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Two-Component Signaling System in Filamentous Fungi and the Mode of Action of Dicarboximide and Phenylpyrrole Fungicides

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1. Introduction

Elucidating the mode of action of fungicides and the mechanism of fungicide resistance is a promising scientific approach to plant protection. However, the fungicidal mode of action or the mechanism of fungicide resistance has not been elucidated for all new fungicides. Furthermore, the mode of the action has not been fully elucidated for some fungicides that have long been on the market. Dicarboximide fungicides, one of the classes of fungicides dealt with in this paper, have also commercially available for a long time. Several theories have been proposed regarding their mode of action, but their bona fide fungicidal mechanism has not been fully elucidated.

Fungi, including phytopathogenic fungi, usually develop thalli and their cells are directly exposed to the environment. The cells inevitably experience several kinds of environmental stresses throughout their life cycles. These stresses include water activity (osmotic) stress and oxidative stress caused by the host response in a host-parasite interaction. To sense and respond to these stresses, fungi possess signal transduction systems and adaptation mechanisms. In the last 10 years, information obtained in the field of genome science on budding yeast (*Saccharomyces cerevisiae*, a leading model organism in fungal science) has enabled us to elucidate signal transduction systems and adaptation mechanisms in filamentous fungi, and further progress led to the clarification of the mode of action of dicarboximide and phenylpyrrole fungicides. These fungicides are closely involved in an osmotic signaling system in filamentous fungi. The fungicides now constitute an essential tool for studying this system. And as a result, this system has attracted great attention as a target of new antifungal agents. Furthermore, in some fungi, this system is involved in the pathogenicity of their hosts. In this paper, we introduce researches on the mode of action of these fungicides, which lead to the identification of the osmotic signaling system in pathogenic filamentous fungi, and related findings.

2. A short history of dicarboximide and phenylpyrrole fungicides

Dicarboximide fungicides were developed between the 1960s and the 1970s. They have strong antifungal properties with respect to many ascomycetes and related anamorphic fungi, including *Botrytis* spp., *Sclerotinia* spp., and *Bipolaris* spp. Owing to their prominent

fungicidal effect, dicarboximide fungicides have been widely used throughout the world, and the emergence of resistant fungi has been reported (q.v. Fungicide Resistance Action Committee, 2010). However, the mode of action and the resistance mechanisms of the dicarboximides have not been well understood. A biochemical study reported that dicarboximides had little effect on respiration or the biosynthesis of sterol, nucleic acids, proteins or chitin (Pappas & Fisher, 1979). However, the application of dicarboxyimides to fungal cells caused hyphal swelling and the bursting of tips (e.g. Eichhorn & Lorenz, 1978). The synthesis of cell walls was stimulated by dicarboximide treatment without any noticeable changes in their constituents (Hisada et al., 1978). The fungicides interfered with fungal membranes but had no effect on ion leakage or water permeability (Yoshida & Yukimoto, 1993). It was also reported that the dicarboximides induced membrane lipid peroxidation in some fungi by interfering with flavin-containing enzymes (Edlich et al., 1988). In addition, their fungicidal toxicity was reduced by piperonyl butoxide and α-tocopherol, which are a cytochrome P-450 inhibitor and an antioxidant, respectively (Leroux et al., 1992). Some dicarboximide resistant isolates of *Alternaria alternata* and *Botrytis cinerea* exhibited slightly enhanced catalase activity in the absence of the fungicides (Steel, 1996; Steel & Nair, 1995). These observations suggested that the mode of action of the fungicides might be related to reactive oxygen species, and the resistance to these fungicides might also be associated with a scavenging mechanism (Steel, 1996). On the other hand, *Neurospora crassa* mutants resistant to dicarboximides showed increased sensitivity to high osmolarity (Grindle, 1983; 1984). In *N. crassa*, at least six genes: *os-1*, *os-2*, *os-4*, *os-5*, *cut*, and *sor(T9)* were involved in the sensitivity to osmotic stress (Mays, 1969; Murayama & Ishikawa, 1973). Of the osmotic sensitive mutants, *os* mutants were resistant to dicarboximide and aromatic hydrocarbons, whereas *cut* and *sor(T9)* mutants were not (Grindle & Temple, 1982). That is, these studies have shown that fungicide resistance and hyper-osmosensitivity constitute the pleiotropic phenotypes of the same mutant gene. The explanations of the resistant mechanisms were incapable of proving these mutant traits.

