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**Review**

**Signaling pathways for stress responses and adaptation in Aspergillus species: stress biology in the post-genomic era**

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**Aspergillus** species are among the most important filamentous fungi in terms of industrial use and because of their pathogenic or toxin-producing features. The genomes of several *Aspergillus* species have become publicly available in this decade, and genomic analyses have contributed to an integrated understanding of fungal biology. Stress responses and adaptation mechanisms have been intensively investigated using the accessible genome infrastructure. Mitogen-activated protein kinase (MAPK) cascades have been highlighted as being fundamentally important in fungal adaptation to a wide range of stress conditions. Reverse genetics analyses have uncovered the roles of MAPK pathways in osmotic stress, cell wall stress, development, secondary metabolite production, and conidia stress resistance. This review summarizes the current knowledge on the stress biology of *Aspergillus* species, illuminating what we have learned from the genomic data in this “post-genomic era.”

**Key words:** *Aspergillus*; stress response; MAPK pathway; TCS system; transcription factor

Filamentous fungi are ubiquitous in nature and are commonly found in and on decaying woods and plants, as well as in the soil. Their saprophytic characteristics are ecologically important as they play a crucial role in carbon and nitrogen recycling in nature. As well as being important components of the ecosystem, many species of filamentous fungi affect human life either beneficially or detrimentally. For instance, some fungi produce a variety of enzymes and organic acids that are used in food and pharmaceutical industries, and in producing fermented foods. A small but significant number of filamentous fungi cause diseases in animals and plants.

Growing on solid substrates seems to be the predominant mode during the lifecycle of filamentous fungi in nature. These fungi generally exist in a mycelial form in plant debris, compost piles, dead insects, and even immunocompromised humans and animals. In such habitats, the fungi must cope with several abiotic and biotic stressors including low or high temperature, dryness, low nutrient levels, oxygen limitation, ultraviolet (UV) radiation, and oxidative stress. Therefore, fungi have evolved mechanisms to sense and respond to the harsh conditions to survive in their environment. When grown on solid substrates, some fungi produce asexual or sexual spores that serve as stress-resistant genetic vehicles.\(^1\) Spore production is triggered by environmental cues such as light, nutrient conditions, temperature, and oxygen levels.\(^2,3\) Spore production is one of the most important ways in which filamentous fungi survive in their surroundings and prosper in their environmental niches.\(^4\)

The genomes of many filamentous fungi have been sequenced and are now available publicly.\(^5-8\) This has led to a better understanding of filamentous fungal biology and improvements in the use of these organisms in various industries. In particular, the wealth of genetic data has enriched our knowledge of fungal stress responses and adaptation mechanisms. In this review, we summarize the latest findings on the stress response and adaptation mechanisms of fungi during culturing. We focus on the genus *Aspergillus* because full genome sequences have been determined for several *Aspergillus* species. Much can be learned about stress biology from genomic analyses of multiple species in the same genus. Such information will lead to further advances in the general biology of fungi and their evolutionary relationships.

**I. Signaling pathways related to stress response and adaptation**

The genome sequences of *A. oryzae*, *Aspergillus nidulans*, and *Aspergillus fumigatus* were published in 2005.\(^5-7\) One of the first findings was that almost all of the stress signaling components identified in yeasts are encoded in the genomes of filamentous fungi. Comparative genomics studies have shown that filamentous
fungi have targets of rapamycin (TOR) signaling and protein kinase A (PKA) signaling pathways, mitogen-activated protein kinase cascades, and calcineurin signaling, as well as upstream signaling machinery (G-protein coupled receptors, G-protein subunits, and two-component signaling (TCS) system). All of these pathways and proteins have been well studied in Saccharomyces cerevisiae. Notably, some components of the signaling systems were found to be duplicated or diversified in filamentous fungi, reflecting their complex multicellular organization. The existence of duplicated/diversified signaling components suggested that filamentous fungi have evolved extensive sensing and signaling mechanisms to adapt to their surrounding environments. Based on the results of comparative studies, stress response mechanisms were further studied using a reverse genetic approach. The following section focuses on MAPK cascades and the upstream TCS system because their roles in the stress response have been clarified in several reverse genome studies over the last decade.

1. Mitogen-activated protein kinase cascades

The MAPK cascade is a highly conserved signaling unit found in fungi, plants, and animals. The prototypical MAPK cascade consists of three different modules: MAPK, MAPK kinase (MAPKK), and MAPK kinase kinase (MAPKKK). MAPKKK activation is followed by phosphorylation of MAPKK, which in turn phosphorylates MAPK. Typically, the phosphorylated MAPK translocates from the cytosol into the nucleus, where it activates (in most cases, phosphorylates) target proteins. The serial activation transmits certain environmental signals to the nucleus and regulates transcription therein leading to appropriate cellular responses. There are five functionally distinct MAPKs in S. cerevisiae: (1) Fus3p (mating-pheromone response pathway), (2) Kss1p (pseudohyphal development pathway), (3) Hog1p (high osmolality glycerol (HOG) pathway), (4) Ste11p/Mpk1p (protein kinase C or cell wall integrity pathway), and (5) Ssk1p (spore wall assembly pathway). Four of these MAPKs (all except Smk1p) are controlled by MAPKKs (Ste7p, Pbs2p, and redundant Mkk1p and Mkk2p) and MAPKKKs (Ste11p, Bck1p, and redundant Ssk2p and Ssk22p) (Fig. 1).

Fig. 1. Schematic diagrams of MAPK signaling pathways in Saccharomyces cerevisiae.

Notes: Five MAPKs, four MAPKKs, and four MAPKKKs have been identified in sequence-based searches. These proteins constitute four signaling pathways involved in the pheromone response, filamentous growth, cell wall integrity, and high osmolality adaptation, although no MAPK or MAPKK has been assigned to regulate Ssk1p MAPK. Mkk1p and Mkk2p, and Ssk2p and Ssk22p are thought to redundantly function in the pathway.

