Chapter 13
Response and Adaptation to Cell Wall Stress and Osmotic Stress in *Aspergillus* Species

Daisuke Hagiwara, Akira Yoshimi, Kazutoshi Sakamoto, Katsuya Gomi, and Keietsu Abe

Abstract *Aspergillus* species are one of the most important filamentous fungi from the point of view of industry, pathogenesis, and mycotoxin production. Some aspergilli are used to produce traditional Japanese fermented foods such as sake, shoyu (soy sauce), and miso. In the fermentation steps, fungi are cultivated on solid substrates (steamed or baked cereal grains), under which conditions fungi produce and secrete a large amount of hydrolytic enzymes. During this solid-state fermentation, fungi must cope with various abiotic stresses including temperature, pH, osmotic stress, and low oxygen. To understand fungal biology and to make more use of fungi for fermentation and enzyme production, intensive research on stress adaptation mechanisms have been performed in *Aspergillus* species. This review focuses on the responses to cell wall stresses and osmotic stresses to which aspergilli should adapt during solid-state cultivation. In a cell wall integrity signaling pathway, the MpkA mitogen-activated protein kinase (MAPK) cascade plays a central role in the regulation of α-1,3-glucan synthase genes and consequently affects fungal cell wall composition. An osmotic stress signaling pathway is composed of the combination of the upstream two-component phosphorelay signaling and the downstream HogA/SakA MAPK cascade, and the signaling pathway is responsible for adaptation to environmental osmotic changes. Here, we provide recent findings on the two stress

D. Hagiwara
Medical Mycology Research Center, Chiba University, Chiba, Japan

A. Yoshimi
NICHe, Tohoku University, Sendai, Miyagi, Japan

K. Sakamoto
National Research Institute of Brewing, Higashi-Hiroshima, Hiroshima, Japan

K. Gomi
Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan

K. Abe (✉)
NICHe, Tohoku University, Sendai, Miyagi, Japan

Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan
e-mail: kabe@niche.tohoku.ac.jp

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response signaling pathways in aspergilli. The accumulated knowledge will be useful for understanding and improving the fermentation processes of industrial aspergilli in solid-state cultivation.

Keywords *Aspergillus* species • Cell wall integrity signaling • HogA/SakA MAPK cascade • MpkA MAPK cascade • Osmotic stress signaling • Solid-state culture • Stress response • α-1,3-Glucan

13.1 Introduction

Filamentous fungi are ubiquitous, commonly found on decaying woods and plants as well as in the soil. They produce a large amount of different kinds of hydrolytic enzymes, which degrade biomass in nature and thus contribute to carbon and nitrogen recycling. The saprophytic property is important for ecosystems, but some plant pathogenic fungi spoil farm crops, which is problematic all over the world. Small but significant numbers of filamentous fungi cause serious infection in humans, to which much attention has been paid as the number of immunocompromised people is increasing because of aging populations or immunocompromising diseases including human immunodeficiency virus (HIV) infection. As a beneficial aspect, some fungi produce a variety of enzymes and organic acids that are applicable to the food and pharmaceutical industry, and they are used for manufacturing fermented foods. Aspergilli includes a wide variety of species such as the industrial fungi *Aspergillus oryzae* and *Aspergillus niger*, the human pathogen *Aspergillus fumigatus*, the aflatoxin producers *Aspergillus flavus* and *Aspergillus parasiticus*, and the model fungus *Aspergillus nidulans*. Because of their industrial, medical, and agricultural importance, the genome sequences of aspergilli have been determined (Machida et al. 2005; Nierman et al. 2005; Galagan et al. 2005; Pel et al. 2007), and the understanding of the biology of filamentous fungi has been extensively advanced in the past decade.

Some *Aspergillus* species, such as *A. oryzae*, *A. sojae*, and *Aspergillus luchuensis*, have been used for hundreds of years in traditional Japanese fermentation industries to produce *sake* (rice wine), *shoyu* (soy sauce), *miso* (soybean paste), and *shōchū* (distilled beverage) (Machida et al. 2008; Hong et al. 2013). *Aspergillus oryzae* has been used for the fermentations because of its notable potential for the secretory production of various enzymes such as amylases and proteases in solid-state cultivation. The ability to secrete such hydrolytic enzymes in solid-state culture is further enhanced compared to submerged (liquid) culture (Oda et al. 2006). Because of this advantage, the solid-state cultivation of *A. oryzae* has been applied to the production not only of fermented foods but also of various enzymes and chemicals (Yu et al. 2003). During solid-state cultivation, fungi have to adapt to various abiotic stresses including temperature, pH, and oxidative and osmotic

conditions on the surface of the solid substrates. Low oxygen is an additional condition to which fungi should adapt inside the solid substrates. At present, our understanding of the stress response biology under solid-state culture conditions is limited. Therefore, it is necessary to intensively accumulate knowledge about this state, which would advance technology in the fermented food, enzyme production, and chemical industries.

Because fungi confront a variety of stresses in nature, well-established mechanisms for sensing and responding to environmental stimuli must be inherent. Of these stress adaptation mechanisms, responses to cell wall stress and osmotic stress, which are also important in solid-state cultivation, have been well studied in several *Aspergillus* species. This review focuses on response mechanisms to the stresses and describes the up-to-date findings and current understanding of the response mechanisms.

13.2 Cell Wall Integrity Signaling System of *Aspergillus* Species

13.2.1 Overview of Cell Wall Integrity (CWI) Signaling

The fungal cell wall is an essential structure that defines the shape of the cell and shields it from environmental stress. In solid-state fermentation, fungi are exposed to a variety of environmental stimuli, including changes in osmolality, temperature, and pH. These stresses primarily act on the fungal cell wall, which generates a need for remodeling or repair. Therefore, understanding the mechanism of cell wall biogenesis is important for fermentation industries.