Phenylpyrrole fungicides, another class of fungicides dealt with in this paper, were developed for agricultural use in the 1990s, with medical antibiotic pyrrolnitrin produced by *Pseudomonas* bacteria, as a lead compound. The compounds have as broad a fungicidal spectrum as the lead compound (Gehmann et al., 1990). The structure of phenylpyrrole fungicides was different from that of dicarboximide fungicides. In addition, there was a difference in the then proposed mode of action. However, in *B. cinerea*, most induced laboratory mutants that were resistant to dicarboximides also showed a cross resistance to phenylpyrroles. Moreover, the pleiotropy of fungicide resistance genes in *B. cinerea* and other plant pathogenic filamentous fungi including *Cochliobolus heterostrophus* was also recognized (Beever, 1983; Faretra & Pollastro, 1991; 1993; Gafur et al., 2001; Leroux et al., 1992; Matsuura & Tsuda, 1993; Yoshimi et al., 2003). Thus, these observations have led to the belief that both classes of the fungicides have the same mode of action, relating to the hyperosmotic adaptation of filamentous fungi. This was indispensable for elucidating the resistant mechanisms and thus understanding the osmotic signaling system in fungi based on a molecular approach.

3. Characteristics of the osmotic signaling system of filamentous fungi

Before discussing the osmotic signaling system of filamentous fungi, we briefly describe the modeled hyperosmotic adaptation mechanism in budding yeast. SLN1, a histidine kinase (HisK), is involved in sensing hyperosmotic stimulation. The transduced signal is

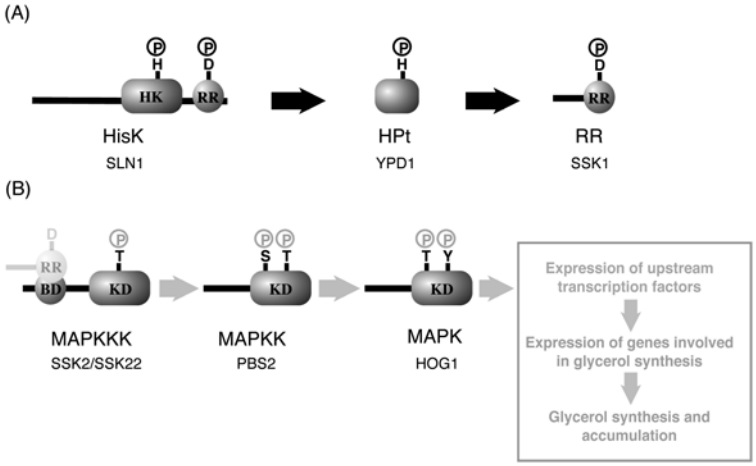

Fig. 1. Schematic of the osmotic signaling pathway of budding yeast
The osmotic signaling pathway consists of two types of signaling systems: (A) a two-component regulatory system and (B) a HOG1 MAPK signaling system.
The figure shows the steady state of the cell (under non-hyperosmotic conditions).
The light area in (B) shows the change in the HOG1 MAPK signaling system under hyperosmotic conditions.
HK: histidine kinase domain, RR: response regulator domain, BD: SSK1 binding domain, KD: kinase domain.

Genome	HisK	HPT	RR*
<Ascomycetes - Yeasts>			
Saccharomyces cerevisiae	1	1	2
Schizosaccharomyces pombe	3	1	2
<Ascomycetes - Filamentous Fungi>			
Neurospora crassa	11	1	2
Aspergillus nidulans	15	1	3
Cochliobolus heterostrophus	21	1	3
Botrytis cinerea	20	1	2
Magnaporthe grisea	10	1	2
Fusarium graminearum	16	1	2
<Basidiomycetes>			
Cryptococcus neoformans	7	1	2

Table1. Number of genes involved in the two-component regulatory system of fungi
* *Rim 15* ortholog is not included.

transmitted to the downstream HOG1 MAP kinase (MAPK) signal system (HOG pathway: Fig. 1B) through a histidine-containing phosphotransfer (HPt) protein YPD1 and a response regulator (RR) protein, SSK1, (a two-component regulatory system: Fig. 1A). Eventually, the activated HOG pathway induces intracellular glycerolgenesis and the cell adapts to the hyperosmotic environment. In this model, hyperosmotic stimulation is transmitted as follows.

When yeast cells are not exposed to hyperosmotic stress, histidine residues in the SLN1 histidine kinase domain present on the cell membrane are autophosphorylated and aspartyl residues in the SLN1 receiver domain are also phosphorylated. This phosphate is transferred to a YPD1 histidine residue and the SSK1 protein is phosphorylated by phosphorylated YPD1. When the cells are exposed to hyperosmotic stress, the phosphorylation of SLN1 is inhibited. Consequently, YPD1 and SSK1 are dephosphorylated. Dephosphorylated SSK1 combines with the SSK2/SSK22 proteins (MAPKK kinases in the HOG pathway), causing the autophosphorylation of these kinases. Phosphorylated SSK2/SSK22 phosphorylates the PBS2 protein (a downstream MAPK kinase), and the phosphorylated PBS2 protein phosphorylates the HOG1 MAPK. The phosphorylated HOG1 protein moves to the nucleus, inducing the gene expression of upstream transcription factors such as HOT1. Eventually, these regulatory factors activate the expression of genes involved in glycerol synthesis (q.v. Hohmann, 2002).

