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Fungal fludioxonil sensitivity is diminished by a constitutively active form of the group III histidine kinase

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ABSTRACT

The fungicide fludioxonil is used to control plant-pathogenic fungi by causing improper activation of the Hog1-type MAPK. However, the appearance of fludioxonil resistant mutants, mostly caused by mutations in the group III histidine kinases, poses a serious problem. Moreover, such mutations cause also hyperosmotic sensitivity and the underlying mechanism has been elusive for a long time. Using *Saccharomyces cerevisiae* as an experimental host, we show that those phenotypes are conferred by a constitutively active form of the group III histidine kinase. Our results explain the different reasons for fludioxonil resistance conferred by its deletion and missense mutation.
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1. Introduction

Two-component systems are composed of a His-Asp phosphorelay from the histidine kinase (HK) to the response regulator. These modules regulate a variety of responses to environmental stimuli and are widely employed in bacteria, fungi, and plants [1,2]. In general, the fungal two-component systems are connected to a high-osmolarity glycerol (HOG) MAPK pathway. The budding yeast *Saccharomyces cerevisiae* HOG pathway is one of the best studied MAPK systems [1,3,4]. It consists of two upstream osmosensing branches (Sln1 and Sho1) and a downstream MAPK cascade (Fig. 1A). Under basal condition, the osmosensor HK Sln1 acts as a negative regulator of the HOG pathway via the phosphotransmitter Ypd1 and the response regulator Ssk1 [5]. Hyperosmotic shock causes a transient inhibition of Sln1, leading to activation of the Hog1 MAPK and expression of osmoresponsive genes [1,3,4]. However, improper activation of the pathway caused by deletion of *SLN1* is lethal [6]. Unlike *S. cerevisiae*, filamentous fungi defective in *SLN1* orthologs are commonly viable [2,7]. This difference is probably due to the presence of multiple HK genes, falling into 11 groups [8].

All fungi, except some yeast species, possess group III HKs which carry a unique N-terminal region consisting of HAMP (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases) domain repeats [2]. Although the group III HKs appear to be positive regulators of the Hog1-type MAPKs [9], it has been unclear until recently how they function as osmosensors. Using DhNik1 from the salt-tolerant yeast *Debaryomyces hansenii*, Meena et al. [10] showed that interactions between the HAMP domains act as an "on–off" osmosensing switch. Furthermore, the group III HK is known to be a target for several fungicides such as fludioxonil [9,11–14]. Fludioxonil treatment causes cell death via improper activation of the Hog1-type MAPK [9,15–17]. Although fludioxonil is used to control harmful plant-pathogenic fungi, the appearance of fludioxonil resistant mutants, mostly caused by mutations in the group III HKs, poses a serious problem [18,19]. Moreover, one of the unsolved issues is the reason why deletion and missense mutation of the group III HK display the same phenotypes, namely fludioxonil resistance and hyperosmotic sensitivity [18,19].

Here, we analyzed how the "on–off" states of DhNik1 affect fludioxonil sensitivity using *S. cerevisiae* as an experimental host. This yeast has only one HK (Sln1) making it feasible to test the effect of specifically DhNik1. We found that a constitutively active form of DhNik1 confers fludioxonil resistance and hyperosmotic sensitivity. Moreover, our results suggest that some of the group III HK missense mutations previously isolated from filamentous fungi also represent constitutively active forms. We discuss the different mechanisms for fludioxonil resistance and hyperosmotic sensitivity conferred by deletion and active form of the group III HK.