Proper cell wall architecture of filamentous fungi requires several cell wall components that are mainly composed of polysaccharides: α-glucans (α-1,3-glucan and α-1,4-glucan), β-glucans (β-1,6-branched β-1,3-glucan), galactosaminogalactan, galactomannan, and chitin (Fig. 13.1) (Fontaine et al. 2000; Kapteyn et al. 1999; Klis 1994; Latgé 2007, 2010). Cell wall biogenesis and a signal transduction system that monitors the cell wall and promotes cell wall remodeling have been studied in detail in *Saccharomyces cerevisiae* (Heinisch et al. 1999; Levin 2005). Sensing of cell wall perturbations requires surface sensors, which are encoded by the cell wall integrity and stress response component (WSC) genes, upstream of the intracellular signal transduction pathway responsible for maintaining cell wall integrity in yeast. The Wsc1p, Wsc2p, and Wsc3p proteins are highly glycosylated plasma membrane proteins that contain an extracellular domain with a cysteine-rich domain, a Ser/Thr-rich region that carries glycosylation sites, and a highly charged C-terminal cytoplasmic region (Levin 2011; Lodder et al. 1999; Rodicio and Heinisch 2010; Verna et al. 1997). Additional cell wall stress sensors are partially redundant to Mid2p and Mlt1p cell-surface proteins. These proteins act as

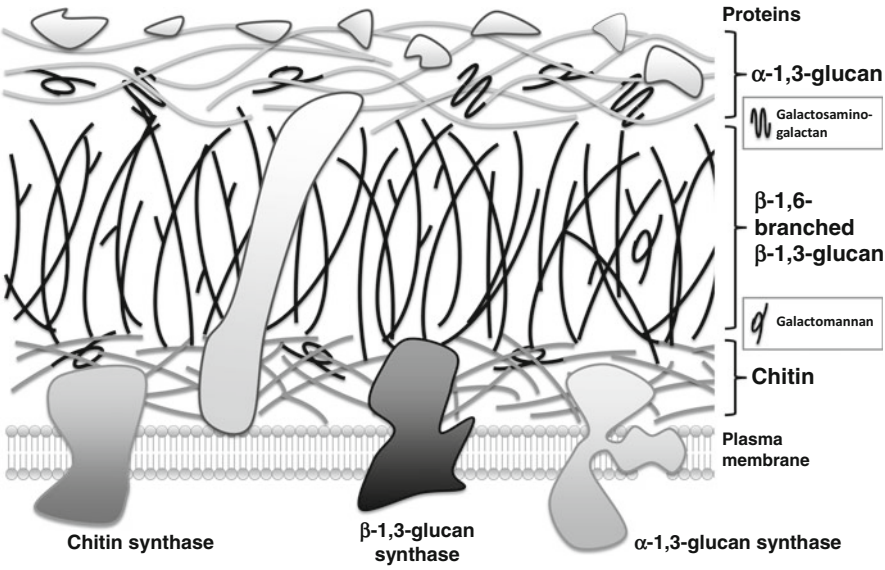

Fig. 13.1 Cell wall organization of *Aspergillus nidulans*. The central core of the cell wall is mainly composed of β-1,6-branched β-1,3-glucan crosslinked to chitin; the amorphous α-1,3-glucan is present in the outer layer of the cell wall. Polysaccharides, such as galactosaminogalactan and galactomannan, and proteins, such as GPI-anchored protein and surface proteins, are also present in the cell wall

mechanosensors and detect cell wall perturbations caused by cell wall stress during growth or pheromone-induced morphogenesis, exposure to high temperature, or changes in osmolality, and they transmit signals to the downstream signaling pathway (Ketela et al. 1999; Rajavel et al. 1999; Verna et al. 1997). The activation of the cell wall integrity (CWI) signaling pathway proceeds through the small G protein Rho1p (Levin et al. 1990; Levin 2011). Rho1p is a small GTPase that is activated by the guanosine nucleotide exchange factors (GEFs) Rom1p, Rom2p, and Tus1p (Levin 2011; Philip and Levin 2001) and is downregulated by the GTPase-activating proteins (GAPs), including Bem2p and Sac7p (Levin 2011; Schmidt et al. 1997). In the active form, Rho1p binds to and activates protein kinase C (Pkc1p) (Kamada et al. 1996), which in turn activates the MAPK (mitogen-activated protein kinase) cascade. The MAPK cascade is a linear pathway that is composed of the MAPK kinase kinase Bck1p (Lee and Levin 1992), a pair of redundant MAPK kinases, Mkk1p and Mkk2p (Irie et al. 1993), and the MAPK Mpk1p/Slt2p (Martin et al. 1993). The Mpk1p phosphorylates and activates the transcription factor Rlm1p, which regulates the transcription of at least 25 genes involved in cell wall biogenesis, including β-1,3-glucan synthase genes and chitin synthase genes (Jung and Levin 1999). In the following sections, recent findings related to CWI signaling in aspergilli are described.