The hyperosmotic adaptation mechanism of filamentous fungi, including plant pathogenic filamentous fungi and human infectious fungi, is similar to but more complicated than that of budding yeast. While budding yeast has Sln1 as a sole HisK gene, filamentous fungi have several HisK genes per genome (Table 1). For example, fungal genome information elucidated eleven HisK genes in *N. crassa*, fifteen in *Aspergillus nidulans*; twenty-one in *C. heterostrophus*; twenty in *B. cinerea*; ten in *Magnaporthe grisea*; sixteen in *Fusarium graminearum*; and seven in *Cryptococcus neoformans*. The gene family in these organisms can be classified into at least ten groups according to the domain structure and the homology of the DNA sequences (Catlett et al., 2003). The ortholog of *Sln1*, which is essential for the hyperosmotic adaptation of budding yeast, is found in many ascomycetes, and is probably the ascomycete specific class of HisK. However, gene disruptants in *Cochliobolus heterostrophus* and *B. cinerea* do not show definite phenotypic changes, and their roles in these fungi are unknown (our unpublished data). In filamentous fungi, the HisK gene, which is classified as Group III, plays an important role in the osmotic stress response.

4. Group-III Hisk and its role in filamentous fungi

In 1997, the *os-1* gene of *N. crassa* was cloned, which bestows dicarboximide resistant and hyper-osmosensitive phenotypes. Sequence analysis revealed that this gene codes HisK, which is known as *nik-1* (Alex et al., 1996; Schumacher et al., 1997). Subsequently, the pleiotropic fungicide resistance genes in *B. cinerea* (*Daf1*; also called *BcOS1* or *Bos1*) and in *C. heterostrophus* (*Dic1*) were cloned (Cui et al., 2002; Oshima et al., 2002; Yoshimi et al., 2004). These two genes also code HisK and share a high homology with *os-1*. However, the structure of these HisKs is very different from that of SLN1 identified in budding yeast.

A genealogic study of the fungal Hisk genes, using fungal genome information, has led to the classification of *os-1*, *Daf1* and *Dic1* orthologs into a unique group (Group III) different from that of *Sln1* (Catlett et al., 2003), and has shown that the Group III HisK gene is generally present in Dikarya, regardless of the classification groups of fungi or their ecological status, e.g., human infectious *Candida albicans* (ascomycete), *C. neoformans*

(basidiomycete); phytopathogenic *M. grisea, Fusarium* spp. (ascomycetes), and *Ustilago maydis* (basidiomycete). The exceptions are budding yeast and fission yeast.

What are the structural characteristics of Group III HisK? SLN1 is believed to have two transmembrane domains at the *N* terminus and to be localized to the membrane. The localization and extracellular region of SLN1 is believed to be essential for sensing extracellular osmotic changes (Ostrander & Gorman, 1999; Reiser et al., 2003). However, a Group III HisK lacks transmembrane domains. A study using a GFP-fusion HisK protein has shown that a Group III HisK is cytoplasmic (Viaud et al., 2006). A domain consisting of around 90 amino acid residues is repeated five to seven times at the *N* terminus of a Group III HisK (Fig. 2). This domain is often found in proteins involved in the signaling system of prokaryotes, and the proteins are called HAMP (**H**istidine kinase, **A**denyl cyclase, **M**ethyl accepting chemotaxis protein, **P**hosphatase). Studies have shown that the HAMP domain is important in relation to the role of the sensor kinase, and is involved in intermolecular interactions (Pollard et al., 2009; Swain & Falke, 2007; Tao et al., 2002). In *N. crassa*, *B. cinerea* and *C. heterostrophus*, the deletions of the HAMP domain or amino acid-substituted mutations resulted in hyper-osmosensitivity and fungicide resistance, clearly indicating that this domain is essential for the function of Group III HisK (Cui et al., 2002; Ochiai et al., 2001; Yoshimi et al., 2004). Futhermore, intramolecular interactions of the HAMP domains of Group III HisK was demonstrated using two hybrid assays (Meena et al., 2010). However, the role and function of the HAMP domains of Group III HisK in molecular interactions is not fully elucidated, and further clarification at the molecular level is still required.

