509 strain.

Phosphorylation of Hog1-type MAP kinase in wild-type and *dic1*-deficient *C. heterostrophus*. To investigate the phosphorylation of Hog1-related MAPK in *C. heterostrophus*, we first cloned the Hog1-related MAP kinase gene (*BmHOG1*: GenBank accession no. AB208438) and disrupted the gene by the homologous recombination method (see Materials and Methods). The phosphorylation of the BmHog1p MAPK was analyzed by Western blotting using an antibody that specifically recognized the phosphorylated forms of p38-type kinase. A duplicate blot was incubated with an antibody raised against the C terminus of Hog1.

The Hog1 antibody detected several bands in the mycelia of *C. heterostrophus*. A 43-kDa band was missing in the mycelia of the *BmHOG1*-deleted mutant DHOG101, whereas this band was detected in the mycelia of the wild-type HITO7711, suggesting that this band represented the BmHog1p MAPK (Fig. 2A). The anti-phospho-p38 antibody clearly detected a band in HITO7711 mycelia after exposure to 0.4 or 0.8 M KCl for 10 min but not in the absence of osmotic stress. The size of the detected band was identical to the size of the band detected by the Hog1 antibody, indicating that the product detected by the anti-phospho-p38 antibody represented the phosphorylated BmHog1p MAPK. NaCl and sorbitol treatment also caused phosphorylation of BmHog1p to the same extent as did KCl treatment (data not shown), which suggests that BmHog1p MAPK was activated under high osmotic stress. The amount of phosphorylated BmHog1p was similar at 10, 30, and 60 min after the exposure to high osmotic stress was initiated (Fig. 2B). The phosphorylation of Hog1-related MAPK was analyzed after a 10-min exposure to the stress compounds.

To investigate whether the group III histidine kinase Dic1p regulates the phosphorylation of BmHog1p MAPK, the phosphorylation of BmHog1p in the E4509 strain was also analyzed (Fig. 2A). As in the wild-type strain, phosphorylated BmHog1p was not detected in E4509 mycelia that were not exposed to osmotic stress. In contrast to the findings in the wild-type strain, some phosphorylated BmHog1p was detected in the mycelia of E4509 exposed to 0.4 M KCl. At the same condition, a trace of phosphorylated BmHog1p was detectable in other *dic1* null strains E4504, N9006, and N9010 (Fig. 4A and data not shown). These data indicate that the histidine kinase Dic1p positively regulates the activation of BmHog1p MAPK. Of note, BmHog1p activation in the *dic1*-deficient strains was seen at a high level of osmotic stress (0.8 M KCl) (Fig. 2A and 4A), suggesting that BmHog1p activation was also controlled by another upstream activator.

We previously reported that phosphorylation of a Hog1-related MAPK was induced by the phenylpyrrole fungicide fludioxonil in certain phytopathogenic fungi, such as *C. heterostrophus* (23). *C. heterostrophus* mutants that were resistant to dicarboximide showed a positive cross-resistance to phenylpyrrole fungicide (45). Thus, we examined whether phosphorylation of BmHog1p was stimulated by dicarboximide fungicides. As shown in Fig. 3A, the dicarboximide fungicide iprodione also led to the activation of BmHog1p MAPK in the wild-type strain, indicating that iprodione and fludioxonil had similar effects on the activation of Hog1-related MAPK.