13.2.2 *Cell-Surface Sensors for CWI Signaling in* Aspergillus *Species*

In *Aspergillus* species, the genes for CWI sensor proteins have been characterized (Dichtl et al. 2012; Goto et al. 2009; Futagami et al. 2011; Futagami and Goto 2012). Goto et al. (2009) identified the *A. nidulans* gene for the cell wall stress sensor WscA from the genome, which is a homologue of Wsc1p in *S. cerevisiae* (Goto et al. 2009). WscA contains a Wsc motif that is rich in cysteine residues, a Ser/Thr-rich region that carries three putative *N*-glycosylation sites, a transmembrane region, and a cytoplasmic region at the C-terminus (Goto et al. 2009). They also suggested that *A. nidulans* WscA is *O*-mannosylated at the conserved Ser/Thr-rich region by PmtA and PmtC, but not by PmtB, which are protein *O*-mannosyltransferases in *A. nidulans*, and that the *O*-glycan attachment has a significant impact on the stability of WscA (Goto et al. 2009; Kriangkripipat and Momany 2009). Protein *O*-glycosylation has a critical role, as demonstrated by the observation that the *pmtA* mutant exhibits an abnormal cell morphology and altered cell wall composition (Oka et al. 2004; Goto 2007). Futagami et al. (2011) determined that WscA and WscB were both *N*- and *O*-glycosylated and localized on the cell surface. They also reported that the *wsc* disruptants (*wscA*Δ and *wscB*Δ) showed reduced colony size, the formation of fewer conidia, and a high frequency of swollen hyphae in hypo-osmotic conditions, whereas the defective phenotype was restored by osmotic stabilization with potassium chloride (Futagami et al. 2011). Moreover, the transcription levels of the α-1,3-glucan synthase genes, *agsA* and *agsB*, are markedly altered in the *wsc* disruptants, resulting in an increase in the amount of alkali-soluble cell wall glucan, including soluble α-1,3-glucan (Futagami et al. 2011). On the other hand, exposure to micafungin, a β-1,3-glucan synthase inhibitor, leads to the activation of the *agsB* transcription in both the wild-type and the *wscA*Δ *wscB*Δ strains. This alteration in expression was dependent on MpkA, which is an orthologue of yeast Mpk1p in *A. nidulans*, indicating that WscA and WscB are not essential for MpkA signaling (Fig. 13.2) (Futagami et al. 2011). In addition, it has been reported that the *A. nidulans* genome possesses a Mid2-like protein, which has structural features that are similar to the CWI sensor protein Mid2p of *S. cerevisiae* (Fig. 13.2) (Futagami et al. 2011; Futagami and Goto 2012). However, the involvement of the protein in *A. nidulans* CWI signaling is yet to be investigated.

In *Aspergillus fumigatus* CWI signaling, the potential CWI sensor proteins Wsc1, Wsc2, Wsc3, and MidA, which are homologues of yeast Wsc1p, Wsc2p, Wsc3p, and Mid2p, respectively, and the Rho GTPases Rho1, Rho2, and Rho4 were functionally characterized (Dichtl et al. 2012). On the basis of experimental data obtained from phenotypic analysis in the mutants of the genes encoding CWI sensor proteins and the Rho GTPases, Dichtl et al. (2012) proposed a model for a CWI signaling pathway in *A. fumigatus*. Cell wall stress induced by Congo red (CR), Calcofluor white (CFW), and heat shock is sensed by MidA. MidA and an unknown sensor activate the MpkA pathway, probably via Rho1 and PkcA. Wsc1

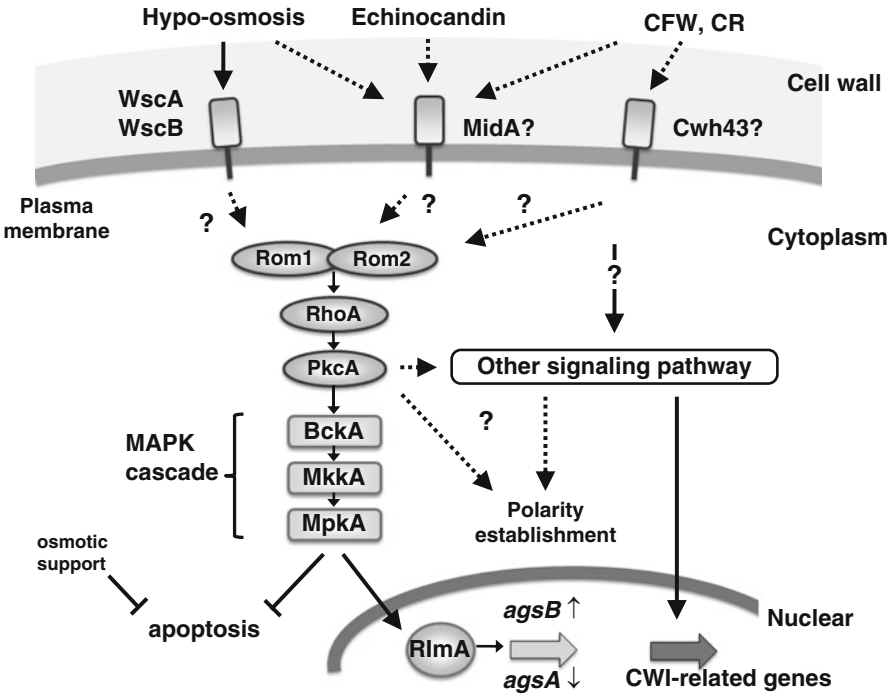

Fig. 13.2 Schematic model of cell wall stress signaling in *A. nidulans*. Based on the study results, we hypothesize that *A. nidulans* has the following cell wall integrity (CWI) signaling system. (1) Putative sensor proteins in the CWI signaling pathway, WscA and WscB, are important in CWI signaling under hypo-osmotic conditions, but WscA and WscB are not essential for MpkA-RlmA signaling. (2) PkcA is involved in the CWI pathway in *A. nidulans*. In addition, PkcA is a factor in the suppression of apoptosis induction via the MpkA pathway, but not in polarity establishment, during hyphal growth independent of the MpkA pathway under heat stress conditions. (3) *AgsA* and *agsB* expression is dependent on MpkA and partly dependent on RlmA. (4) Other CWI-related genes, such as *fksA*, *gelA*, *gelB*, *chsA*, *chsB*, *chsC*, *chsD*, *csmA*, and *csmB*, are independent of the MpkA-RlmA system. The CWI pathway mainly regulates the transcription of α-1,3-glucan biogenesis-related genes. The transcripts of β-1,3-glucan and chitin biogenesis-related genes are mainly regulated by other unknown signals that might be activated by a cell wall stress such as echinocandin (micafungin) treatment

is specifically required to be tolerant of β-1,3-glucan synthase inhibitors, such as caspofungin, which is an echinocandin antifungal drug. Wsc1, Wsc3, and MidA are redundantly required to promote radial growth and conidiation, possibly via the MpkA pathway. Rho2 and Rho4 seem to not directly contribute to MpkA phosphorylation, but both are important for CWI signaling. Moreover, Rho4 is essential for septum formation and contributes to tolerance of β-1,3-glucan synthase inhibitors (Dichtl et al. 2012).