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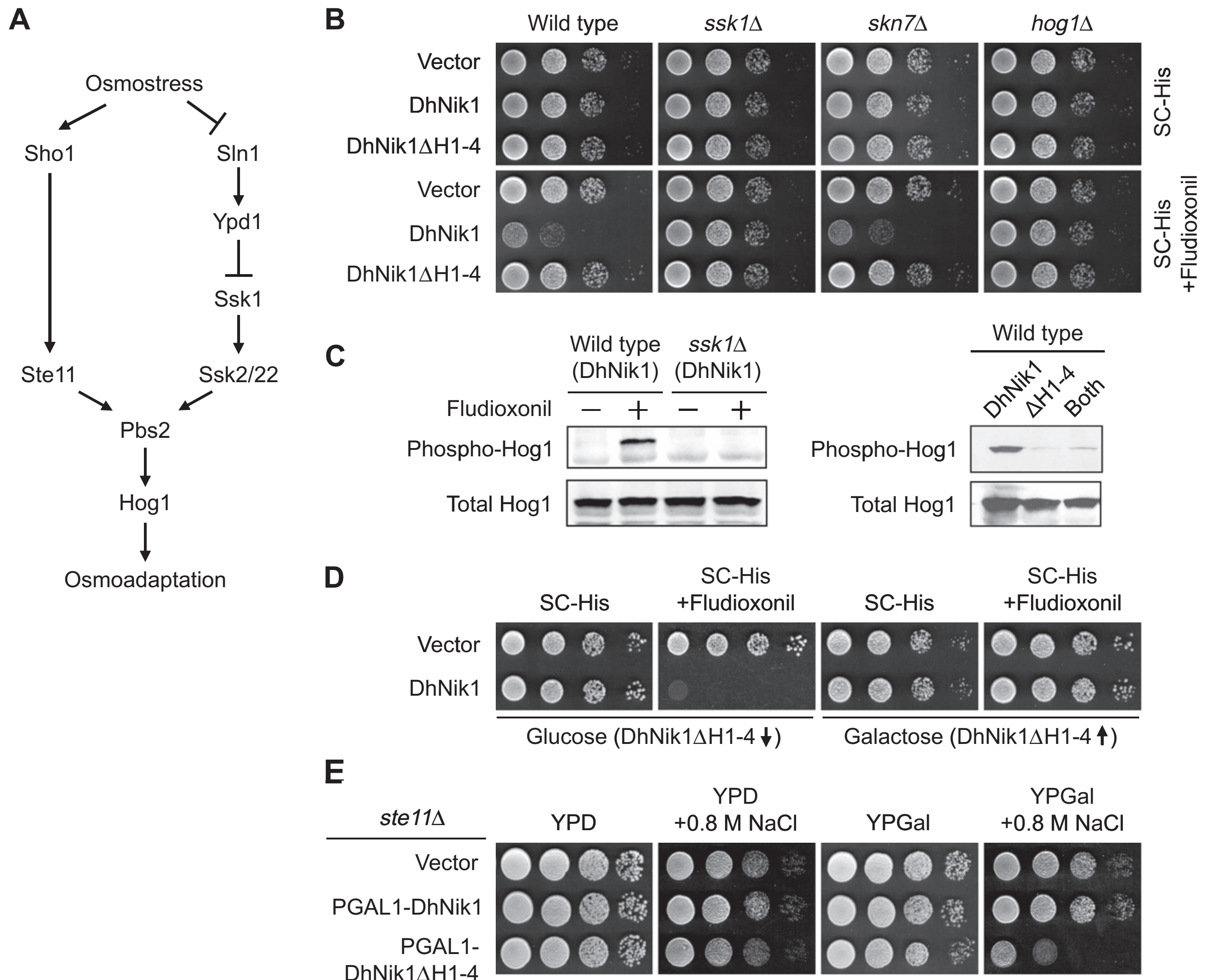

Fig. 1. Distinct effects of heterologous expression of DhNik1 and DhNik1ΔH1-4 on *S. cerevisiae*. (A) The *S. cerevisiae* HOG pathway consists of two upstream osmosensing branches (Sln1 and Sho1) and a downstream MAPK cascade (Ssk2/Ssk22 and Ste11 MAPKKKs, Pbs2 MAPKK, and Hog1 MAPK). See text and references for details. (B) Expression of DhNik1 but not DhNik1ΔH1-4 confers fludioxonil sensitivity on *S. cerevisiae* in an Ssk1-Hog1-dependent manner. The indicated yeast cells carrying pRS423, pRS423-DhNIK1, or pRS423-DhNik1ΔH1-4 were grown on SC-His plates with or without 20 μg/ml fludioxonil for 2 days at 30 °C. (C) Hog1 phosphorylation upon fludioxonil treatment depends on DhNik1 via Ssk1 (Left), but the phosphorylation is diminished by DhNik1ΔH1-4 (Right). Cells expressing DhNik1 or/and DhNik1ΔH1-4 were grown in SC medium to log phase. Before and after fludioxonil treatment (15 min), cells were collected and immediately frozen in liquid nitrogen. Total cell extract was examined for Western blot analysis using anti phospho-p38 antibody (Phospho-Hog1) and anti-Hog1 antibody (Total Hog1). (D) Co-expression of DhNik1 and DhNik1ΔH1-4 diminishes fludioxonil sensitivity. Wild-type cells carrying two plasmids, YIpGAL-DhNik1ΔH1-4, and pRS423 or pRS423-DhNIK1, were grown on glucose (repressing condition) or galactose (inducing condition) plates as in (B). (E) Expression of DhNik1ΔH1-4 confers hyperosmotic sensitivity on the *ste11Δ* strain. Cells of the *ste11Δ* strain carrying pRS406, YIpGAL-DhNIK1, or YIpGAL-DhNik1ΔH1-4 were grown on YPD or YPGal plates with or without 0.8 M NaCl for 2–3 days at 30 °C.