Homology searches revealed that there are three types of MAPK proteins and three MAPKK and MAPKKK proteins encoded in the genomes of filamentous fungi, including A. nidulans, A. oryzae, A. fumigatus, and Aspergillus niger (Table 1). This finding suggested that while the Kss1p (pseudohyphal development) pathway and Ssk1p (spore wall assembly) pathway are absent, three MAPK cascades function in the stress response of filamentous fungi: (1) the Fus3 (pheromone) pathway, (2) the Hog1 (HOG) pathway, and (3) the Mpk1 (cell wall integrity) pathway (Fig. 2). One component of the HOG pathway, a MAPK protein, is duplicated in A. nidulans and A. fumigatus, (each has HogA/SakA and MpkC) and triplicated in A. oryzae and A. niger (each has HogA/SakA, MpkC, and MpkD). This suggests that the signal transduction cascade of A. oryzae and A. niger might be more complex than that of A. nidulans and A. fumigatus.

The physiological roles of the three MAPK cascades in Aspergillus species have been investigated. The MpkB MAPK in the pheromone-responsive pathway was reported to play a role in hyphal growth, conidia formation, conidia viability, sexual development, and autolysis in A. nidulans. MpkB was also required for the control of secondary metabolite production and growth upon micafungin treatment. The upstream component SteC MAPKKK, a homolog of S. cerevisiae Ste11, has been characterized. Compared with the wild type, the deletion mutant of steC showed a slower growth rate, formation of more branched hyphal tips, altered conidiophore morphology, and inhibited heterokaryon formation and cleistothecium development. Recently, Bayram et al. demonstrated that the SteC–Ste7–MpkB complex with an adaptor protein Ste50 mediates development and secondary metabolism in A. nidulans. Together, these findings indicated that the pheromone-responsive pathway in Aspergillus is responsible for multiple biological processes.

The second MAPK cascade is the cell wall integrity pathway. This pathway is activated in response to several environmental stimuli in S. cerevisiae, resulting in increased expressions of genes encoding integral cell wall proteins and enzymes involved in cell wall biogenesis. Based on the results of studies on this pathway in S. cerevisiae, the corresponding Aspergillus MpkA MAPK cascade was characterized by several groups. The A. nidulans mpkA deletion mutant showed significant defects in conidia germination and in polarized growth, and markedly delayed colony growth. The mutant was sensitive to two cell wall perturbing reagents; micafungin and calcofluor white (CFW). These phenotypes suggested that MpkA is involved in maintaining the cell wall and promoting cell wall remodeling.

Notably, the genes that are regulated in the cell wall integrity pathway differ between A. nidulans and S. cerevisiae. Whereas the Mpk1p cascade controls the expressions of FKS1 (encoding β-1,3-glucan synthase) and CHS3 (encoding chitin synthase) in S. cerevisiae, the expressions of the corresponding genes in A. nidulans (fksA encoding β-1,3-glucan synthase and a gene-encoding chitin synthase) are independent of MpkA. Another intriguing finding was that MpkA regulated the expressions of two α-1,3-glucan synthase.
genes, agsA and agsB.\textsuperscript{26} This may be because α-1,3-glucan is one of the major cell wall polysaccharides in *Aspergillus*, but not in *S. cerevisiae*. The evolutionarily different functional roles of the cell wall integrity pathways are key factors in understanding the physiology of filamentous fungi.

The third MAPK cascade is the HOG pathway. This pathway plays a pivotal role in the osmotic stress response in *S. cerevisiae*.\textsuperscript{9} The *S. cerevisiae* HOG pathway consists of Ssk2p/Ssk22p MAPKKs, Pbs2p MAPKK, and Hog1p MAPK, and is regulated by two different upstream branches; the TCS system and the Sho1p pathway (Fig. 3). Signaling of the Sho1p branch depends on an interaction with Pbs2p MAPKK to regulate Hog1p MAPK. Hence, the two upstream routes converge at Pbs2p MAPKK. In contrast, the *A. nidulans* PbsB protein, an ortholog of Pbs2p, lacks the Pro-rich motif that is required for binding to the Sre-homology3 (SH3) domain of Sho1p. This suggests that PbsB does not interact with the ShoA branch (which corresponds to the Sho1p branch). In fact, PbsB was unable to transduce the signal from the Sho1p branch in a complementation experiment in *S. cerevisiae*, but it transduced the signal from the TCS branch.\textsuperscript{27} Taken together, these results showed that the connection between the Sho1p branch and Pbs2p is not conserved in *A. nidulans*.

The physiological functions of the HOG pathway have been intensively analyzed by a genetic deletion approach in wide variety of filamentous fungi.\textsuperscript{28–30} *A. nidulans* possesses SskB MAPKKK, PbsB MAPKK, and HogA/SakA MAPK in the HOG pathway (Fig. 2 and 3). The *A. nidulans* MAPKK cascade is summarized based on previous studies. Pheromone-responsive pathway consists of SteC, Ste7, and MpkB. High osmolarity glycerol (HOG) pathway consists of SskB, PbsB, HogA/SakA, and MpkC. Cell wall integrity pathway consists of BckA, MkkA, and MpkA. Known upstream regulators, target transcription factors (TF), and regulated genes are shown. SM: secondary metabolite, OS: osmotic stress, WSC: wsc motif-containing proteins.

<table>
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<th>MAPK</th>
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<th>Regulated</th>
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<td>AN10153 (SskB)</td>
<td>AN3422 (Ste7)</td>
<td>AN4189 (MkkA)</td>
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<td>AO090003000662</td>
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<td>AO090020000060</td>
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</tr>
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<td>BckA</td>
<td>SskB</td>
<td>PbsB</td>
<td>HogA/SakA, MpkC</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAPKK</td>
<td>MAPK</td>
<td>Upstream system</td>
<td>Cell wall integrity pathway</td>
</tr>
<tr>
<td>G-protein signaling system</td>
<td>WSC-sensing, Protein kinase C</td>
<td>Two-component signaling (TCS) system</td>
<td></td>
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</tr>
<tr>
<td>Pheromone-responsive pathway</td>
<td>Cell wall integrity pathway</td>
<td>High osmolarity glycerol (HOG) pathway</td>
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</tbody>
</table>

Table 1. Proteins of *Aspergillus* MAPK cascades.