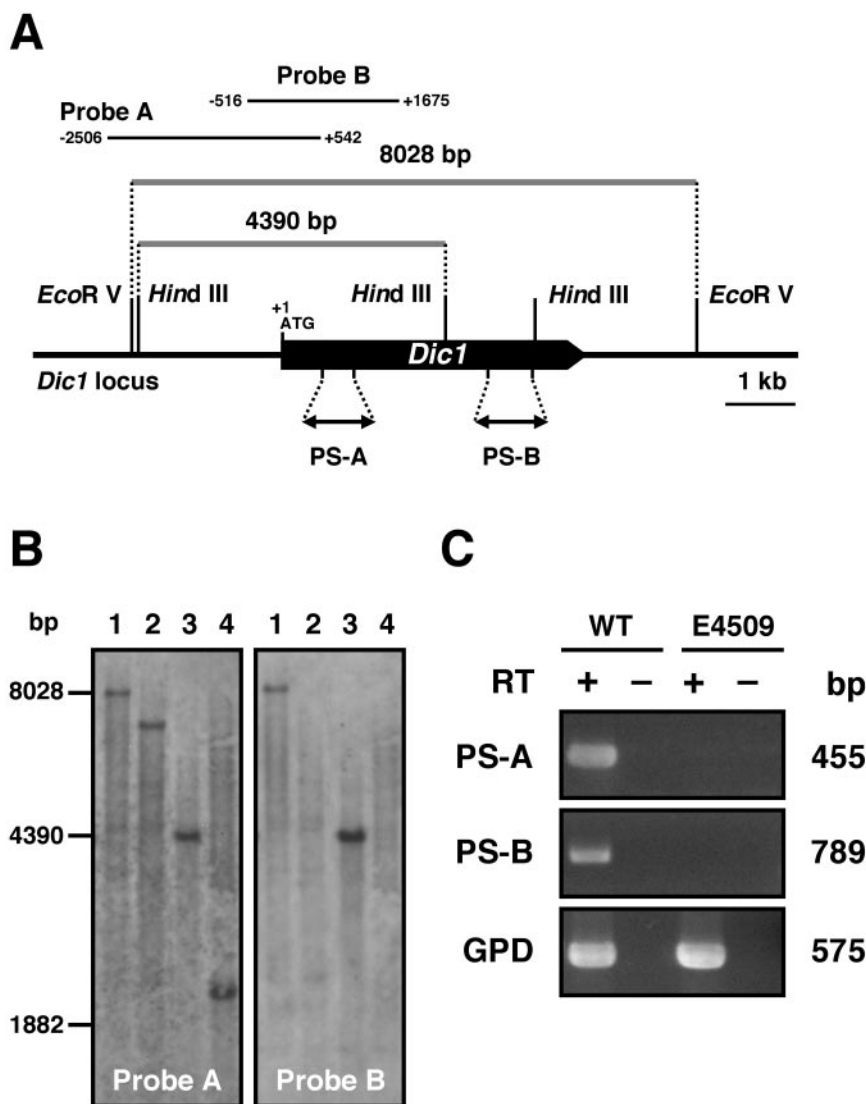


FIG. 1. *dic1* deficiency in strain E4509 of *C. heterostrophus*. (A) Schematic representation of the chromosomal *DIC1* locus in the wild-type strain HITO7711. The restriction enzyme sites, the point at which the probes hybridize, and the site amplified by RT-PCR are indicated. (B) Southern analysis of the *dic1* locus in the wild-type and *dic1* mutant strains. Chromosomal DNA of the wild-type strain HITO7711 (lanes 1 and 3) and of the *dic1* mutant strain E4509 (lanes 2 and 4) was digested with EcoRV (lanes 1 and 2) or HindIII (lanes 3 and 4). An approximately 3-kb DNA fragment was first used as the probe (probe A), and, after reprobing, an approximately 2-kb DNA fragment (probe B) was hybridized. (C) *Dic1* gene expression in the wild-type and *dic1*-deficient strains. RNA isolated from the wild-type strain HITO7711 (WT) and the *dic1*-deficient strain E4509 was used for cDNA synthesis. The cDNA synthesized (+) or not synthesized (-) with reverse transcriptase was used as the template for RT-PCR. The RT-PCR products amplified by two specific primer sets (PS-A and PS-B) and the primers for the *C. heterostrophus* *GPD* gene, a positive control, were separated by electrophoresis.

Next, we compared the patterns of BmHog1p phosphorylation by iprodione in wild-type and *dic1*-defective strains (Fig. 3B and 4B). In the mycelia of the wild-type strain, BmHog1p was phosphorylated after exposure to iprodione at a concentration as low as 1 $\mu\text{g/ml}$. In the mycelia of *dic1*-deficient strains, phosphorylated BmHog1p was not observed following exposure to less 10 $\mu\text{g/ml}$ of iprodione. This indicates that BmHog1p MAPK is activated through Dic1p by iprodione at a concentration of 1 $\mu\text{g/ml}$. In contrast, BmHog1p phosphorylation was detected in the mycelia of *dic1*-deficient strains that were treated with 100 $\mu\text{g/ml}$ iprodione, demonstrating that high concentrations of iprodione can cause activation of BmHog1p in cells without Dic1p histidine kinase.