2. Materials and methods

2.1. Yeast media and growth conditions

Standard media were used for yeast culture and selection of transformants. SC (synthetic complete: 2% glucose, 0.67% yeast nitrogen base without amino acids, and supplemented with amino acids to satisfy nutritional requirements), YPD (1% yeast extract, 2% peptone, and 2% glucose), YPGal (1% yeast extract, 2% peptone, and 2% galactose). NaCl or fludioxonil (Sigma–Aldrich) was added in the media to examine sensitivity of yeast cells. For growth assays, cells were pregrown for 2 days on SC plates, resuspended in water to OD_{600} = 0.1, and 5 μl of a 10-fold dilution series were spotted onto agar plates supplemented with osmotica or fludioxonil as indicated. Growth was monitored for 2–3 days at 30 °C.

2.2. Yeast strains and plasmids

The yeast strains and plasmids used in this study are listed in Tables S1 and S2, respectively. Yeast transformation was performed as described previously [20]. Galactose-inducible DhNik1 or DhNik1ΔH1-4 expression plasmids were constructed by simultaneous insertion of two fragments (*GAL1* promoter and *DhNIK1(ΔH1-4)*) into pRS406 (for integration at *ura3*) or pRS404 (for integration at *trp1*). A *lacZ* reporter plasmid (YIpSSRE-lacZ) carrying the synthetic Skn7 response element (SSRE) was constructed by overlap extension PCR-mediated mutagenesis into the zero-background reporter plasmid YIpMELβ2 [21] using primers P-SSRE(F) and P-SSRE(R) (Table S3). Missense mutant plasmids (YIpPGAL-DhNIK1 backbone) were constructed by QuikChange mutagenesis (Stratagene; primers shown in Table S3).

2.3. Western blot

Cells expressing DhNik1, DhNik1ΔH1-4, or both were grown in SC liquid medium to log phase. Before and after fludioxonil treatment (15 min), cells were collected by centrifugation and immediately frozen in liquid nitrogen. Total cell extract was examined for Western blot analysis using anti-phospho-p38 antibody (Cell Signaling Inc.) and anti-Hog1 antibody (Y-215: Santa Cruz Biotechnology) as described previously [10].

2.4. Reporter assay

Cells carrying the reporter plasmid were grown in YPGal liquid media with or without stress (0.8 M NaCl or 20 μg/ml fludioxonil) to log phase at 30 °C, and then β-galactosidase activity was determined using the Yeast β-Galactosidase Assay Kit (Pierce) as described previously [22]. Values represent the mean and standard deviation of at least five replicas and were expressed in Miller units. 1 Miller unit = $1000 \times (A_{420})/(T \times V \times A_{660})$, where T is the reaction time (in minutes) and V is the volume of culture used in the assay (in milliliters).