What are the functional differences between Group III HisKs and SLN1 in budding yeast? As mentioned at the beginning of the paper, when budding yeast cells are exposed to hyperosmotic stress, HOG1 MAPK is phosphorylated and glycerol accumulates in the cells. Glycerol accumulation as a result of hyperosmotic stimulus is also observed in filamentous fungal cells, e.g. *N. crassa* and *C. heterostrophus* (Fujimura et al., 2000; Tanaka et al., 2006). Moreover, fungicide treatment also induces glycerol accumulation in these filamentous fungal cells. In filamentous phytopathogenic fungi, i.e. *Colletotrichum lagenarium*, *C. heterostrophus* and *B. cinerea*, phenylpyrrole, fungicide treatments abnormally induce the phosphorylation of the HOG1 MAPKs of those fungi (Kojima et al., 2004). However, in mutants lacking the Group III HisKs of those filamentous fungi, the phosphorylation of HOG1 MAPK and glycerol accumulation due to exposure to hyperosmotic stress or the fungicides are not observed (Fujimura et al., 2000; Yoshimi et al., 2005). That is, in filamentous fungi, a Group III HisK functions as a responsible upstream factor in an osmotic signaling system, to sense and transduce osmotic signals, in the same way as SLN1 in budding yeast. In addition, budding yeast is innately insensitive to dicarboximide and phenylpyrrole fungicides. However, the introduction of *Hik1*, a Group III HisK gene of *M. grisea*, results in phenotypic alterations: transformants are no longer tolerant to the fungicide and the phosphorylation of HOG1 MAPK occurs due to the fungicide treatment (Motoyama et al., 2005b). These results indicate that Group III HisK is an essential factor that regulates the phosphorylation of HOG1 MAPK in response to both hyper-osmolarity and the fungicides. They also suggest that Group III HisK itself, or the phospho-relaying interactions of Group III HisK in intramolecules or intermolecules with other factors, e.g. HPt and RRs, are the targets of the fungicides. The fungicides mimic hyperosmotic stress that forces fungal cells to induce physiological adaptation via Group III HisK as if the cells were in a hyperosmotic condition, although they are not exposed to hyperosmotic stress.

Until now, the fungicide categorized as an aromatic hydrocarbon, the aforementioned antibiotic pyrrolnitrin, and bacteria-derived polyketide antibiotic ambruticin were considered to have similar modes of action to dicaroboximides and phenylpyrroles (Motoyama et al., 2005b; Okada et al., 2005; Vetcher et al., 2007).

Budding yeast possesses a sole HisK, SLN1 and its mutant exhibits lethality (Posas et al., 1996). The reason for this is believed to be that in mutants defective in *Sln1*, the HOG1 MAPK is constitutionally activated, and improper physiological changes are induced. However, in filamentous fungi, mutants defective in Group III HisKs do not exhibit lethality. This difference is probably not due to the type of HisK involved or the difference in the genes controlled by the downstream HOG pathway, but to the difference between a single and a multiple HisK-based two-component regulatory system. It is very interesting and important to know how several HisKs interact with and regulate HPt and RRs in the signaling systems, because this will enable us to understand the modes of action of dicarboximide and phenylpyrrole fungicides at the molecular level.

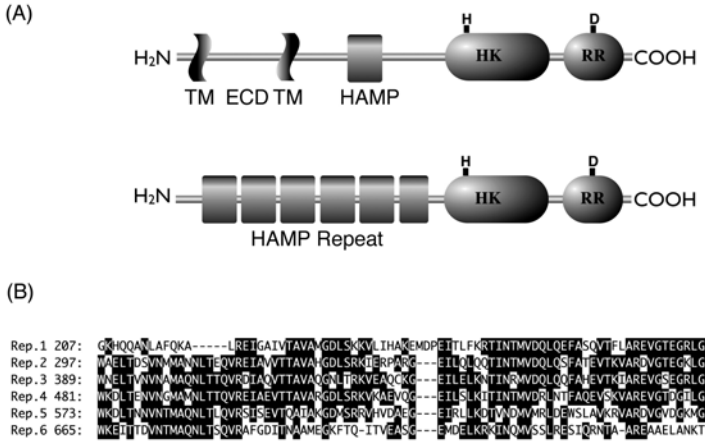

Fig. 2. (A) Schematic of the structure of a SLN1 histidine kinase and a Group III histidine kinase, (B) an example of the HAMP sequence (*Cochliobolus heterostrophus* Dic1). HAMP of Group III HisK is repeated five times in ascomycetous yeasts such as *Candida*, six times in filamentous Ascomycetes and seven times in Basidiomycetes.
TM: transmembrane domain, ECD: extracellular region of SLN1, HMAP: see the main text