Phosphorylation of Os-2p MAP kinase in the wild-type and *os-1* mutant of *N. crassa*. The *N. crassa* osmotic-sensitive mutants *os-1*, *os-2*, *os-4*, and *os-5* are resistant to dicarboximide and phenylpyrrole fungicides (13, 14, 18). Schumacher et al. (36) reported that the *os-1* gene encodes the putative histidine kinase, which was identical to the *NIK1* gene product reported by Alex et al. (1). It was reported that the *os-2* gene encodes a MAPK that is orthologous to *S. cerevisiae* Hog1p (47). To examine whether Os-2p MAPK activation was induced by the fungicides and was controlled by the histidine kinase Os-1p, we determined the levels of active, phosphorylated Os-2p in the wild-type and the *os-1* mutant strains after exposure to the fungicides and osmotic stress (Fig. 5). A protein with a molec-

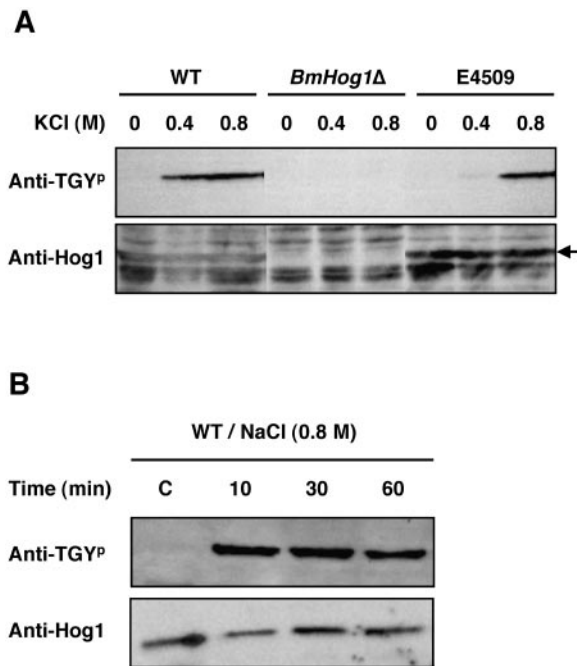


FIG. 2. Phosphorylation of *C. heterostrophus* BmHog1p MAPK induced by osmotic stress. (A) BmHog1p phosphorylation in the wild-type strain, the *BmHOG1* disruption strain, and the E4509 strain. Mycelia of the strains HITO7711 (WT), DHOG101 (*BmHOG1Δ*), and E4509 were used for analysis. Prepared mycelia of the strain tested were incubated in CM with or without 0.4 and 0.8 M KCl for 10 min. The cells were harvested, and total protein extracts were prepared as described in Materials and Methods. Protein samples (50 μ g) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes for Western blot analysis. Phosphorylated BmHog1p was detected using anti-dually phosphorylated p38 antibody (indicated by anti-TGYP^P). The total amount of BmHog1p was measured using anti-Hog1 C terminus antibody (indicated by anti-Hog1). (B) Continuous activation of BmHog1p by NaCl in the wild-type strain. Mycelia of HITO7711 (WT) were incubated in CM with 0.8 M NaCl for 10, 30, and 60 min. Prepared mycelia were incubated in CM without NaCl as a control (indicated by C). The procedures for Western blot analysis were the same as those described above.

ular weight similar to that of BmHog1p was clearly detected in the mycelia of the wild-type strain 74-OR8-1a after exposure to iprodione and fludioxonil, but was not present in the unexposed mycelia or in the UCLA80 strain (Fig. 5A). The UCLA80 strain was null with nonsense point mutations in the *os-2* gene (47). The same results were obtained using another *os-2* null mutant strain, UCLA93 (data not shown). These results suggest that the protein detected by the anti-phospho-p38 antibody represents the phosphorylated Os-2p MAPK in *N. crassa*.

In the *os-1* strain Y256M209, which has a null mutation at the *os-1* locus (30), the phosphorylated Os-2p was not or barely detectable following exposure to 10 μ g/ml iprodione and fludioxonil. Phosphorylated Os-2p was detectable in the *os-1* strain after exposure to 100 μ g/ml of the fungicides, but the amount of phosphorylated Os-2p was significantly reduced in the mutant strain compared with that in the wild-type strain. Similarly, phosphorylated Os-2p was clearly detected in the mycelia of the wild-type strain after exposure to 0.4 M KCl (Fig. 5B) and was also detected in the mycelia of the *os-1* strain