3. Results

3.1. Distinct effects of heterologous expression of DhNik1 and DhNik1ΔH1-4 on S. cerevisiae

First, we determined whether heterologous expression of DhNik1 confers fludioxonil sensitivity on *S. cerevisiae* as previously shown for *Magnaporthe grisea* Hik1, *Alternaria brassicicola* AbNik1, and *Candida albicans* CaNik1 [12,23,24]. We examined also a putative "on" state mutant, DhNik1ΔH1-4, which lacks the N-terminal HAMP1–4 domains (carrying HAMP5 domain only) and complements the *sln1-ts4* mutation [10]. As shown in Fig. 1B, expression of DhNik1 conferred fludioxonil sensitivity on the wild-type and *skn7Δ* (another response regulator under the control of Ypd1) strains but not on the *ssk1Δ* and *hog1Δ* strains. This result confirms that the fludioxonil sensitivity conferred by DhNik1 depends on Hog1 via Ssk1. In contrast, expression of DhNik1ΔH1-4 did not confer fludioxonil sensitivity on any of those strains (Fig. 1B). Next, we performed Western blot to analyze Hog1 phosphorylation in cells expressing DhNik1, DhNik1ΔH1-4, or both. Upon fludioxonil treatment, Hog1 was phosphorylated in cells expressing DhNik1, but not in cells expressing DhNik1ΔH1-4 or in *ssk1Δ* mutant cells (Fig. 1C). Interestingly, Hog1 was not phosphorylated in cells co-expressing DhNik1 and DhNik1ΔH1-4 upon fludioxonil treatment (Fig. 1C), and the cells were indeed resistant to fludioxonil (Fig. 1D). These results show that fludioxonil treatment activates the HOG pathway in a DhNik1- and Ssk1-dependent manner and that these effects are diminished by DhNik1ΔH1-4. These results prompted us to determine whether expression of DhNik1ΔH1-4 inhibits the osmotic signal of the Sln1 branch. To examine this idea, we used a *ste11Δ* mutant in which Hog1 activation solely depends on the Sln1 branch. The *ste11Δ* cells expressing DhNik1ΔH1-4 but not the cells expressing DhNik1 were sensitive to hyperosmotic stress (Fig. 1E). This result is consistent with the finding that yeast cells expressing a constitutively active Sln1 mutant (lacking the Sho1 branch) are sensitive to hyperosmotic stress [25,26]. Taken together, these