13.2.3 *Protein Kinase C Pathway in* Aspergillus nidulans

In addition to CWI sensor proteins and Rho GTPases, the PKCs have been isolated from several filamentous fungal species. For example, *Neurospora* PKC is suggested to be essential for viability and is involved in a light-signaling pathway (Arpaia et al. 1999; Franchi et al. 2005). With regard to *Aspergillus* species, an *A. nidulans* PKC-encoding gene, *pkcA* (a counterpart of yeast *pkc1*), is suggested to be essential for its viability even under osmotic stabilization, whereas the lethality caused by deletion of yeast *pkc1* is suppressed by osmotic stabilization (Herrmann et al. 2006; Ichinomiya et al. 2007; Ronen et al. 2007; Teepe et al. 2007). Repression of *pkcA* expression led to hypersensitivity to cell wall-defective agents, such as caspofungin and CFW, and defects in the cell wall structure, suggesting that PkcA is involved in the CWI pathway in *A. nidulans* (Fig. 13.2) (Ronen et al. 2007; Teepe et al. 2007). It was shown that PkcA localized to the hyphal apices, forming septa, and tips of phialides (Teepe et al. 2007). In addition, PkcA is suggested to be associated with numerous functions, including conidiation, germination, secondary metabolism, and farnesol-induced cell death (Colabardini et al. 2010; Herrmann et al. 2006; Ichinomiya et al. 2007; Ronen et al. 2007; Teepe et al. 2007). Katayama et al. (2012) constructed and characterized temperature-sensitive mutants of *pkcA* of *A. nidulans*. These mutants exhibited apoptotic phenotypes at 42 °C, a restrictive temperature, although the mutants showed almost normal growth and conidiation at 30 °C. They also suggested that PkcA functions in the suppression of apoptosis induction via the MpkA pathway. However, polarity establishment during hyphal growth under heat stress conditions, which involves PkcA, is independent of the MpkA pathway (Fig. 13.2). Direct deletion mutants of *pkcA* have not yet been constructed in any filamentous fungi, and the evidence collected thus far suggests that *pkcA* is essential in *A. nidulans*.

13.2.4 *MAP Kinase Pathway for CWI Signaling in* Aspergillus *Species*

The genes encoding a counterpart of yeast Mpk1p (Slt2p) have also been characterized in aspergilli (Bussink and Osmani 1999; Fujioka et al. 2007; Jain et al. 2011). Deletion analysis of *A. nidulans mpkA* has suggested that the kinase is involved in conidial germination and in polarized growth (Bussink and Osmani 1999). In *A. fumigatus*, MpkA is involved in the response against reactive oxygen species, siderophore production during iron starvation, and the production of secondary metabolites (Jain et al. 2011). Besides these physiological functions, the involvement of MpkA in CWI signaling has been demonstrated in *Aspergillus* species.

Fujioka et al. (2007) constructed disruptant strains of *A. nidulans, mpkA*, *rlmA*, and *Answi4*/*Answi6* (orthologues of *SWI4*/*SWI6*, which encodes the Mpk1p-activating transcription factor Swi4p–Swi6p complex in *S. cerevisiae*): *mpkAΔ*,

rlmAΔ, *Answi4Δ*, and *Answi6Δ* strains (Fujioka et al. 2007). The transcriptional regulation of cell wall-related genes and *mpkA* via CWI signaling was investigated in the disruptants under cell wall stress induced by micafungin, a β-1,3-glucan synthase inhibitor. The transcription of most cell wall-related genes except two α-1, 3-glucan synthase genes (*agsA* and *agsB*) is transiently upregulated by micafungin treatment, but this action is independent of MpkA, RlmA, and AnSwi4-AnSwi6, suggesting that transcription of the β-1,3-glucan synthase gene *fksA* and several chitin synthase genes (*chsA–chsD*, *csmA*, and *csmB*) is regulated by non-MpkA signaling (Fig. 13.2). Transcription of *agsB*, which encodes a major α-1,3-glucan synthase, depends mainly on MpkA–RlmA signaling (Fig. 13.2) (Fujioka et al. 2007). The *agsA* gene is scarcely transcribed in *A. nidulans* wild-type strains, but its transcription is weakly upregulated in the *mpkAΔ* and *rlmAΔ* strains (Fig. 13.2). Fujioka et al. (2007) further reported that the GUS reporter gene controlled by the *mpkA* promoter was expressed in the wild-type and *rlmAΔ* strains but not in the *mpkAΔ* strain, suggesting that *mpkA* transcription is autoregulated by CWI signaling via MpkA but is independent of RlmA and AnSwi4-AnSwi6. In contrast to the prominent roles of Rlm1p and Swi4p-Swi6p in the maintenance of CWI in *S. cerevisiae*, neither RlmA nor AnSwi4-AnSwi6 in *A. nidulans* is a major transcription factor that controls the expression of *mpkA* or most cell wall-related genes (except the α-1,3-glucan synthase genes *agsA* and *agsB*) as the target of MpkA, and expression of *mpkA* is autoregulated by CWI signaling via an unknown transcription factor that is the target of MpkA. The transcriptional regulation of most genes involved in the biosynthesis of β-1,3-glucan and chitin seems to be regulated by an unknown signaling pathway that is activated by cell wall stresses (e.g., treatment with micafungin) rather than CWI signaling via MpkA in *A. nidulans* (Fujioka et al. 2007).