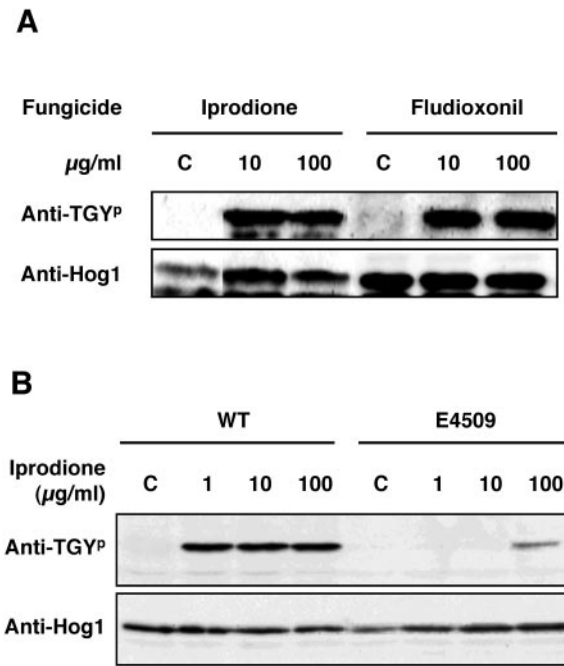


FIG. 3. Phosphorylation of the *C. heterostrophus* BmHog1p MAPK by iprodione and fludioxonil. (A) BmHog1p phosphorylation by iprodione and fludioxonil in the wild-type strain. Mycelia of HITO7711 (WT) were incubated in CM with 10 or 100 μ g/ml iprodione or fludioxonil for 10 min. Prepared mycelia were incubated in CM with ethanol and dimethyl sulfoxide as a control (indicated by C). The cells were harvested, and total protein extracts were prepared as described in Materials and Methods. Protein samples (50 μ g) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes for Western blot analysis. Phosphorylated BmHog1p was detected using anti-dually phosphorylated p38 antibody (indicated by anti-TGYP^P). The total amount of BmHog1p was measured using anti-Hog1 C terminus antibody (indicated by anti-Hog1). (B) BmHog1p phosphorylation by iprodione in the wild-type strain and the *dic1*-deficient strain. Mycelia of HITO7711 (WT) and E4509 were used for analysis. Prepared mycelia were incubated in CM with 1, 10, or 100 μ g/ml iprodione for 10 min. Prepared mycelia of the strain tested were incubated in CM without iprodione as a control (indicated by C). The procedures for Western blot analysis were the same as those described above.

under the KCl treatment, but the amount of phosphorylated protein detected in the mutant strain was significantly reduced compared with that in the wild type. Similar results were obtained using the *os-1* null mutant strain NM233t (data not shown). These results suggest that the activation of Os-2p is positively regulated by the histidine kinase Os-1p/Nik1p.

DISCUSSION

We analyzed the phosphorylation pattern of the Hog1-type MAPK BmHog1p in *C. heterostrophus* wild-type and *dic1*-deficient strains after exposure to osmotic stress or dicarboximide and phenylpyrrole fungicides. The results revealed that the activation of BmHog1p-MAPK was positively regulated by the protein encoded by *DIC1* (Fig. 6A). Similar results were obtained from the analyses of phosphorylated Os-2p MAPK in the wild-type strain and in the *os-1* mutant of *N. crassa*.

The *DIC1* and *os-1* genes each encode a putative two-component histidine kinase that consists of HAMP domain re-