<table>
<thead>
<tr>
<th>A. nidulans</th>
<th>A. oryzae</th>
<th>A. fumigatus</th>
<th>A. niger</th>
<th>Ortholog of <em>S. cerevisiae</em></th>
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</thead>
<tbody>
<tr>
<td>MAPKK</td>
<td>MAPKK</td>
<td>MAPK</td>
<td>Target TF</td>
<td>Regulated</td>
</tr>
<tr>
<td>SteC</td>
<td>BckA</td>
<td>SskB</td>
<td>PbsB</td>
<td>HogA/SakA, MpkC</td>
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<td>Ste7</td>
<td>MkkA</td>
<td>MpkA</td>
<td>MpkA</td>
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<tr>
<td>MAPKKK</td>
<td>MAPKK</td>
<td>MAPK</td>
<td>Upstream system</td>
<td>Cell wall integrity pathway</td>
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<tr>
<td>G-protein signaling system</td>
<td>WSC-sensing, Protein kinase C</td>
<td>Two-component signaling (TCS) system</td>
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<tr>
<td>Pheromone-responsive pathway</td>
<td>Cell wall integrity pathway</td>
<td>High osmolarity glycerol (HOG) pathway</td>
<td></td>
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</tbody>
</table>

Notes: Three MAPK cascades of *A. nidulans* are summarized based on previous studies. Pheromone-responsive pathway consists of SteC, Ste7, and MpkB. High osmolarity glycerol (HOG) pathway consists of SskB, PbsB, HogA/SakA, and MpkC. Cell wall integrity pathway consists of BckA, MkkA, and MpkA. Known upstream regulators, target transcription factors (TF), and regulated genes are shown. SM: secondary metabolite, OS: osmotic stress, WSC: wsc motif-containing proteins.

Fig. 2. Components of three MAPK cascades and associated mechanisms in *Aspergillus nidulans*. The third MAPK cascade is the HOG pathway. This pathway plays a pivotal role in the osmotic stress response in *S. cerevisiae*.\textsuperscript{8,9} The *S. cerevisiae* HOG pathway consists of Ssk2p/Ssk22p MAPKKs, Pbs2p MAPKK, and Hog1p MAPK, and is regulated by two different upstream branches; the TCS system and the Sho1p pathway (Fig. 3). Signaling of the Sho1p branch depends on an interaction with Pbs2p MAPKK to regulate Hog1p MAPK. Hence, the two upstream routes converge at Pbs2p MAPKK. In contrast, the *A. nidulans* PbsB protein, an ortholog of Pbs2p, lacks the Pro-rich motif that is required for binding to the Sre-homology3 (SH3) domain of Sho1p. This suggests that PbsB does not interact with the ShoA branch (which corresponds to the Sho1p branch). In fact, PbsB was unable to transduce the signal from the Sho1p branch in a complementation experiment in *S. cerevisiae*, but it transduced the signal from the TCS branch.\textsuperscript{27} Taken together, these results showed that the connection between the Sho1p branch and Pbs2p is not conserved in *A. nidulans*.
be highly phosphorylated in conidia, even after prolonged incubation (up to 7 days) in water at 4 °C.\(^{32}\) These results suggested that the HOG pathway is responsible for conidial stress tolerance in \textit{A. nidulans}.

The \textit{A. fumigatus} deletion mutant of SakA (HogA) also showed growth inhibition under osmotic stress. SakA was shown to be phosphorylated (activated) in response to osmotic shock and certain types of fungi-icide in a TCS system-dependent manner.\(^{33}\) \textit{A. fumigatus} SakA regulates germination in response to nitrogen availability, and was shown to be phosphorylated upon nitrogen or carbon starvation during vegetative growth.\(^{34}\) Recently, \textit{sskB} expression in \textit{A. fumigatus} was shown to be regulated directly by the calcium-responsive transcription factor (TF) CrzA.\(^{35}\) The deletion mutant of \textit{sskB} was sensitive to excess calcium, suggesting that the calcium signaling pathway is involved in regulating the HOG pathway. Interestingly, the \textit{sskB} deletion mutant showed attenuated virulence in a mouse infection model, supporting the view that the HOG pathway plays a role in the pathogenicity of \textit{A. fumigatus}.

Besides HogA/SakA, an additional MAPK, MpkC, is present in the \textit{Aspergillus} HOG pathway. The deletion mutant of MpkC in \textit{A. fumigatus} showed growth defects on minimal medium with sorbitol or mannitol as the sole carbon source.\(^{36}\) Recently, Hagiwara et al.\(^{37}\) reported that the \textit{sakA} deletion mutant produced conidia with normal stress resistance (unlike the stress-sensitive conidia produced by the \textit{A. nidulans hogA} mutant) and that the \textit{mpkC}sakA double deletion mutant produced stress-labile conidia with reduced trehalose content. Collectively, these results showed that the HOG pathway of \textit{Aspergillus} species functions in various physiological processes to adapt to the surrounding environment.

\textbf{I.ii. TCS system}

The TCS (also known as His-Asp phosphorelay signaling) system, which was first described in bacteria, is a common signal transduction mechanism that exists in organisms ranging from bacteria to fungi and plants, but not in animals.\(^{38-41}\) The prototypical system in bacteria consists of two types of common signal transducers (hence, “two-component”); a histidine kinase (HK) and a response regulator (RR). Eukaryotes have an additional component, a histidine-containing phosphor-transmitter (HPT); hence, their signaling system has three components (HK-HPT-RR). Each component has a conserved motif with an invariant amino acid residue, His or Asp, and phosphor-groups are transferred (relayed) between the components at these amino acid residues (His to Asp). The eukaryotic HK has both a kinase domain (with a conserved His) and a receiver domain (with a conserved Asp), and is known as a hybrid-HK. The kinase domain is autophosphorylated from ATP, most likely via conformational changes in response to certain stimuli. In turn, the phosphor-group is transferred to the Asp residue in the receiver domain. The HPT contains a conserved His and the RR has a receiver domain with a conserved Asp. Hence, the
fungal TCS system consists of a multi-step phosphoryl-transfer (His-Asp-His-Asp) among the three components.40,41)

The TCS systems in *S. cerevisiae* and *Schizosaccharomyces pombe* have been well characterized. *S. cerevisiae* has one HK (Slt1p), three RRs (Ssk1p, Skn7p, and Rim15p), and one HPT (Ypd1p). *S. pombe* has three HKs (Phk1–Phk3), three RRs (Mcs4, Prr1, and Cek1), and one HPT (Mpr1) (Fig. 3).42-44) In yeasts, these systems are involved in osmotic and oxidative stress responses and in regulating the mitotic cell cycle and sexual development.45-48) Genomic analyses of *A. nidulans*, *A. oryzae*, and *A. fumigatus* revealed that they contain 15, 15, 11, and 13 HK genes, respectively (Table 2). Thus, HK genes are more abundant in filamentous fungi genomes than in yeast genomes,49) suggesting that signaling networks might be more complex in filamentous fungi than in yeasts.