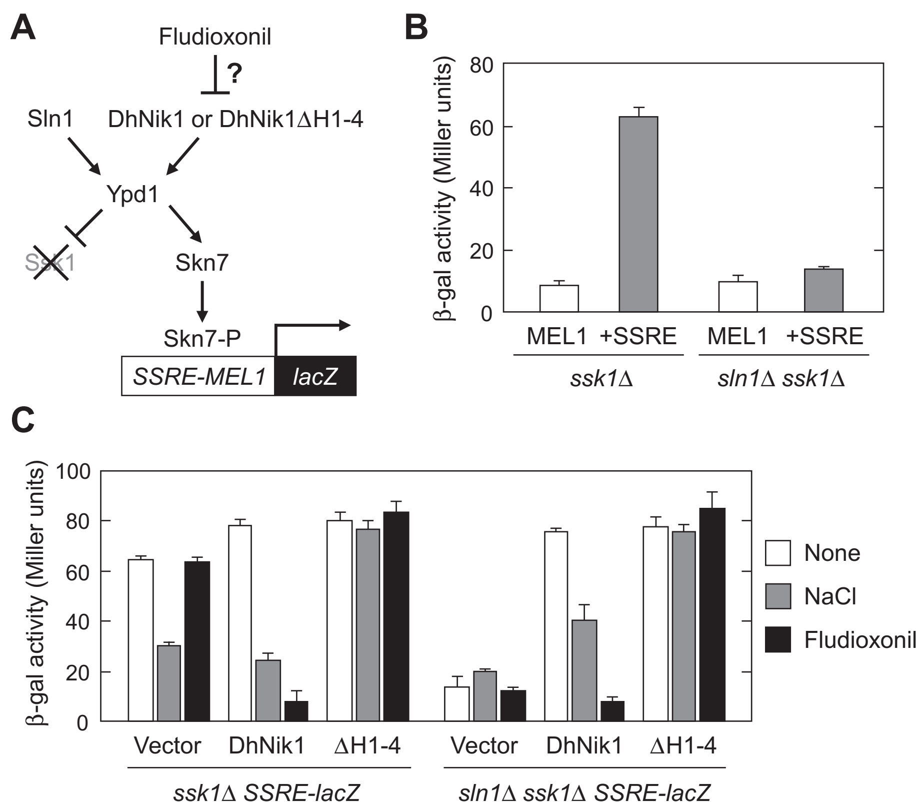

Fig. 2. Histidine kinase activity of DhNik1ΔH1-4 is neither affected by osmostress nor by fludioxonil. (A) A *lacZ* reporter system carrying a synthetic Skn7 response element (SSRE) to monitor HK activity in vivo. (B) The *SSRE-lacZ* reporter activity depends on the presence of HK. *ssk1Δ* and *sln1Δ ssk1Δ* cells carrying YIpMELβ2 (MEL1) or YIpSSRE-lacZ (+SSRE) were grown in YPGal liquid media to log phase at 30 °C, and then β-galactosidase activity was determined. Values represent the mean and standard deviation of at least five replicas. (C) HK activity of yeast cells expressing DhNik1 is inhibited by osmostress and fludioxonil, while that of DhNik1ΔH1-4 is not affected. The *SSRE-lacZ* reporter activity of the *ssk1Δ* and *sln1Δ ssk1Δ* strains carrying YIplac204, YIpGAL-DhNIK1(T), or YIpGAL-DhNik1ΔH1-4 (T) were determined after growth in YPGal liquid media with 0.8 M NaCl or 20 μg/ml fludioxonil.

results suggest that DhNik1ΔH1-4 is a constitutively active form that is neither inhibited by osmotic stress nor by fludioxonil.

3.2. Histidine kinase activity of DhNik1ΔH1-4 is neither affected by osmostress nor by fludioxonil

To verify that activity of DhNik1ΔH1-4 is not inhibited by osmostress or fludioxonil, we constructed a *lacZ* reporter system to monitor HK activity in vivo. The *lacZ* reporter plasmid (YIp-SSRE-lacZ) carries a synthetic Skn7 response element (SSRE) [27,28], which is targeted by phospho-Skn7 (Fig. 2A). First, we measured reporter activity of the *ssk1Δ* and *sln1Δ ssk1Δ* strains in which YIpMELβ2 (lacking SSRE) or YIpSSRE-lacZ was integrated at the *ura3* locus. The *ssk1Δ* strain carrying YIpSSRE-lacZ showed more than ~7-fold higher reporter activity than that carrying YIp-MELβ2, while reporter activity in the *sln1Δ ssk1Δ* strain was very low (Fig. 2B). Thus, the *SSRE-lacZ* reporter activity depends on the presence of SSRE sequence and HK activity. Next, we determined whether osmostress and fludioxonil treatment affect HK activity of cells expressing DhNik1 or DhNik1ΔH1-4 (Fig. 2C). The *SSRE-lacZ* reporter activity in *ssk1Δ* or *sln1Δ ssk1Δ* mutants expressing DhNik1 was reduced under hyperosmotic condition in the same way as Sln1 [27], showing that DhNik1 acts as a negative regulator upon osmostress. The *SSRE-lacZ* reporter activity of the *ssk1Δ* or *sln1Δ ssk1Δ* strains expressing DhNik1 in the presence of fludioxonil was as low as that of the *sln1Δ ssk1Δ* strains carrying the vector. On the other hand, reporter activity of DhNik1ΔH1-4 cells was hardly affected by osmostress or by fludioxonil. These results clearly show that HK activity of DhNik1ΔH1-4 is neither inhibited by osmostress nor by fludioxonil. Moreover, the fact that fludioxonil treatment inhibits reporter activity of DhNik1 cells regardless of absence or presence of Sln1 suggests that inhibited DhNik1 adversely affects also Sln1 kinase activity or a step downstream of Sln1.

3.3. Mutations of conserved amino acids among the group III histidine kinases cause fludioxonil resistance and hyperosmotic sensitivity