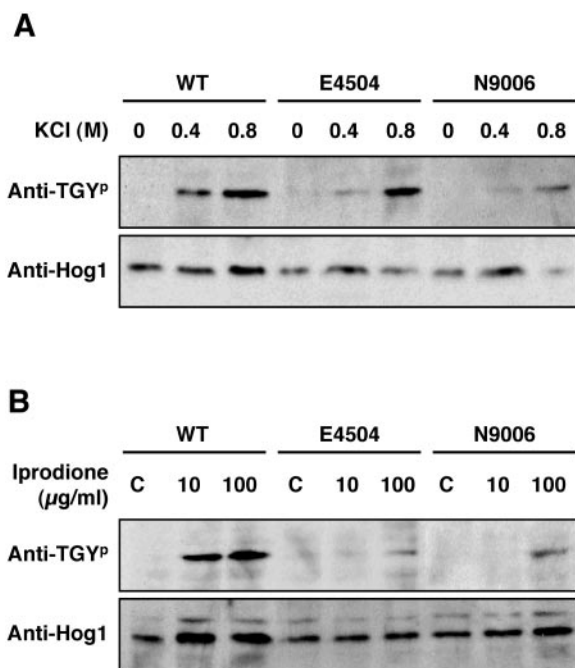


FIG. 4. Phosphorylation of *C. heterostrophus* BmHog1p MAPK in the wild-type strain and the *dic1* mutants E4504 and N9006. (A) BmHog1p phosphorylation by osmotic stress. Mycelia of the strains HITO7711 (WT), E4504 and N9006 were used for analysis. Prepared mycelia of the strain tested were incubated in CM with or without 0.4 and 0.8 M KCl for 10 min. The cells were harvested, and total protein extracts were prepared as described in Materials and Methods. Protein samples (50 µg) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes for Western blot analysis. Phosphorylated BmHog1p was detected using anti-dually phosphorylated p38 antibody (indicated by anti-TGYP). The total amount of BmHog1p was measured using anti-Hog1 C terminus antibody (indicated by anti-Hog1). (B) BmHog1p phosphorylation by iprodione in the wild-type strain and the *dic1*-deficient strains. Mycelia of HITO7711 (WT), E4504, and N9006 were used for analysis. Prepared mycelia were incubated in CM with 10 or 100 µg/ml iprodione for 10 min. Prepared mycelia of the strain tested were incubated in CM without iprodione as a control (indicated by C). The procedures for Western blot analysis were the same as those described above.

peats, a phosphoacceptor histidine kinase domain, and a response regulator domain (1, 38, 46). Filamentous ascomycetes, including *N. crassa*, *C. heterostrophus*, *G. moniliformis*, and *B. fuckeliana*, have been found to possess several histidine kinase genes that were subsequently classified into 11 groups (5, 16). The histidine kinase genes that were homologous to *S. cerevisiae* *SLN1* were categorized into group VI, and both the *dic1* and *os-1* genes were categorized into group III. Group VI histidine kinases are thought to be putative membrane proteins because they possess two potential transmembrane helices in their N-termini. In contrast, no membrane-spanning domain is predicted in the group III proteins, which suggests that Dic1p and Os-1p regulate osmotic signal transmission in a manner different from that mediated by Sln1p in *S. cerevisiae*. Indeed, Dic1p and Os-1p are positive regulators of Hog1-related MAPK in *C. heterostrophus* and *N. crassa*, whereas *S. cerevisiae* Sln1p negatively regulates the activation of Hog1 MAPK.

Group III histidine kinase genes were also found in other filamentous fungi such as *A. nidulans*, *Candida albicans*, and *M.*