5. Other HisKs involved in the osmotic signaling system

It is known that in some fungi, such as *C. neoformans*, HisKs other than Group III HisKs are involved in the hyperosmotic stress response. Mutants deficient in the *Tco1* (also called *CnNIK1*) gene coding a Group III HisK in this fungus show strong phenylpyrrole resistance but do not become hyper-osmosensitive. Tco2, a HisK belonging to a class specific to this fungus, is believed to control the hyperosmotic stress response of this fungus (Bahn et al., 2006). However, the *Tco2* gene disruptants show weak phenylpyrrole resistance, and therefore Tco1 and Tco2 are believed to have overlapping functions associated with the HOG pathway. In *C. heterostrophus* and *B. cinerea*, Group III HisKs control the hyperosmotic

stress response, regardless of the type of osmolytes (Yoshimi et al., 2003; Izumitsu et al., 2007). However, *Hik1* gene disruptants in *M. grisea* are highly sensitive to hyperosmotic stress with sorbitol, but not with certain solutes (KCl, NaCl and glycerol). In *M. grisea*, the involvement of HisK(s) other than Hik1 or an unknown hyperosmotic adaptation mechanism for these solutes has been suggested (Motoyama et al., 2005a).

6. RR involved in the osmotic signaling system and the mode of action of the fungicides

In *Neurospora crassa*, mutants of *os-1*, a Group III HisK gene, and those of *os-2*, a HOG1 MAPK gene, exhibit almost the same hyperosmotic sensitivity and fungicide resistance (Fujimura et al., 2000). However, HOG1 MAPK gene disruptants in *C. heterostrophus* and *B. cinerea* do not show as strong a hyperosmotic sensitivity and fungicide resistance as the Group III HisK gene (Fig. 3; Izumitsu et al., 2010). This suggests that in these plant pathogenic filamentous fungi, a signal stream from Group III HisK is divergent in down streams. There is the possibility of signal branching at each step of the two-component regulatory system and the HOG1 MAPK signaling pathway. In this work, signal divergence is shown to be the result of the involvement of the two RRs (Table 1) composing the two-component regulatory system in the hyperosmotic stress response.

The RR protein is a key element in the two-component signaling system. It governs output responses via its phosphorylation level, which is under the control of an upstream regulator HisK (West & Stock, 2001). Two conserved RR proteins homologous to Ssk1 and Skn7 in *S. cerevisiae* have been identified in several fungal species (Catlett et al., 2003). However, the specific responses that these proteins govern have only been characterized in certain yeast species, not in filamentous fungi (Bahn et al., 2006; Cottarel 1997; Krems et al., 1996; Nakamichi et al., 2003; Posas et al., 1996; Singh et al., 2004). The role of the response regulators in the filamentous fungus was first characterized in a study using *C. heterostrophus* RR disruptant (Izumitsu et al., 2007).

Ssk1 mutants of *C. heterostrophus* showed increased sensitivity to hyperosmotic stress and moderate dicarboximide and phenylpyrrole resistance, implying that *Ssk1* plays a role in osmotic adaptation and fungicide sensitivity. Although the role of Ssk1 in a two-component signaling system and a high-osmolarity glycerol pathway has been well characterized in yeast, only one functional analysis has been conducted on the Ssk1 homologue in filamentous fungi. In that report, an *Aspergillus nidulans ssk1* (*sskA*) mutant showed an osmosensitive phenotype and a deficiency of HOG1 MAPK phosphorylation (Furukawa et al., 2005). The data from the study using *C. heterostrophus* suggested that the Ssk1-type response regulator plays roles in high-osmolarity adaptation and in the mode of action of the fungicides. The results obtained with *C. heterostrophus* also indicate that the other response regulator, Skn7, plays a role in the osmotic adaptation and moderate resistance of Ssk1 and Skn7 RRs are involved in high-osmolarity adaptation and fungicide sensitivity. However, the two proteins show different mechanistic functions in the response pathway. The disruption of the *Ssk1* gene prevents the phosphorylation of HOG1 MAPK in both the high-osmolarity stress and the fungicide treatments, whereas the *Skn7* mutation does not affect the phosphorylation of HOG1 MAPK (Fig. 4). Various morphological observations of the *Ssk1* and *Skn7* mutants compared with wild-type cells after the application of the fungicides also indicated a difference in function between the two RRs (Fig. 5). The wild type develops heavily swollen hyphae with inflated cells, and hyphal growth is strongly