Mutations that cause both fludioxonil resistance and hyperosmotic sensitivity have been reported in filamentous fungi such as *Neurospora crassa nik-1/os-1* [18] and *Cochliobolus heterostrophus dic1* [19]. The *N. crassa* Nik1 mutations are divided into nonsense mutations and missense mutations (Fig. 3A). Two of the missense mutations (A578V and G580R) are located in the HAMP5 domain and well conserved among the group III HKs (Fig. 3B). We speculated that some of those mutations (except non-functional ones) cause constitutive activation. To examine this idea, we constructed DhNik1 mutants (A360V and G362R in HAMP4, V407P between HAMP4 and 5) that correspond to the *N. crassa* Nik1 mutants (A578V, G580R, L625P). We concluded that $DhNik1^{G362R}$ is a constitutively active form on the basis of the following observations. First, expression of these DhNik1 mutants suppressed the lethality of *sln1-ts4* mutation at 37 °C (not shown), indicating that they are functional kinases. Second, cells of the *ste11Δ* mutant expressing $DhNik1^{G362R}$ or $DhNik1^{A360V}$ were sensitive to hyperosmotic stress and resistant to fludioxonil (Fig. 3C). Finally, the *SSRE-lacZ* reporter assay showed that $DhNik1^{G362R}$ is not strongly inhibited by osmostress or by fludioxonil (Fig. 3D). The difference in fludioxonil resistance between $DhNik1^{A360V}$ and $DhNik1^{G362R}$ is consistent with a previous report for the *N. crassa* $Nik1^{A578V}$ and $Nik1^{G580R}$ mutants [18]. These results suggest that at least *N. crassa* $Nik1^{A578V}$ and $Nik1^{G580R}$ are also constitutively active forms and that fludioxonil resistance is conferred in different ways by deletion and missense mutations of the group III HK.