As stated above, the TCS system regulates the HOG MAPK cascade in yeast. A comparative genomics analysis suggested that one of the *Aspergillus* RRs, SskA (corresponding to *S. cerevisiae* Skk1p), likely activates SskB MAPKK by direct interaction, and consequently activates the HOG pathway.50,51) Genetic analyses of *A. nidulans* and *A. fumigatus* revealed that SskA is dispensable for phosphorylation of the HogA/SakA MAPK. The deletion of SskA resulted in retarded growth under high osmolality conditions.27,33)

The other characterized RR is an Skn7-type RR (SrtA in *A. nidulans* and Skn7 in *A. fumigatus*) that is not involved in regulating the HOG pathway in *Aspergillus* species.47) The Skn7-type RRs were shown to be required for the oxidative stress response, as is the case in *S. cerevisiae* and *S. pombe*.46-49) According to the findings of studies on yeasts,50,51) the Skn7-type RR of filamentous fungi may function in association with an AP-1-type TF, a highly conserved regulator of the oxidative stress response. The deletion mutant of *A. fumigatus skn7* showed remarkable sensitivity to oxidative stress in *vitro*; however, virulence of the mutant was comparable to that of the parental strain in a mouse infection model.52) This study suggested that the oxidative stress response is not necessarily related to the pathogenicity of *A. fumigatus*. In future studies, nonetheless, it will be important to elucidate whether upstream components such as HKs and HPTs are involved in the oxidative stress response and in the activation of the Skn7-type RR in filamentous fungi.

Other types of RR have been found and characterized in *A. nidulans* (SskB and SskC) and *A. oryzae* (AoRim15) (Table 2). The *A. nidulans* mutants of SskB and SskC showed no distinguishable phenotypes.46) In *A. oryzae*, AoRim15 was shown to be involved in the resistance to heat and oxidative stresses in conidia.53) The *Aorim15* mutant showed reduced conidiation and sclerotia formation, suggesting that AoRim15 plays a

<table>
<thead>
<tr>
<th>Family</th>
<th><em>A. nidulans</em></th>
<th><em>A. oryzae</em></th>
<th><em>A. fumigatus</em></th>
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**Notes:** IDs and ortholog relationships are as listed at AspGD (http://www.aspgd.org/).

*1: Asp residue required for accepting phosphor-group is replaced with Glu in receiver domain.

*2: Name is as listed in Hayashi et al. [45].

*3: Although N-terminus of HK protein shows moderate similarity to that of other HKs in the group, C-terminus sequence is missing. ID is shown in parentheses since it does not appear to function as a HK.

*4: N-terminus sequence is missing.

*5: Names of HKs are as listed in Hagiwara et al. [33].

*6: Sc, Sp, and Ca indicate *S. cerevisiae*, *S. pombe*, and *Candida albicans*, respectively.
role in the development of *A. oryzae*. Although AoRim15 is regarded as an RR because it has a receiver domain similar to that of Ssk1-type or Skn7-type RRs, the Asp residue that is required to accept the phosphor-group is replaced with Glu in the receiver domain of AoRim15. Instead, it contains PAS and protein kinase domains. These bioinformatics data raise the possibility that AoRim15 does not function in a TCS system.

The genomes of Aspergillus species encode 11–15 HKs, which have been investigated to some extent. First, the HKs of four *aspergilli* were classified into nine families (HK1–HK9) based on amino acid sequence similarity (Table 2). The HK1 family is orthologous to *S. cerevisiae* Snf1p, and the HK4 and HK5 families are orthologous to *S. pombe* Phk1/Phk2 and Phk3, respectively. This comparative genomics evidence suggests that the TCS system of filamentous fungi has evolved from that of yeasts in a somewhat reciprocal fashion. Homology searches revealed that HK6 is the prevalent HK among filamentous fungi, and it is also found in basidiomycetes. Together, these findings suggest that the TCS system of filamentous fungi has evolved from that of yeasts in a somewhat reciprocal fashion. Homology searches revealed that HK6 is the prevalent HK among filamentous fungi, and it is also found in basidiomycetes. Together, these findings suggested that TCS systems may have evolved both differentially and conservatively in each species to adapt to their environmental niche.

The HKs in the HK1 family are thought to play a role in osmotic stress responses because of their similarity to the *S. cerevisiae* Snf1p. Thus, the *A. nidulans* HK1 protein TcsB has been investigated in detail. Heterologous expression of the *A. nidulans* tcsB gene complemented the lethality of a *S. cerevisiae* strain defective in *SNF1*. Contrary to expectations, however, the tcsB deletion mutant did not show any detectable phenotypic defects on standard and osmotic stress media, and TcsB was not required for the phosphorylation of the HogA MAPK in response to osmotic and oxidative stresses. In a recent report on *A. fumigatus*, TcsB was shown to be involved in phosphorylation of the SakA MAPK in response to osmotic and oxidative stresses. In the HK1 family, the tcsB gene is expressed in a wide range of conditions, including osmotic stress, 

The HK3 family contains *A. nidulans* TcsA and *A. fumigatus* Fos1. The tcsA mutant did not produce conidia on standard medium, but the defect in conidiation was suppressed by addition of 1 M sorbitol to the medium. TcsA is required for the nuclear localization of the basic helix–loop–helix TF, DevR, which is involved in conidiation formation under standard growth conditions. Conversely, the fos1 deletion mutant did not show detectable defects in either hyphal growth or morphology on solid medium. However, the expression of fos1 was increased in a SakA-dependent manner in response to osmotic stress, suggesting that Fos1 may be involved in regulating the Hog pathway in *A. fumigatus*. This is an interesting example of how HKs in the same family can show different roles in different Aspergillus species.