In *Aspergillus niger*, the genes that encode glutamine:fructose-6-phosphate amidotransferase (*gfaA*) and α-1,3-glucan synthase (*agsA*) are induced in response to stress at the cell wall (Damveld et al. 2005a; Ram et al. 2004). In silico analysis of the promoter region of the two genes revealed the presence of putative DNA-binding sites targeted by the transcription factors RlmA and MsnA that are orthologues of the stress-responsive transcription factors Rlm1p and Msn2p/Msn4p in *S. cerevisiae*. Promoter analysis using a GUS reporter indicated that induction of *agsA* in response to stress at the cell wall depends fully on a single putative RlmA-binding site in its promoter region (Damveld et al. 2005a). Deletion of the *rlmA* gene in *A. niger* eliminates the induction of *agsA* and results in reduced induction of *gfaA* during cell wall stress induced by CFW. The increase in cell wall chitin content in the presence of CFW is also affected in the *rlmA* deletion strain. In addition, the deletion strain is more sensitive to agents that induce cell wall stress. The results indicate that *A. niger* responds to cell wall stress by transcriptional activation of cell wall-reinforcing genes, including *agsA* and *gfaA*, by RlmA.

In *A. oryzae*, understanding of the CWI signaling pathway has been advanced by a functional study of the *kexB* gene encoding a subtilisin-like processing protease KexB that is homologous to *S. cerevisiae* Kex2p (Mizutani et al. 2004). The *kexB* disruptant (Δ*kexB*) forms shrunken colonies with poor generation of conidia on Czapek–Dox (CD) agar plates and hyperbranched mycelia in CD liquid medium.

The phenotypes of the Δ*kexB* strain are restored under high-osmolality conditions in both solid and liquid culture. Gene expression profiles of the Δ*kexB* and wild-type strains were analyzed by using *A. oryzae* cDNA microarrays (Mizutani et al. 2004). Transcription levels of the *mpkA* gene, which encodes a putative MAPK involved in the CWI signaling pathway, is significantly higher in Δ*kexB* cells than in wild-type cells. Constitutively higher levels of phosphorylated MpkA are also observed in Δ*kexB* cells in CD plate culture. High osmotic stress remarkably downregulates the level of *mpkA* transcripts and the phosphorylated form of MpkA in Δ*kexB* cells, concomitantly suppressing the aforementioned morphological defects (Mizutani et al. 2004). The Δ*kexB* cells also contain higher levels of transcripts for cell wall-related genes that encode β-1,3-glucan synthase, β-1, 3-glucanosyltransferases, and chitin synthases. Taken together, these results suggest that KexB is required to maintain normal cell wall structure or integrity, and that the KexB defect induces disordered CWI signaling. To confirm whether the higher levels of transcripts of cell wall-related genes in *A. oryzae* Δ*kexB* cells depend on MpkA or non-MpkA signaling, it is further necessary to construct an *A. oryzae kexB*Δ *mpkA*Δ strain.

13.2.5 Targets of CWI Signaling in Aspergillus *Species*

Because filamentous fungi, including aspergilli, seem to use the MpkA MAPK pathway mainly to regulate the transcription of α-1,3-glucan synthase genes (Fujioka et al. 2007), the biological functions of α-1,3-glucan have been investigated. Originally, the importance of cell wall α-1,3-glucan relative to fungal virulence has been studied in several human pathogenic fungi, such as *Blastomyces dermatitidis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* (Hogan and Klein 1994; Reese and Doering 2003; Rappleye and Goldman 2006), and the plant pathogenic fungus *Magnaporthe grisea* (Fujikawa et al. 2009). To reveal biological functions of α-1,3-glucan in the *Aspergillus* species, α-1,3-glucan synthase genes have been characterized (Beauvais et al. 2005; Damveld et al. 2005b; Maubon et al. 2006). *A. fumigatus* contains three AGS genes, *ags1* to *ags3* (Fig. 13.3). *A. fumigatus ags1*, which is an orthologue of *A. nidulans agsB* (Fig. 13.3), is involved in the formation of 50 % of the cell wall α-1,3-glucan, whereas disruption of *ags2*, which is an orthologue of *agsA* (Fig. 13.3), had no detectable effect on glucan levels (Beauvais et al. 2005). Disruption of the third gene, *ags3*, which has no orthologue in *A. nidulans* (Fig. 13.3), results in the overexpression of *ags1*, which may serve to compensate for the lost enzyme activity and maintain normal cell wall composition (Maubon et al. 2006). In addition, the disruption of *ags3* in *A. fumigatus* causes hypervirulence, whereas the disruption of *ags1* and *ags2* did not affect virulence (Beauvais et al. 2005; Maubon et al. 2006). Furthermore, a triple-mutant strain of *A. fumigatus* lacking the three α-1,3-glucan synthase genes (*ags1*, *ags2*, and *ags3*) was generated, and the growth of the triple mutant in plate culture was similar to that of the parental strain (Henry et al. 2011). The triple mutant showed slightly