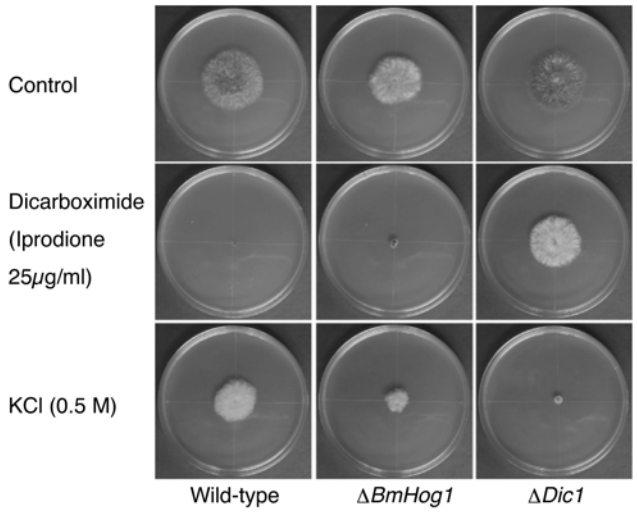

Fig. 3. Resistance to the dicarboximide fungicide, iprodione, and sensitivity to hyperosmotic condition with KCl in the wild-type, HOG1-MAPK (*BmHog1*) disruptant and Group III HisK (*Dic1*) disruptant of *Cochliobolus heterostrophus*

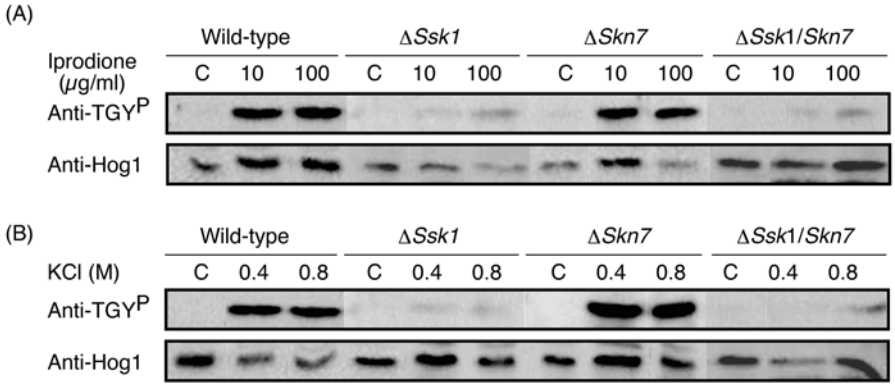

Fig. 4. HOG1-MAPK phosphorylation in the *Cochliobolus heterostrophus* wild-type strain, the *Ssk1* mutant strain, the *Skn7* mutant strain, and the *Ssk1/Skn7* double-mutant strain induced by the fungicide and osmotic stress. (A) Prepared mycelia of the strain tested were incubated in CM medium with or without 10 and 100 μg/ml iprodione for 10 min. Phosphorylated BmHog1 was detected using anti-dually phosphorylated p38 antibody (indicated by Anti-TGY^P). The total amount of BmHog1 was measured using anti-Hog1 C-terminus antibody (indicated by Anti-Hog1). (B) Prepared mycelia of the strain tested were incubated in CM medium with or without 0.4 and 0.8 M KCl for 10 min

inhibited by the fungicides, whereas both of the mutants showed partially restricted growth of hyphae, indicating incomplete fungicidal activity. In addition, the *Skn7* mutant develops swollen hyphae and inflated cells similar to those of the wild type, and the *Ssk1* mutant does not. Applications of dicarboximide and phenylpyrroles to *N. crassa* and *C. heterostrophus* mycelia cause abnormal accumulations of cellular glycerol, resulting in cell inflation and hyphal swelling (Tanaka et al., 2006; Zhang et al., 2002). These results suggest that only Ssk1 controls HOG1 MAPK-phosphorylation, which, under osmotic and fungicide stress conditions, seems to result in the accumulation of cellular glycerol. Moreover, Skn7 appears to play other roles in high-osmolarity adaptation and fungicide sensitivity that are independent of the activation of HOG1 MAPK.