The HK4 and HK5 families are orthologous to the HKs of *S. pombe* that are involved in the oxidative stress response, mitotic cell cycle control, and sexual development. The transcriptions of *A. fumigatus* phkA and phkB (corresponding to HK4 and HK5 proteins, respectively) increased in response to osmotic shock. However, the growth of the deletion mutants of phkA or phkB genes under high osmolarity stress conditions was comparable to that of the wild-type (WT) *A. fumigatus* strain. In both *A. fumigatus* and *A. nidulans*, the phkA and phkB deletion mutants did not show reduced growth under oxidative stress conditions, compared with that of the parental strains. The physiological roles of the HKs in Aspergillus species remain unknown.

The HK in the HK6 family has a characteristic motif, a repeated HAMP domain, at its N-terminus. Although the function of the HAMP domain is elusive, it is highly conserved in fungi. In *A. nidulans*, the gene encoding the HK in the HK6 family resulted in a retarded growth on high osmolality medium. This result led to the hypothesis that instead of HK1, HK6 might regulate the Hog pathway in the response and adaptation to osmotic conditions. Indeed, both in *A. nidulans* and *A. fumigatus*, disruption of *nikA* encoding a HK6 family protein resulted in a retarded growth on high osmolality medium. Although the function of the HAMP domain is elusive, it is highly conserved in fungi.

Besides its role in the osmotic stress response, the HK in the HK6 family has been implicated in the response to phosphorylase and dicarboximide classes of fungicides. Two representatives of these fungicides, fludioxonil and iprodione, are widely used to protect crops from range of plant pathogenic fungi. Deletion of the gene encoding the HK in the HK6 family resulted in resistance to these fungicides in both *A. nidulans* and *A. fumigatus*, as well as in some plant pathogenic fungi. Upon treatment with these fungicides, the HOG pathway is activated in an SskA-dependent manner in *A. fumigatus* and *A. nidulans*. In the *A. fumigatus* nikA deletion mutant, the fludioxonil (0.1–1 μg/mL)-induced SakA MAPK phosphorylation level was lower than that in the wild type. However, the level of SakA MAPK phosphorylation was similar in the nikA mutant and wild type when treated with a relatively high concentration (10 μg/mL) of fludioxonil. These data suggested that an excessive dose of fludioxonil may affect the HOG pathway through HKs other than Nika. Considering all of the evidence for the roles of Nika in the responses to fungicides and osmotic stress, it appears that Nika plays a major role in regulating the HOG pathway in Aspergillus species, although there remain many open questions.

The HK7 family is composed of phytochrome proteins that play a major role in red light perception. The *A. nidulans* phytochrome FphA has red light-dependent autophosphorylation activity, and functions in the balance between asexual and sexual development and in mycotoxin production in response to red light. FphA forms a complex consisting of the developmental regulator VeA and white collar-like, blue light-signaling...
proteins LreA (WC-1) and LreB (WC-2). Thus, FphA plays a central role in photosensory systems.

In the TCS system circuitry of yeasts, HPt is an intermediate in signaling between HK and RR, where all HKs (Shl1p in S. cerevisiae and Phk1, Phk2, and Phk3 in S. pombe) are thought to function upstream of HPt (Fig. 3). In contrast, Aspergillus species have 11–15 HKs and only a single HPt, which raises the question as to whether all of the HKs interact with HPt to transmit their signals. One possible explanation for the high multiplicity of the upstream HK proteins in Aspergillus species is that HK functions may be specialized in each developmental stage or in response to certain environmental stimuli. This was partly proven by comprehensive transcriptional analyses of an array of HKs expressed at various developmental stages and during stress responses in both A. nidulans and A. fumigatus. For instance, A. nidulans nikA was upregulated during the asexual stage and preferentially expressed in conidial heads and stalks; ypdA was upregulated in response to osmotic shock and fluodioxin treatment and was expressed in conidia; A. fumigatus fos-1, phkA, phkb, and phfk6 were upregulated in response to osmotic shock; A. fumigatus phk1 was upregulated during the asexual stage; and A. fumigatus phk5 was upregulated under oxygen-limited conditions.

The HPt is found in all fungal species, which highlights its importance in the TCS system. Indeed, S. cerevisiae has a single HPt, Ypd1p, whose gene YPD1 is an essential gene; mutants lacking this gene showed hyper-phosphorylation of the Hog1p MAPK, which was lethal to cells. In contrast, the S. pombe HPt, Mpr1/Spy1, is not essential for their growth but is essential in viability. This result indicated that the HPt protein is an essential gene; mutants lacking this gene showed constitutive stress responses and was lethal to cells.

In A. fumigatus, repression of ypdA expression resulted in impaired growth, suggesting that it plays an essential role in viability (unpublished data, Hagiwara et al.). In contrast, the HPt gene in the rice blast pathogenic fungus Magnaporthe oryzae, Moypd1, was shown to be dispensable for viability. These recent data show that the importance of HPt in viability varies among different yeasts and filamentous fungi.

I.iii. TF downstream of MAPK

Studies on yeast MAPK cascades have also revealed that several TFs downstream of the MAPKs play an important role in cellular responses through transcriptional changes. In this decade, the TFs of filamentous fungi have been intensively investigated in several species. Comparisons of studies of TFs between yeasts and filamentous fungi have provided insights into evolutionarily differentiated functions of the MAPK cascade. This section summarizes the accumulated knowledge on TFs, especially those involved in the three MAPK cascades that exist in Aspergillus fungi, gained from comparative genomics analyses.

S. cerevisiae Ste12p is a target of Fus3p MAPK in the mating–pheromone response pathway, and of Kss1p MAPK in the pseudohyphal development pathway. The Ste12-like protein SteA in A. nidulans lacks the Kss1p-interacting domain present in Ste12p, and instead has C-terminal Cy/H2 Zn2+ finger domains that are not present in Ste12p. The A. nidulans deletion mutant of steA showed normal conidiation but did not produce cleistothecia (fruiting bodies), suggesting that SteA is an important regulator of sexual development. In contrast, the A. oryzae steA deletion mutant showed no detectable phenotypes.

However, overexpression of steA resulted in defective hyphal growth and conidiation, and over-secretion of cell wall degrading enzymes. Hence, SteA may function as a regulator of cell wall metabolism in A. oryzae. It should be noted that any physical interaction of SteA with the pheromone-responsive pathway MAPK, MpkB, is yet to be determined in the aspergilla (Fig. 2). Thus, the output mechanism for the MpkB MAPK cascade remains unknown.