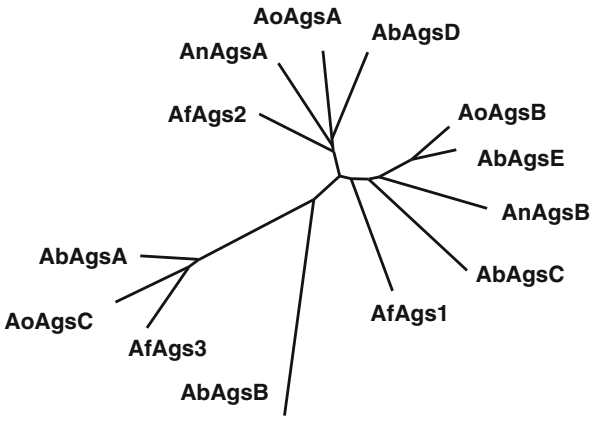

Fig. 13.3 Phylogenetic tree of the **α**-1,3-glucan synthases in *Aspergillus* species. The tree was constructed using the neighbor-joining method based on the alignment of the amino acid sequences. *An A. nidulans*, *Ao A. oryzae*, *Af A. fumigatus*, *Ab A. niger*

decreased conidiogenesis, as did the single *ags1* and *ags2* mutants (Beauvais et al. 2005; Henry et al. 2011), and the lack of cell wall α-1,3-glucan led to an increase in β-1,3-glucan and chitin levels in mycelia of the triple mutant (Henry et al. 2011). *A. niger* has five α-1,3-glucan synthases encoded by *agsA* to *agsE* (Fig. 13.3). The expression of *agsA* (an orthologue of *A. fumigatus ags3*) and *agsE* (an orthologue of *A. fumigatus ags1* and *A. nidulans agsB*) was induced in the presence of cell wall stress-inducing compounds such as CFW, sodium dodecyl sulfate, and caspofungin (Damveld et al. 2005b). In *A. nidulans*, several mutants for the α-1,3-glucan synthase genes *agsA* and *agsB* were constructed (Yoshimi et al. 2013). The *agsA* disruption strains did not show markedly different phenotypes from those of the wild-type strain. The *agsB* disruption strains and the double-disruption strains showed increased sensitivity to CR and lysing enzymes (Yoshimi et al. 2013). In addition, the *agsB* disruption strains formed dispersed hyphal cells under liquid culture conditions regardless of the *agsA* genetic background (Yoshimi et al. 2013). Biochemical analysis of the cell wall polysaccharides revealed that the disruption of *agsB* led to almost complete loss of cell wall α-1,3-glucan, which was mainly composed of linear α-1,3-glucan with a structure that is similar to that of mutan, a biofilm component that is produced by the oral bacterium *Streptococcus mutans* with its glucanosyltransferase reaction (Yoshimi et al. 2013). Recently, He et al. (2014) demonstrated that *agsA* was mainly expressed during conidiation, and the *agsB* disruptant showed increased sensitivity to CFW but not to CR in *A. nidulans*. In contrast to the results of He et al., Yoshimi et al. (2013) reported that the *agsB* disruption strain showed increased sensitivity to CR, but not to CFW, and the amount of CR adsorption to the hyphae of the *agsB* disruptant strain was significantly greater than that of the wild-type strain. Both CR and CFW interact with various polysaccharides, although β-1,3-glucan shows a strong interaction with CR but a weak interaction with CFW. In addition, the amount of CR adsorbed to α-1,3-glucan is significantly less than the amount adsorbed to β-1,3-glucan or chitin (Yoshimi et al. 2013). It is reasonable to hypothesize that the loss of α-1,3-glucan from the cell wall

led to increased exposure of β-1,3-glucan on the cell surface and the resulting increased sensitivity to CR. The differences between the results of the two research groups in relation to the sensitivity to CR and CFW might be related to the fact that the two groups used different parental *A. nidulans* strains. These observations indicate that α-1,3-glucan is involved not only in fungal virulence but also in multiple functions in cell wall biogenesis, such as the maintenance of normal growth characteristics and protection against a certain cell wall stress in *Aspergillus* species.

13.3 Osmotic Stress Signaling in *Aspergillus* Species

13.3.1 Overview of the High Osmolality Glycerol (HOG) Pathway

In solid-state cultivation, which is used in the fermentation industry, *A. oryzae* grows on solid substrates including steamed rice grain, roasted wheat grain, and steamed ground soybean (Abe and Gomi 2007). These conditions make the organism produce a large amount of enzymes that hydrolyze starch and proteins into sugars and peptides/amino acids, respectively. Thus, fungi are thought to be exposed to elevated osmotic stress in a microenvironment during solid-state cultivation.

Some fungi are known to synthesize glycerol as a main osmolyte to adapt to the surrounding hyperosmotic environment. In *S. cerevisiae*, the production of glycerol is initiated by the conversion of dihydroxyacetone phosphate through a two-step reaction of glycerol-3-phophate dehydrogenase (Gpd1p, Gpd2p) and glycerol-3-phosphatase (Gpp1p, Gpp2p) (Albertyn, et al. 1994; Norbeck et al. 1996). The expression of *GPD1* and *GPP2* and subsequent production of glycerol are under control of the Hog1p MAPK cascade that consists of the MAPK kinase kinases Ssk2p/Ssk22p, MAPK kinase Pbs2p, and MAPK Hog1p (Hohmann 2002). The Hog1p MAPK cascade is regulated by two different upstream branches, the Sho1p shunt and a two-component signaling (TCS) system, and is activated via these pathways in response to hyperosmotic stresses (Fig. 13.4) (Maeda et al. 1994, 1995; Posas et al. 1996). The *S. cerevisiae* TCS system has one membrane-anchored histidine kinase, Sln1p, which seems to act as a sensor for environmental osmotic conditions. In hypo-osmotic conditions, Sln1p is phosphorylated and, in turn, a phospho group is relayed from Sln1p to the downstream response regulator (RR), Ssk1p, via an intermediate protein Ypd1p (Fig. 13.4). The phosphorylated form of Ssk1p inactivates the downstream Hog1p MAPK cascade (Posas et al. 1996; Posas and Saito 1998). In hyperosmotic stress conditions, Sln1p shows phosphatase activity and thus deprives the phospho group from Ypd1p-Ssk1p components. The dephosphorylated form of Ssk1p activates the Hog1p MAPK cascade, which in turn facilitates glycerol biosynthesis for osmotic adaptation.

In *Aspergillus* species, counterparts of the Hog1p MAPK cascade and TCS system have been extensively studied. We provide up-to-date findings of the aspergilli HOG pathway in the following sections.