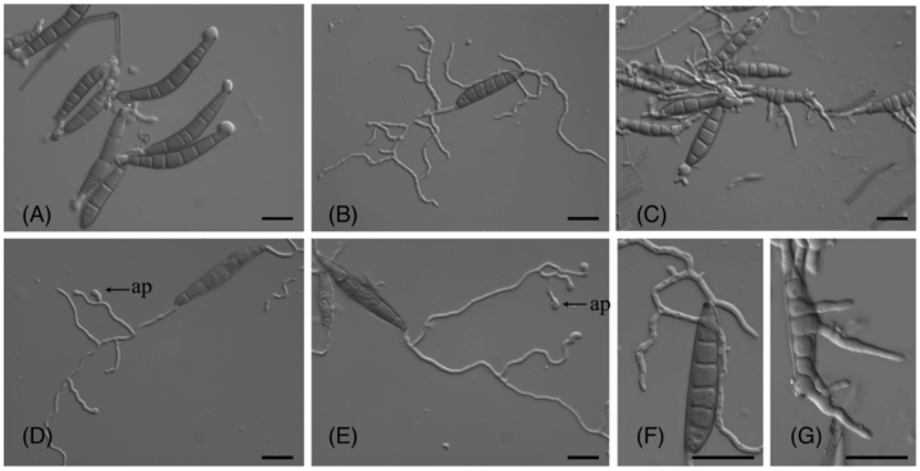

Fig. 5. Effect of dicarboximide treatment on the *Cochliobolus heterostrophus* wild-type strain, the *Ssk1* mutant strain, the *Skn7* mutant strain, and the *Ssk1/Skn7* double-mutant strain. (A) Wild-type strain incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (B), (E), (F) *Ssk1* mutant incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (C), (G) *Skn7* mutant incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (D) *Ssk1/Skn7* double-mutant strain incubated in CM medium containing 10 µg/ml iprodione for 6 h at 25˚C. (E) Untreated wild-type strain. No differences were observed between the untreated wild-type strain and the untreated mutant strains (data not shown). ap = appressorium. Scale bars = 50 µm

The phenotypes of the *Ssk1*, *Skn7*, and *Dic1* (Group III HisK) mutants are comparable but not identical. As mentioned above, Dic1 is the HisK responsible for osmotic adaptation and fungicide sensitivity in this fungus (Yoshimi et al., 2004; Yoshimi et al., 2005). All the phenotypic characteristics of the *Ssk1* and *Skn7* mutants are also observed in the Group III HisK mutants. In contrast to the *Ssk1* and *Skn7* single mutants, the *Ssk1/Skn7* double-mutant cells clearly show higher resistance to the fungicides than either single-mutant strain alone. Furthermore, the double-mutant strains are much more sensitive to the osmotic stress than the single-mutant strains. The dose–response of the *Ssk1/Skn7* double mutant to high osmolarity and fungicide exposure parallels that of the Group III HisK mutant. The above data clearly indicate that there are two osmotic signaling pathways in this fungus: a Group

III HisK => SSK1 => HOG pathway and a Group III HisK => SKN7 pathway. These two pathways are believed to contribute to hyperosmotic adaptation and the onset of fungicidal action equally and additively.

The discovery of the involvement of two osmotic signaling pathways in the down streams of Group III HisK provide a new insight into the mode of action of dicarboximide and phenylpyrrole fungicides. As mentioned in the above section, Group III HisK is probably a primary target of these fungicides or a core mediator of their fungicidal action, and the main mode of action of these fungicides is the abnormal phosphorylation of the HOG1 MAPK controlled by Group III HisK and consequent improper gene expression. Our results strongly indicate that improper signal mediation by the "Group III HisK => Skn7" pathway, which will cause the abnormal expression of the genes needed for hyper-osmotic adaptation, along with the "Group III HisK => SSK1 => HOG" pathway, is also required for the full activity of these fungicides. Moreover, with *B. cinerea*, the improper activation of "Group III HisK => SKN7" alone is sufficient to arrest colonial growth (Izumitsu et al., 2010). These facts implied that not only the primary target but also the genes abnormally expressed under the controls of each pathway are also important if we are to understand the mode of action of the fungicide. The identification of the genes that provide the greatest fungicidal activity will promote the development of new fungicides.

Today, the involvement of two osmotic signaling pathways in the down streams of Group III HisK is widely recognized in several filamentous fungi, e.g. *A. nidulans*, *M. grisea* (Aguirr et al., 2008; Hagiwara et al., 2007). However, in *M. grisea,* the Group III HisK => SSK1 => HOG pathway plays a major role in the hyperosmotic adaptation and the Group III HisK => SKN7 pathway plays a minor role, owing to the difference between the phenotypes of the two pathways (Motoyama et al., 2008). Fig. 6 shows a generalized osmotic signaling system in filamentous fungi.

7. Interesting characteristics regulated by the osmotic signaling system

The above discussion focused on cellular adaptation to osmotic stress and fungicidal action. However, some studies have shown that the osmotic signaling system of filamentous fungi is involved in various other important functions.