The A. cerevisiae HOG pathway has several TFs including Hot1p and Sko1p which interact with Hog1p MAPK. Sko1p is a bZIP-type TF in the ATF/CREB family that represses the expressions of a set of osmotic stress-responsive genes under low-osmolarity conditions. In response to high osmotic stress, Hog1p phosphorylates Sko1p, causing it to switch forms. This results in the derepression and activation of osmotic stress-responsive genes. Hot1p, a positive regulator that interacts with Hog1p, is also involved in modulating the expressions of osmotic stress-responsive genes (Fig. 3). In S. pombe, phosphorylated Spc1 MAPK in the HOG pathway translocates from the cytoplasm to the nucleus to phosphorylate and activate a bZIP TF, Atf1 (Fig. 3). Based on the results of these studies, researchers searched for candidate TFs in the HogA MAPK cascade in Aspergillus genomes. A comparative transcriptome analysis revealed that AtfA, an ortholog of S. cerevisiae Sko1p and S. pombe Atf1, functions in the A. nidulans HOG pathway, where most of osmotic stress-responsive gene expressions were shown to be dependent on AtfA. Furthermore, Lara-Rojas et al. showed that HogA MAPK interacts with the AtfA protein upon oxidative and osmotic stresses, as well as in conidia. However, the atfA mutant did not show distinguishable growth defects under strong osmotic and oxidative stress conditions. This suggests that AtfA has a minor role in adaptation to osmotic and oxidative stresses, and that unidentified TFs may function in thehog pathway (Fig. 3).

Importantly, AtfA was shown to play as crucial a role as that of HogA in conidia viability, suggesting that HogA–AtfA is involved in conidia stress tolerance (Fig. 3). Involvement of AtfA in the Hog pathway and in conidia stress tolerance was also found in A. fumigatus.
In the cell wall integrity pathway, S. cerevisiae Mpk1p phosphorylates and activates Rlm1p TF, which regulates at least 25 genes involved in cell wall biogenesis. 24 The filamentous fungi orthologs of these TFs were investigated in A. nidulans and A. niger. Disruption of \textit{rlmA} in \textit{A. nidulans} resulted in sensitivity to the cell wall perturbing reagent CFW, suggesting that RlmA plays a role in the cell wall stress response.26 Regulation of the expression of \(\alpha\)-1,3-glucan synthase genes, \textit{agsA} and \textit{agsB}, was shown to be dependent on both \textit{RlmA} and MpkA MAPK. Similarly, the \textit{A. niger rlmA} deletion mutant was sensitive to CFW, and RlmA was required for upregulation of the expressions of \textit{agsA} and \textit{gfaA} (encoding glutamine:fructose-6-phosphate amidotransferase). 23 Taken together, these results showed that RlmA functions downstream of the MpkA MAPK cascade and is responsible for adaptation to cell wall stress conditions in the \textit{aspergilli}.

II. Stress biology of hyphae

The most obvious morphological features of filamentous fungi are their hyphal form and multicellularity. The hyphae are highly polarized and branched to broaden their habitat and to obtain sufficient nutrients for survival. In general, the multicellular hyphal form is the predominant form of \textit{Aspergillus} fungi when grown in natural environments including soil and compost. Hence, \textit{Aspergillus} fungi would have evolved sophisticated stress adaptation mechanisms to allow hyphae to survive under various environmental stress conditions encountered in their surroundings. This section summarizes recent discoveries on the response and adaptation mechanisms of the hyphal form of \textit{Aspergillus} fungi against abiotic stresses.

II.i. Osmotic stress response

Some fungi synthesize glycerol as the main osmolyte to adapt to high-osmolality conditions. 93–95 In \textit{S. cerevisiae}, glycerol production begins with the conversion of dihydroxyacetone phosphate (DHAP) through a two-step reaction catalyzed by glycerol-3-phosphate dehydrogenase (Gpd1p, Gpd2p) and glycerol-3-phosphatase (Gpp1p, Gpp2p). 59 As described above, hyperosmotic stress activates the \textit{S. cerevisiae} HOG pathway. This in turn increases the transcriptions of \textit{GPD1} and \textit{GPP2} and facilitates glycerol production. In \textit{A. nidulans}, the homologs for glycerol biosynthesis-related genes are \textit{GfdA}, \textit{GfdB}, and \textit{GppA}. 96 The \textit{gfdA} mutant showed decreased glycerol accumulation during growth under osmotic stress conditions, but it did not show greater sensitivity to high osmolality stress. 97 The expression of another \textit{A. nidulans} GPD homolog, \textit{GfdB}, was shown to increase in response to osmotic shock in a HogA-MAPK-cascade-dependent manner, but its functional role remains undetermined. 98 In \textit{A. fumigatus}, glycerol was accumulated under a NaCl-derived osmotic stress treatment in a \textit{SakA} MAPK- and PbsB MAPKK-dependent manner. 37 The mutants lacking \textit{sakA} or \textit{pbsB} showed stronger growth inhibition under high-NaCl conditions, suggesting that accumulated glycerol in \textit{A. fumigatus} hyphae plays a role in stress adaptation. For other \textit{Aspergillus} species, polyols such as glycerol, erythritol, arabitol, or mannitol were found in hyphae as compatible solutes under osmotic stress conditions. 99–103 In such conditions, whereas \textit{A. niger} mainly produces glycerol, \textit{Aspergillus repens} and \textit{Aspergillus flavus} produce a mixture of polyols. The main osmolyte accumulated in hyphae seems to vary among different \textit{Aspergillus} species.

II.ii. Oxidative stress response

Aerobic organisms must maintain a reduced cellular environment. Reactive oxygen species are spontaneously generated from oxygen metabolism in the cells. Hence, living organisms constantly sense and adapt to changes in the redox balance. One of the central fungal regulators of antioxidant responses is AP-1 TF. As mentioned above, in \textit{S. cerevisiae} and \textit{S. pombe}, the Skn7-type RRs (Skn7p and Prr1, respectively) interact with AP-1 TFs (Yap1p and Pap1, respectively). This mediates the expressions of genes encoding several antioxidants and antioxidant enzymes, including cystolic catalase (Ctt1p and Ctt1), superoxide dismutase (Sod1p and Sod1), thioredoxin (Trx2p and Trx1), thioredoxin reductase (Trr1p and Trr1), and thioredoxin peroxidase (Tsa1p and Tpx1). 104–106 The AP-1 family TF and Skn7-type RR have been well characterized in \textit{A. nidulans} and \textit{A. fumigatus}. All of the proteins are required for growth under oxidative stress conditions. 46, 52,107,108 Notably, their mutants showed oxidative stress sensitivity at the hyphal stage but not in conidia, suggesting that another mechanism against oxidative stress exists in conidia.