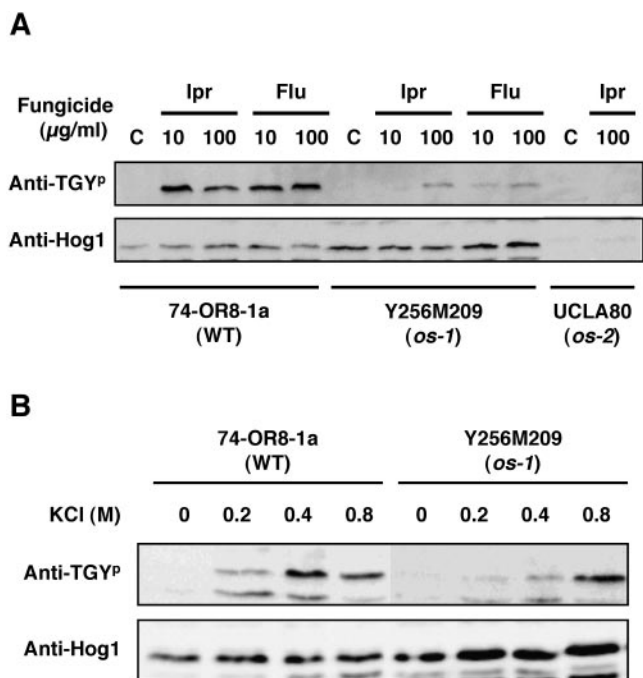


FIG. 5. Phosphorylation of Os-2p MAPK of *N. crassa*. (A) Treatment with fungicides. Mycelia of the wild-type strain 74-OR8-1a (WT) and the *os-1* mutant Y256M209 (*os-1*) were used for analysis. Prepared mycelia of the strain tested were incubated in liquid Vogel's medium with 10 or 100 µg/ml iprodione (Ipr) for 10 min or with 10 or 100 µg/ml fludioxonil (Flu) for 10 min. Prepared mycelia were incubated in CM without iprodione and fludioxonil as a control. Mycelia of the *os-2* mutant UCLA80 (*os-2*) were also exposed to liquid Vogel's medium with or without 100 µg/ml iprodione. The cells were harvested, and total protein extracts were prepared as described in Materials and Methods. Protein samples (50 µg) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes for Western blot analysis. Phosphorylated Os-2p was detected using anti-dually phosphorylated p38 antibody (indicated by anti-TGYP). The total amount of BmHog1p was measured using anti-Hog1 C terminus antibody (indicated by anti-Hog1). Os-2p was detected using anti-dually phosphorylated p38 antibody (indicated by anti-TGYP). The total amount of BmHog1p was measured using anti-Hog1 C terminus antibody (indicated by anti-Hog1). (B) Under osmotic stress. Mycelia of the wild-type strain 74-OR8-1a (WT) and the *os-1* mutant Y256M209 (*os-1*) were used for analysis. Prepared mycelia of the strain tested were incubated in liquid Vogel's medium with 0.2, 0.4, or 0.8 M KCl for 10 min. Prepared mycelia were incubated in liquid Vogel's medium without KCl as a control. The procedures for Western blot analysis were the same as those described above.

grisea (Table 1). In addition, mutations of the group III histidine kinase genes were responsible for osmotic sensitivity in several fungi (8, 26, 27, 30, 31, 46). These observations suggest that the group III histidine kinase in filamentous fungi commonly functions in osmotic sensing.

There are several reports concerning the functional analysis of Hog1-related MAPK in filamentous fungi. In *N. crassa*, one of the osmotic-sensitive genes, *os-2*, encodes a Hog1-related MAPK and could complement the osmotic-sensitive phenotype of an *S. cerevisiae* *hog1* mutant (47). The *C. lagenarium* Hog1-related MAPK, Osc1p, is involved in responses to hyperosmotic stress and is translocated to the nucleus after exposure to osmotic stress (23). In addition, *C. heterostrophus* BmHog1p MAPK was activated by hyperosmotic stress. In