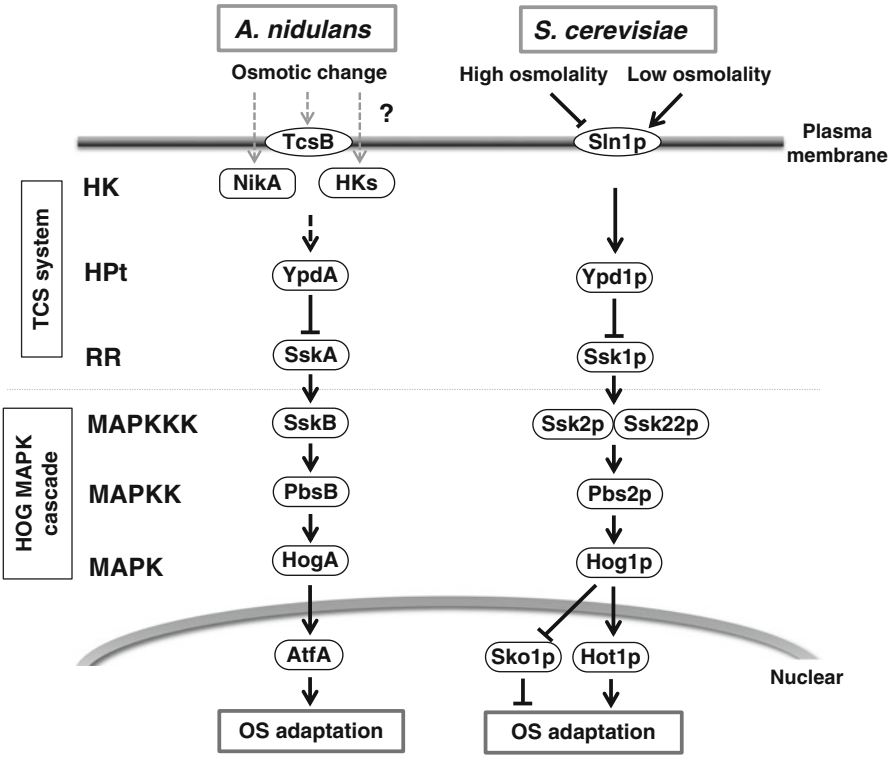

Fig. 13.4 Schematic model of osmotic stress signaling in *A. nidulans*. Osmotic stress signaling [the high-osmolality glycerol (HOG) pathway] involves the two-component system (TCS) and HOG mitogen-activated protein kinase (MAPK) cascade in *A. nidulans*, which corresponds to the well-studied *Saccharomyces cerevisiae* (*S. cerevisiae*) HOG pathway. *OS* osmotic stress

13.3.2 TCS System for Osmotic Stress Signaling in Aspergillus *Species*

The TCS (also known as His-Asp phospho-relay signaling) system, which was first described in bacteria, is a common signal transduction mechanism found in organisms ranging from bacteria to fungi and higher plants, but it is not in animals (Mizuno 1998). The eukaryotic TCS system consists of three types of common signal transducers, a histidine kinase (HK), a histidine-containing phospho transmitter (HPt), and an RR, resulting in a multistep phospho-relay signal. Each component has an invariant amino acid residue, His or Asp, within a conserved motif, and a phospho group is transferred (relayed) from His to Asp or Asp to His in response to external stimuli (Appleby et al. 1996).

BLAST of the genome sequences of *A. nidulans*, *A. oryzae*, and *A. fumigatus* revealed that 13 to 15 HKs, 3 RRs, and 1 HPt were found in their genomes.

Considering that the model yeasts *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* have 1, 3, and 3 HKs, respectively, the larger number of HKs found in the aspergilli genomes is in sharp contrast to the small number of HKs in the yeasts (Kobayashi et al. 2007). This divergence suggests that signaling networks might be more complex in filamentous fungi.

Of the multiple *Aspergillus* HKs, TcsB is an orthologue of *S. cerevisiae* Sln1p and was thought to have a crucial role in the osmotic stress response. In contrast to expectation, the *tcsB* deletion mutant of *A. nidulans* and *A. fumigatus* does not exhibit any detectable phenotypic defects on osmotic stress medium, and *tcsB* is not required for the phosphorylation of the HogA/SakA MAPK, which is a counterpart of Hog1p, in response to osmotic stress (Furukawa et al. 2002, 2005; Du et al. 2006). In a recent report, *A. fumigatus* TcsB is involved in the phosphorylation of the SakA MAPK in response to a cold shock stress, and it is required for growth under high-temperature conditions (Ji et al. 2012). Although the involvement of TcsB in certain stress responses has been reported, a sensor for osmotic conditions, which functions in the HOG pathway, is yet to be identified.

Among filamentous fungi, NikA is a widely conserved HK that has a characteristic motif, a repeated HAMP domain, in its N-terminus. Although the function of the HAMP domain has remained elusive so far, some reports suggested a potential role of the domain in the perception of osmotic conditions (Meena et al. 2010; El-Mowafy et al. 2013). Indeed, in both *A. nidulans* and *A. fumigatus*, disruption of the *nikA* gene results in a growth defect on plate medium containing high osmolality stress (Hagiwara et al. 2009b, 2013). This result raised the hypothesis that, instead of TcsB, NikA protein might regulate the HOG pathway in response and in adaptation to osmotic conditions. However, phosphorylation of the SakA MAPK in response to osmotic stress occurs irrespective of NikA in *A. fumigatus*, whereas the SskA RR is indispensable for the phosphorylation of SakA (Hagiwara et al. 2013). This finding suggests that the other HKs may contribute to the response and adaptation to the osmotic changes through the HOG pathway in the fungus.