II.iii. Response to hypoxic conditions

Molecular oxygen plays an essential role in respiratory ATP synthesis and is also critical for the biosynthesis of sterols and hemes. Studies on \textit{S. cerevisiae} showed that cells sense oxygen availability at least partly through cellular heme and sterol levels in the hypoxic response. 109,110 Filamentous fungi require oxygen to proliferate, but the mechanisms of hypoxia adaptation remain largely unstudied. Some genome-wide analyses of \textit{Aspergillus} species have revealed that glycolysis is upregulated under hypoxic conditions. 111,112 This upregulation of glycolysis may contribute to the generation of ATP via substrate-level phosphorylation.

Although the details of the signaling mechanism remain unclear, recent studies on \textit{A. fumigatus} have provided evidence that a sterol response element binding protein (SREBP), SrbA, is a key regulator for adaptation to hypoxic conditions. 113,114 SrbA is a TF containing a bHLH motif and plays a role in ergosterol biosynthesis by regulating various metabolic genes. A strain lacking SrbA showed impaired growth under hypoxic conditions. As is the case in \textit{S. cerevisiae}, ergosterol biosynthesis is likely associated with the oxygen level in \textit{A. fumigatus}. SrbA proteins are widely conserved among filamentous fungi. In fact, SrbA is required for growth under hypoxic conditions in \textit{A. oryzae} (unpublished data, Gomi et al).
Recent studies have demonstrated that adaptation to hypoxic conditions is intimately associated with the pathogenicity of two human pathogenic fungi, *A. fumigatus* and *Cryptococcus neoformans*.115) The SREBP proteins (SrbA of *A. fumigatus* and Sre1 of *C. neoformans*) are required for virulence and growth under hypoxic conditions.113,116) In the mice infection model for invasive mycoses, fungal cells encounter hypoxic microenvironments at the sites where they penetrate the epithelial cell layers of the lung and invade the lung tissues. Thus, adaptation to oxygen-limited conditions is a requirement for the survival of fungal pathogens in vivo. Several groups have conducted genome-wide transcriptional analyses of *A. fumigatus* during the hypoxia response.117,118) Together, the results of those studies showed that a wide array of cellular mechanisms (i.e. cell wall metabolism, ergosterol biosynthesis, amino acid transport, nitrogen metabolism, and glycolysis) are affected by the transition from normoxic to hypoxic conditions. Notably, the expression of *fhk5* encoding an HK8 family HK strongly increased under hypoxic conditions.119) The *fhk5* null mutant showed significant altered morphology only when ethanol was the sole carbon source in hypoxic culture, suggesting that Fhk5 plays a specific role in ethanol utilization under hypoxic conditions.

II.iv. Response to elevated temperature

Temperature is one of the most important factors that fungi must cope with in the natural environment. For instance, the temperature increases to more than 50 °C in compost piles due to fermentation heat. Although *Aspergillus* species are generally considered to be mesophiles, *A. fumigatus* is characterized by its moderate thermostolerance and is able to grow at 50 °C.118) In the mice infection model for virulence, growth in hypoxic culture, and adaptation to hypoxia, *A. fumigatus* showed that six hydrophobins (RodA, and *A. fumigatus* has led to enormous economic losses around the world. Therefore, the stress biology of conidia is a topic that should attract great attention in the next decade. In this section, we focus on the molecular mechanisms of conidial stress tolerance (summarized in Fig. 4).

III. Stress biology of conidia

Formation of conidia is thought to be one strategy for sessile fungi to adapt to the limited resources at a given place. Conidia are, in general, stress-tolerant propagules, and *Aspergillus* fungi vigorously produce air-borne conidia under appropriate conditions. The conidia of *A. fumigatus* are etiological agents for aspergillosis, in which inhaled conidia colonize and germinate in the lungs of immunocompromised patients. Contamination of harvested crops and food products by conidia of some mycotoxigenic species such as *A. flavus* and *Aspergillus parasiticus* has led to enormous economic losses around the world. Therefore, the stress biology of conidia is a topic that should attract great attention in the next decade. In this section, we focus on the molecular mechanisms of conidial stress tolerance (summarized in Fig. 4).

III.i. Conidium structure for stress resistance

The conidia of *Aspergillus* species have thick cell walls composed of α-glucan, β-glucan, and chitin. The conidia cell walls are further covered with hydrophobin on the outermost layer. The rodlet layer of hydrophobins confers hydrophobicity to the conidia, and masks the cell wall components to prevent immune cells from immediately detecting the inhaled conidia inside lungs.120,121) A recent study on the hydrophobins of *A. nidulans* showed that six hydrophobins (RodA, and *DewA–E*) are involved in the hydrophobicity of the conidia and that RodA plays a major role in rodlet layer formation. Both *dewD* and *dewE* are expressed in

strain was obtained by overexpressing the trehalose-6-phosphate synthase gene.126) To further explore the heat shock response, transcriptional and proteomic analyses were conducted in *A. fumigatus*.127,128) Albrecht et al. demonstrated that 54 proteins were upregulated following a shift from 30 °C to 48 °C, 12 of which were assigned as chaperones (HSP60, HSP70, and HSP90, etc.).128) Additionally, enzymes involved in the oxidative and nitrosative stress response (AFUA_4G09110: cytochrome c peroxidase Ccp1; AFUA_4G03410: flavohemoprotein; AFUA_5G90910: nitroreductase family protein) were upregulated in the temperature shift. It is notable that many proteins involved in carbohydrate metabolism (glycolysis and pentose phosphate pathways) were found to be upregulated by heat shock. This suggested that de novo energy production and balancing the redox state are necessary in the heat shock response of *A. fumigatus*. A recent transcriptome study was conducted on *Aspergillus kawachii*, in which the culture temperature was decreased from 40 to 30 °C to mimic the industrial culture conditions for fermentation of the Japanese distilled spirit, *shochu*.129) Upon lowering of the temperature, the expression levels of genes involved in the glycerol, trehalose, and pentose phosphate pathways, as well as HSP genes, were downregulated. This trend is opposite to that of *A. fumigatus* under heat shock treatment described above.
vegetative hyphae, but the other four hydrophobin genes are not. Instead, they are strongly induced during the asexual stage.