In *B. cinerea*, a Group III HisK gene disruptant forms very few conidia (Viaud et al., 2006). This characteristic can also be found in a downstream HOG1 MAPK gene disruptant (Segmüller et al., 2007). In these two disruptants, sclerotia are formed in very large quantities. In *B. cinerea,* a sclerotium is recognized as a survival organ for overwintering, and is prerequisite for sexual reproduction (Faretra et al., 1987). That is, it is suggested that in this fungus, the osmotic signaling system serves as a switch between asexual and sexual reproduction modes. In addition to this role in morphogenesis, the osmotic signaling system of *B. cinerea* plays a critical role in plant infection. Both the Group III HisK and HOG1 MAPK disruptants are largely non-pathogenic to host plants (Izumitsu et al., 2010; Segmüller et al., 2007; Viaud et al., 2006). The fact that in *B. cinerea*, the mutations of the fungicide resistance genes interferes with its pathogenicity, and morphogenesis may provide a scientific basis for understanding the well-known fact that there is a loss of fitness associated with dicarboximide resistance, resulting in a decrease in the frequency of resistant strains when dicarboximides are no longer applied (q.v. Fungicide Resistance Action Committee, 2010). In addition to this fungus, genes involved in the osmotic signaling system: *Tco1* and *Skn7* in *C. neoformans* (Bahn et al., 2006), *CaNik1* (also called as *Cos1*) and *Ssk1* in *C. albicans* (Calera et al., 2000; Yamada-Okabe et al.,

1999), *Ssk1* in *M. grisea (*Motoyama et al., 2008), and *AbNik1* in *Alternaria brassicicola* (Cho et al., 2009), are known to confer pathogenicity.

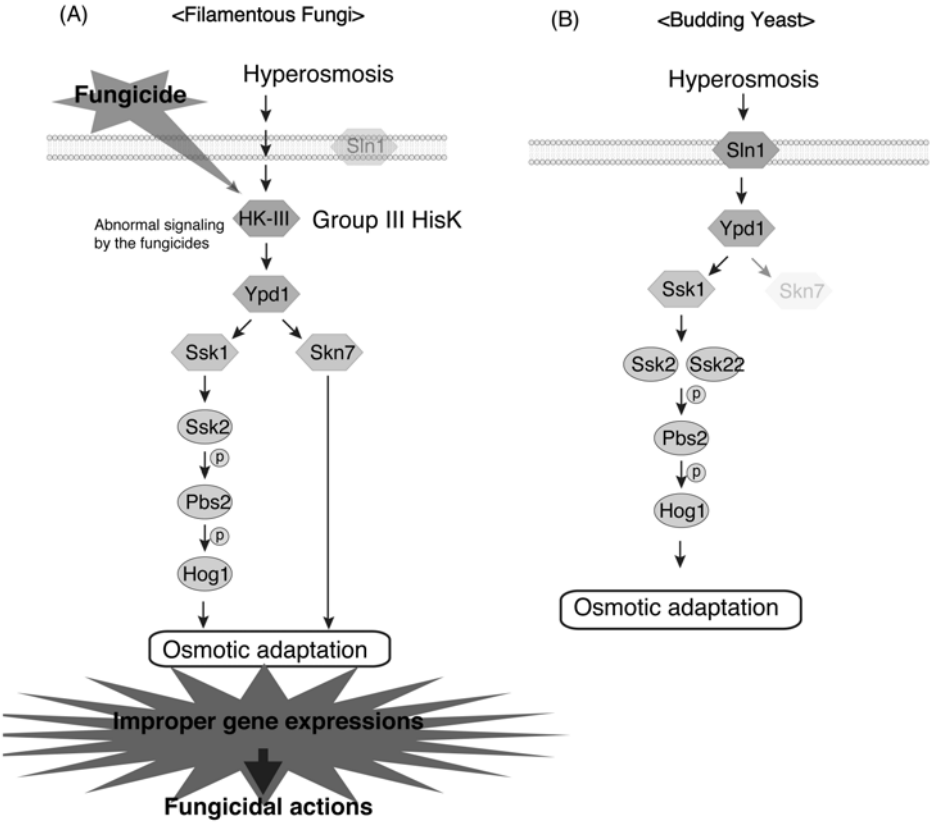

Fig. 6. Model illustration of the osmotic signaling systems of filamentous fungi (A) and budding yeast (B).

8. Concluding remarks

The osmotic signaling system of filamentous fungi consists of a two-component regulatory system and a HOG1 MAPK signaling system, as does the budding yeast used in the modeling of the osmotic signaling system. This system seems to be evolutionarily conserved among fungi, and yet has diverse functions and roles. This diversity is believed to be the result of the evolution of the environmental adaptability and survival strategies of each fungal species. The progress and accumulation of work on fungal genome science is promoting functional studies on the species without genomic sequence data. In addition, comparative studies using many species will lead to an understanding of the diverse roles of the signaling system. This knowledge is expected to be used to provide basic information that will aid the development of both new fungal control methods and new fungicides.

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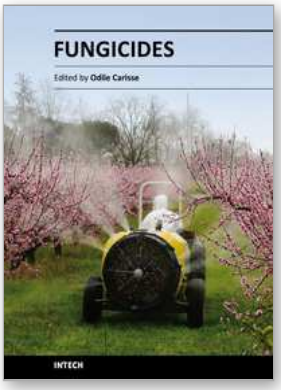

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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