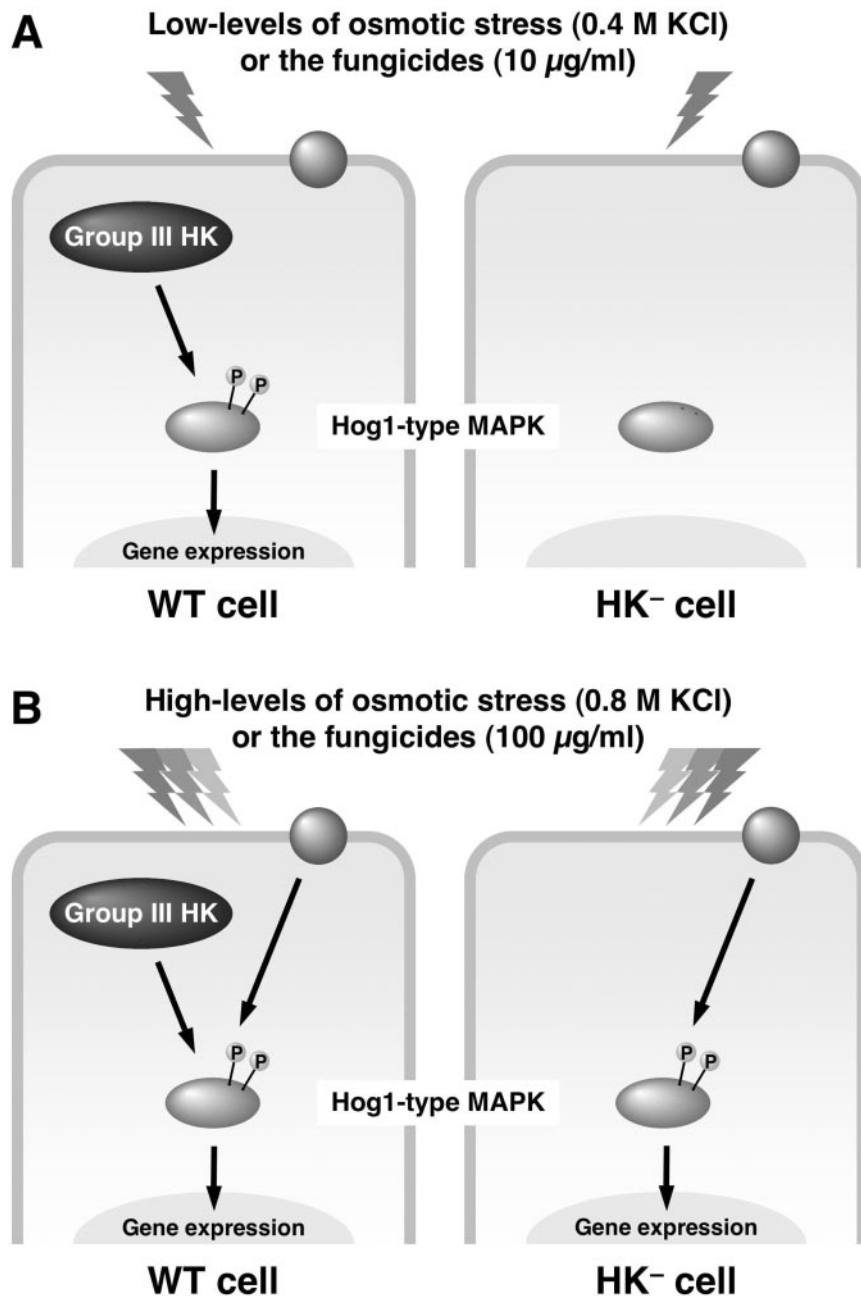


FIG. 6. Models of Hog1-related MAPK phosphorylation. (A) Phosphorylation patterns of Hog1-related MAPK in a wild-type cell and a cell without group III histidine kinase (HK) at low levels of osmotic stress or fungicide. (B) Phosphorylation patterns of Hog1-related MAPK in a wild-type cell and a cell without group III histidine kinase at high levels of osmotic stress or fungicide.

these filamentous fungi, the amino acid sequences of the Hog1-related MAPKs have been well deduced (Table 1). Therefore, it is feasible that the Hog1-type MAPK of filamentous fungi generally functions to transmit extracellular osmotic signals to the nucleus. As mentioned above, our data showed that Hog1-type MAPK activation was controlled by the group III histidine kinase in both *C. heterostrophus* and *N. crassa* (Fig. 5A). Therefore, we believe that filamentous fungi have a common mechanism to sense and respond to extracellular osmotic changes, namely, the group III histidine kinase acting as a positive regulator of Hog1-type MAPK in filamentous fungi.

The results obtained in this study also revealed that *C. heterostrophus* and *N. crassa* might possess another upstream activator of Hog1-type MAPK because even the *dic1*-deficient strains and the *os-1* mutants showed MAPK phosphorylation following exposure to high levels of the fungicides and osmotic stress (Fig. 6B). The genomes of *C. heterostrophus* and *N. crassa* have been found to contain 21 and 11 histidine kinase genes, respectively (5, 16). Although Dic1p and Os1p are involved in fungicide resistance and osmotic adaptation in each fungus, the functions of the other histidine kinases remain unknown. In the opportunistic pathogen *C. albicans*, *CaNIK1*/

TABLE 1. Known group III histidine kinases and Hog1-related MAP kinases in filamentous fungi

Fungal species	Gene name	% Amino acid sequence identity ^a	GenBank accession no.	Reference(s)
Group III histidine kinase				
<i>Neurospora crassa</i>	<i>Nik1/os-1</i>		U53289	1, 37
<i>Cochliobolus heterostrophus</i>	<i>DIC1</i>	63	AB095748	46
<i>Botrytis cinerea</i>	<i>BcOS1/daf1</i>	78	AF435964	8, 31
<i>Magnaporthe grisea</i>	<i>HIK1</i>	83	AB041647	27
<i>Candida albicans</i>	<i>CaNIK1/COS1</i>	51	AB006363	2, 28
<i>Gibberella moniliformis</i>	<i>GmNIK1</i>	79	AY456038	5
<i>Aspergillus nidulans</i>	Not named	67	Sequencing Project ^b	
Hog1-related MAP kinase				
<i>Neurospora crassa</i>	<i>os-2</i>		AF297032	47
<i>Cochliobolus heterostrophus</i>	<i>BmHog1</i>	90	AB208438	Unpublished
<i>Colletotrichum lagenarium</i>	<i>OSC1</i>	95	AF184980	23
<i>Magnaporthe grisea</i>	<i>OSM1</i>	95	AB162820	9
<i>Aspergillus nidulans</i>	<i>SAKA</i>	80	AF282891	21
<i>Saccharomyces cerevisiae</i>	<i>HOG1</i>	77	L06279	4
<i>Schizosaccharomyces pombe</i>	<i>SPC1/STY1</i>	82	X89262	39

^a Amino acid sequence identities were calculated by DNAsis Maximum Matching program for Os-1p (histidine kinase) and Os-2p (MAP kinase).