Melanin, which is incorporated into the cell walls, gives the conidia of filamentous fungi better rigidity and resistance to certain environmental stresses.\(^{125}\) For example, in the conidia of *A. niger*, dihydroxyphenylalanine (DHN)-melanin confers strength against heat and UV stresses.\(^{133,134}\) The conidia of *A. fumigatus* also contain DHN-melanin, which is thought to provide protection from environmental stresses such as UV radiation and heat.\(^{135}\) Furthermore, DHN-melanin protects *A. fumigatus* conidia against phagocytic killing by inhibiting the acidification mechanism of phagolysosomes.\(^{126,137}\) More intriguing observations were reported recently by Brakhage’s group, who demonstrated that the pigment-less, white conidia of the *pbsP* mutant were ingested more frequently than WT conidia by the soil-living amoeba *Dictyostelium discoideum*.\(^{138}\) Hence, DHN-melanin not only plays an essential role in infections of mammalian hosts, but also contributes to avoidance of amoeba predation.

These data on conidial structure support the idea that fungi might have evolutionarily strengthened the shield on the surface of their conidia against enemies encountered in their environment. Despite their significance, however, the molecular mechanisms regulating hydrophobin production and melanin formation remain elusive.

### III.ii Stress resistance-conferring molecules inside conidia

A conidia-specific catalase (CatA) was shown to play crucial role in tolerance against oxidative and thermal stress in *A. nidulans* and *A. fumigatus* conidia.\(^{139,140}\) *A. oryzae* also has a CatA ortholog that is specifically expressed in conidia.\(^{141}\) The expression of *catA* genes is dependent on AtfA TF and its upstream pathway, the TCS–HOG pathway, in *Aspergillus* species.\(^{31,37,46,91,142}\) Besides AtfA, there are some paralogs in each fungus, and only *A. oryzae* AtfB has been characterized. Disruption of *atfB* resulted in conidia sensitivity to oxidative stress and decreased *catA* expression in conidia.\(^{143}\) However, these phenotypes were moderate compared with those observed in the *atfA* deletion mutant.\(^{142}\) These results suggested that AtfA plays a major role and AtfB plays an additional role in conidia stress tolerance in *A. oryzae*. The genomes of all *Aspergillus* species sequenced to date contain multiple *atf* genes (AspGD, http://www.aspergillusgenome.org/). For instance, *A. oryzae* has three *atf* genes (*atfA*: AO0900030000685; *atfB*: AO090120000418; and *atfC*: AO090010000747), *A. nidulans* has three *atf* genes (*atfA*: AN2911; *atfB*: AN6849; and *atfC*: AN8643) and *A. fumigatus* has four *atf* genes (*atfA*: AfuAgi311330; *atfB*: AfuAgi52960; *atfC*: AfgI1G17360; and *atfD*: AfgI6g12150), whose encoded proteins share a bZIP-type DNA-binding domain that is highly conserved among *atf* genes. This implies that multiplication and functional differentiation of *atf* genes might be evolutionarily important for these fungi to survive in their surrounding environments.

Another important determinant for stress tolerance of conidia is the accumulation of compatible solutes such as trehalose and mannitol. Trehalose and mannitol function as stress protectants in conidia of some *Aspergillus* species.\(^{124,125,144}\) The *atf* genes (*atfA*: AN8643) and *atfB* genes (*atfA*: AN8643) of *A. oryzae* have four *atf* genes (*atfA*: AfgI1G131330; *atfB*: AfgI52960; *atfC*: AfgI1G17360; and *atfD*: AfgI6g12150), whose encoded proteins share a bZIP-type DNA-binding domain that is highly conserved among *atf* genes. This implies that multiplication and functional differentiation of *atf* genes might be evolutionarily important for these fungi to survive in their surrounding environments.

III.iii Conidial stress resistance mechanisms

The conidial stress resistance mechanisms of the fungi are depicted. (A) Representative stresses that are encountered by fungal hyphae in the natural habitat are shown. For each stress, main adaptation mechanisms of the fungi are depicted. (B) Representative mechanisms conferring stress resistance on conidia are shown. As described in the text, the TCS–HOG MAPK cascade–AtfA signaling pathway regulates the production of conidia-specific catalase and compatible solutes.

![Fig. 4. Summary of stress biology of hyphae and conidia in *Aspergillus* fungi.](image)

**Notes:** (A) Representative stresses that are encountered by fungal hyphae in the natural habitat are shown. For each stress, main adaptation mechanisms of the fungi are depicted. (B) Representative mechanisms conferring stress resistance on conidia are shown. As described in the text, the TCS–HOG MAPK cascade–AtfA signaling pathway regulates the production of conidia-specific catalase and compatible solutes.

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**Fig. 4.** Summary of stress biology of hyphae and conidia in *Aspergillus* fungi.

**Notes:** (A) Representative stresses that are encountered by fungal hyphae in the natural habitat are shown. For each stress, main adaptation mechanisms of the fungi are depicted. (B) Representative mechanisms conferring stress resistance on conidia are shown. As described in the text, the TCS–HOG MAPK cascade–AtfA signaling pathway regulates the production of conidia-specific catalase and compatible solutes.

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**IV. Concluding remarks**

In this decade, in the post-genomic era, many studies have focused on interpreting the genomic information obtained for filamentous fungi. Consequently, a large body of data on genes and proteins is now available.
Progress in molecular biology has allowed us to further understand how organisms live in, and adapt to, their natural habitats. These improvements in our fundamental understanding will further improve industrial processes including biomaterial production and food fermentation. In the next decade, next-generation sequencing technology promises enormous amounts of data for genomes, genes, and proteins, allowing for comparisons not only among species, but also among strains or among genera. This will undoubtedly result in further progress in several technologies that affect aspects of human life; crop protection, food preservation, food fermentation, chemical production, and prevention of infectious diseases.

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