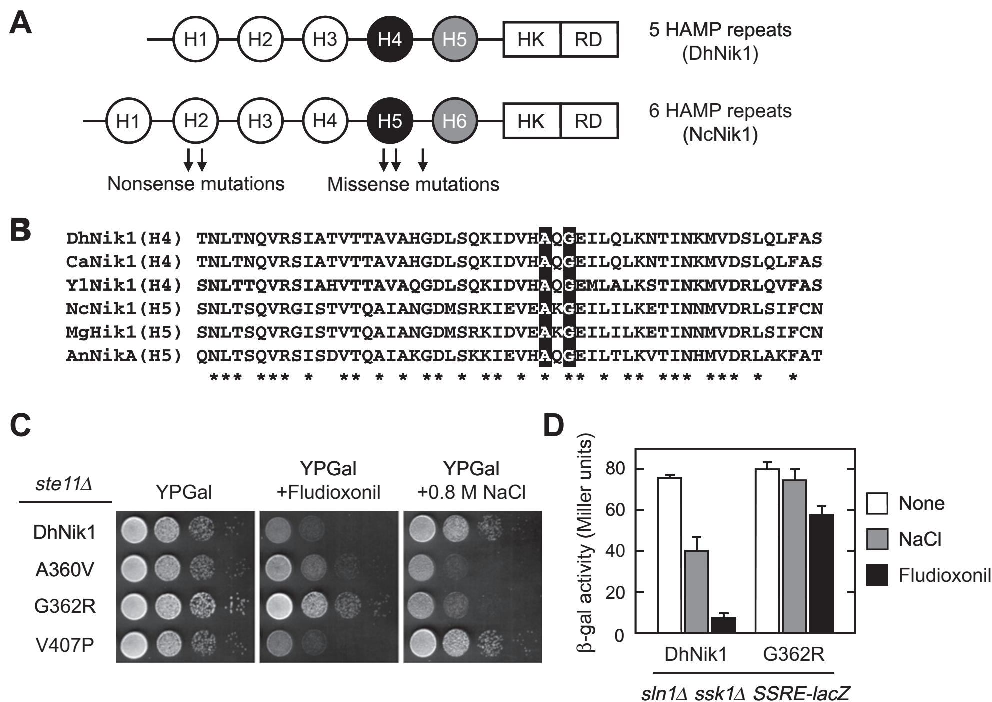

Fig. 3. Mutations of conserved amino acid among the group III histidine kinases cause fludioxonil resistance and hyperosmotic sensitivity. (A) Different numbers of the HAMP domain in yeasts and filamentous fungi. Positions of the point mutations isolated in *N. crassa* Nik1 are indicated by arrows. (B) Alignment of amino acid sequence of the HAMP4 (yeasts) and HAMP5 (filamentous fungi) domains. Dh, *Debaryomyces hansenii*; Ca, *Candida albicans*; Yl, *Yarrowia lipolytica*; Nc, *Neurospora crassa*; Mg; *Magnaporthe grisea*; An, *Aspergillus nidulans* or (*niger*). Mutation of the highlighted Ala and Gly residues causes fludioxonil resistance and hyperosmotic sensitivity in *N. crassa* [18]. (C) Expression of $DhNik1^{G362R}$ confers fludioxonil resistance and hyperosmotic sensitivity on *S. cerevisiae*. *ste11Δ* cells expressing DhNik1, $DhNik1^{A360V}$, $DhNik1^{G362R}$, or $DhNik1^{V407P}$ were grown for 2–3 days at 30 °C. (D) HK activity of $DhNik1^{G362R}$ is not strongly inhibited by osmostress or fludioxonil. *SSRE-lacZ* reporter activity was measured as described in Fig. 2.

4. Discussion

Since deletion of the group III HK results in diminished phosphorylation of the Hog1-type MAPK upon stress, these kinases have been thought to be positive regulators in filamentous fungi [9]. However, the following observations from a previous report and our *SSRE-lacZ* reporter assays indicate that group III HKs function as negative regulators: (i) expression of DhNik1 complements an *sln1* mutation [10], (ii) DhNik1ΔH1-4 blocks Hog1 phosphorylation upon osmostress [10], (iii) the HK activity of DhNik1 is inhibited by osmostress as is the case for Sln1 (Fig. 2C). To our knowledge, this study provides the first evidence that HK activity of group III HKs is inhibited by osmostress and by fludioxonil. Based on these results, we believe that these DhNik1 mutants can provide a phosphate moiety to Ypd1 even under kinase inhibiting conditions and that this is the reason for hyperosmotic sensitivity and fludioxonil resistance caused by expression of DhNik1ΔH1-4 or DhNik1^{G362R}. Strikingly, fludioxonil diminished kinase activity of Sln1 only in the presence of DhNik1 (Fig. 2C), suggesting that the effect of group III HKs can override the function of Sln1. This observation could be explained if one postulates that inhibited DhNik1 tightly binds Ypd1 and prevents access of Sln1 to Ypd1.

Our results shown in Fig. 3 suggest that some of the previously known missense mutations are also constitutively active forms and that the HAMP4 domain of group III HKs with 5 HAMP repeats is functionally equivalent to the HAMP5 domain with 6 HAMP repeats. Since many of the isolated fludioxonil resistant mutations locate to the HAMP domains and result in different degrees of resistance [18,19], the observed effects may be caused by variable strength of the HAMP domain interactions and interference of such interactions by fludioxonil. DhNik1ΔH1-4 and DhNik1^{G362R} cells are insensitive to fludioxonil, but they show somewhat different HK activity in the presence of fludioxonil. These results suggest that those DhNik1 mutants completely or partially lack a negative regulation of their HK activity. During preparation of this manuscript, Buschart et al. [24] reported that the *C. albicans NIK1* mutant lacking the HAMP domain (corresponding to HAMP4 of DhNik1) does not confer fludioxonil sensitivity on *S. cerevisiae*, suggesting that the role of HAMP domains is conserved among the group III HKs. However, those and our data do not necessarily mean that the specific HAMP domain is the direct target of fludioxonil. Further studies on the regulatory mechanism of the HAMP domains may contribute to the development of novel fungicides against the group III HKs as well as elucidation of the inhibitory mechanism by fludioxonil.

The reason for fludioxonil resistance caused by deletion of the group III HK genes is rather straightforward, but we are still missing why its deletion also causes hyperosmotic sensitivity in some filamentous fungi [18,19]. Since activation of the Hog1-type MAPK in filamentous fungi depends solely on the two-component system [2,29], there is no doubt that those two-component systems are insensitive to osmostress in the absence of the group III HK. The two-component system may be negatively regulated by other (non-osmosensor) HKs in the absence of the group III HKs. Such a scenario raises additional questions. How do the group III HKs cause osmotic activation of the Hog1-type MAPK in the presence of multiple (non-osmosensor) HKs? Why do the multiple HKs not suppress the lethality caused by inhibition of the group III HK upon fludioxonil treatment? Elucidation of these mechanisms is needed to understand how fludioxonil causes improper activation of the Hog1-type MAPK via the group III HK. Reconstitution analysis of the two-component system with multiple HKs in *S. cerevisiae* could address those questions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.05.057.

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