^b <http://www.broad.mit.edu>.

COS1 is a homologue of *C. heterostrophus DIC1* and is required for osmotic tolerance (2, 40). In addition to *CaNIK1/COS1*, *C. albicans CaSLN1*, which is related to *S. cerevisiae SLN1*, causes slight growth retardation at high osmolarity (28). These findings suggest that *C. albicans* employs these histidine kinases to adapt to extracellular osmotic changes. Therefore, it is possible that another histidine kinase, such as an Sln1p homologue in *C. heterostrophus* and *N. crassa*, can act as an alternative activator of the Hog1-type MAPK for the group III histidine kinases.

In *S. cerevisiae*, the upstream part of the HOG pathway is thought to have two branches, both of which regulate Pbs2p MAPKK (25). One branch contains the putative membrane protein Sho1p and the MAPKKK Ste11p. The other upstream branch contains a His-Asp phosphorelay signaling complex composed of Sln1p, Ypd1p, and Ssk1p (24). It was reported that these two-branches have different response characteristics to high osmolarity (25). Our results show that only a high concentration of the osmoticum and the fungicides led to the activation of Hog1-related MAPK in the cells without the histidine kinase. This suggests that the response of the histidine kinase to osmolarity is different from that of another activator of Hog1-related MAPK and raised the possibility that upstream mechanisms similar to the Sho1p branch might be used to respond to environmental osmotic changes in filamentous fungi. We are planning to conduct functional analyses of the candidate activators in *C. heterostrophus* and *N. crassa*.

Dicarboximide and phenylpyrrole fungicides are used to control a variety of important plant pathogenic fungi (17, 35). Despite intensive study (8, 10, 11, 13, 14, 26, 45, 46, 47), the primary targets of these fungicides have not been identified. In *N. crassa*, osmotic-sensitive mutants such as *os-1*, *os-2*, *os-4*, and *os-5* show increased resistance to dicarboximides and phenylpyrroles, and these osmotic loci encode the group III histidine kinase, Hog1-related MAPK, Ssk22-related MAPKKK, and Pbs2-related MAPKK, respectively. In several phytopathogenic fungi, resistance against dicarboximide and phenylpyrrole fungicides in the field and/or laboratory was caused by mutations of the group III histidine kinase genes, and the gene

mutants often showed osmolarity-sensitive phenotypes. Moreover, the Hog1-type MAPK of some filamentous fungi was abnormally activated by exposure to phenylpyrrole fungicide (23). Therefore, it is now thought that these fungicides interact with the protein(s) related to the osmotic signaling pathway.

Our data obtained in this study are consistent with this hypothesis because *C. heterostrophus* BmHog1p and *N. crassa* Os-2p MAPK were also improperly activated by the fungicides, and the amount of phosphorylated MAPK was significantly reduced in the fungicide-resistant mutants of *C. heterostrophus* and *N. crassa* when the cells were exposed to low-levels of the fungicides (Fig. 6A). However, MAPK phosphorylation was detected even in the resistant mutants upon exposure to high levels of the fungicides (100 µg/ml). This suggests that the fungicides can lead to abnormal activation of the MAPKs even when the histidine kinases do not act as intermediates (Fig. 6B).

Currently, we have no evidence regarding the potential presence of another effector(s), different from the effector for osmotic stress, which leads to the activation of the Hog1-related MAPK in response to fungicide exposure. The hyphal growth rate of the *C. heterostrophus dic1* strains used in this study was not reduced by 50% at the fungicide concentration of 100 µg/ml (median effective concentration > 100 µg/ml) (45). Nevertheless, the MAPK was improperly activated by the fungicides at this concentration, which suggests that the antifungal activity of the fungicides is not caused only by the abnormal activation of Hog1-related MAPK. Further studies are needed to elucidate the relationship between the fungicide activity and the signal transduction pathway of osmotic sensing.

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