13.3.3 HogA/SakA MAPK Cascade in the Osmotic Stress Response in Aspergillus *Species*

With regard to osmotic stress signaling, the HogA/SakA MAPK cascade plays a central role among aspergilli and other filamentous fungi (Bahn 2008). *A. nidulans* possesses the SskB MAPK kinase kinase, the PbsB MAPK kinase, and the HogA MAPK in the HogA MAPK cascade (Fig. 13.4). The *sskB*, *pbsB*, and *hogA* deletion mutants show growth inhibition under high osmolality. In response to osmotic shock, the HogA MAPK is phosphorylated in an SskA RR-, SskB-, and PbsB-dependent manner (Furukawa et al. 2005). Along with *A. nidulans*, the *A. fumigatus* SakA MAPK cascade is composed of the SskB MAPK kinase kinase, the PbsB MAPK kinase, and the SakA MAPK. The *sakA* and *pbsB* deletion mutants show

retarded growth under high osmolality conditions (Hagiwara et al. 2013, unpublished data). SakA is phosphorylated in response to osmotic shock in an SskA RR-dependent manner (Hagiwara et al. 2013). Importantly, these studies on two *Aspergillus* HOG pathways indicated that the activation of the HogA/SakA MAPK cascade is exclusively dependent on the TCS system (at least the SskA RR, although the responsible HK is undetermined). In contrast to *S. cerevisiae*, the ShoA (counterpart of Sho1p) shunt is not responsible for the regulation of the HogA/SakA MAPK cascade in *Aspergillus* species. This view is supported by the fact that *A. nidulans* PbsB protein lacks the Pro-rich motif that is required for binding to the Src-homology 3 domain of Sho1p (Furukawa et al. 2005). Taken together, osmotic stress signaling in the HOG pathway involves the HogA/SakA MAPK cascade following the TCS system in aspergilli.

Transcriptome analysis in response to osmotic shock was conducted in *A. nidulans* using a DNA microarray (Hagiwara et al. 2009a). The study identified 181 and 85 genes as osmotic stress-upregulated (>2 times) genes and osmotic stress-downregulated (<0.5 times) genes, respectively. Of those, 123 genes (68 %) and 22 genes (26 %) were dependent on HogA, respectively, which revealed that the HOG pathway is a central regulator for environmental osmolality changes. In the global expression study, the expression profile in response to a treatment of fludioxonil, a filamentous fungi-specific fungicide widely used in agricultural applications, was also investigated. A mode of action of fludioxonil had been thought to be the HOG pathway from studies on several filamentous fungi. Indeed, transcriptome data indicated that treatment with fludioxonil activates the *Aspergillus* HOG pathway, and a large number of genes were expressed differentially in a HogA-dependent manner. Notably, the genes that were differentially expressed largely overlapped with those that were differentially expressed with fludioxonil treatment or osmotic shock, suggesting that the HOG pathway is shared by the fludioxonil and osmotic stress responses in the fungus.

13.3.4 Targets of the HOG Pathway in Aspergillus *Species*

The *Aspergillus* HOG pathway is activated in osmotic stress and is involved in growth under high osmolality conditions, which at least partly shows how the cells perceive environmental osmolality changes. The next question is the adaptive mechanism of how cells adapt to the extracellular osmotic conditions. As stated, *S. cerevisiae* accumulates glycerol as an osmolyte in the cell when treated with a high concentration of sodium chloride as osmotic stress, which serves as osmotic adaptation. In a similar fashion, *A. fumigatus* accumulates a marked amount of glycerol in hyphae in response to osmotic stress treatment, and the glycerol de novo biosynthesis is dependent on the SakA MAPK cascade (Hagiwara et al., unpublished data). It appears that the HOG pathway functions in osmotic adaptation by glycerol accumulation as an osmolyte in the fungus.

A. nidulans has homologues for the glycerol biosynthesis-related genes GfdA and GfdB (presumed glycerol-3-phophate dehydrogenases) and GppA (a presumed

glycerol-3-phosphatase) (Miskei et al. 2009). Although the inactivation of *gfdA* results in decreased glycerol accumulation during growth under osmotic stress conditions, the mutant does not show an apparent sensitivity to high osmolality stress (Fillinger et al. 2001). Another *A. nidulans* GPD homologue is GfdB, the expression of which is increased in response to osmotic shock in a HogA MAPK cascade-dependent manner, but the involvement of the protein in osmotic adaptation remains to be determined (Furukawa et al. 2007).

Transcription factors that function downstream of the HOG pathway in aspergilli have not been identified except AtfA, which is an orthologue of *S. pombe* Atf1. From the transcriptome comparison analysis already stated here, AtfA was found to function in the *A. nidulans* HOG pathway (Hagiwara et al. 2009a). Notably, the upregulation of *gfdB* and other genes that are under the control of HogA MAPK are largely dependent on AtfA. The deletion mutant of *atfA* shows growth retardation on plates containing high osmolality stress. From these results, the TCS system-HogA MAPK cascade-AtfA transcription factor signaling pathway has a crucial role in the osmotic stress response and adaptation in *A. nidulans* (Fig. 13.4).

The inferred osmotic stress signaling pathway also is crucial in stress tolerance in conidia of *Aspergillus* species. Sakamoto et al. (2009) reported that conidia from an *A. oryzae atfA* deletion mutant showed a decreased germination rate and a higher sensitivity to oxidative stress. Indeed, the expression of a conidia-specific catalase gene, *catA*, and trehalose content were lower in conidia of the *atfA* mutant than the control strain. The decreased conidia viability was also observed in *atfA* mutants of *A. nidulans* and *A. fumigatus* (Hagiwara et al. 2008, unpublished data). Importantly, keeping conidia alive is a key technology enabling the cultivation and distribution of fungi in fermentation industries. Therefore, the osmotic stress signaling pathway appears to contribute to applications of industrially important *Aspergillus* species.

13.4 Concluding Remarks

In this decade, studies of stress responses and adaptation mechanisms in filamentous fungi have been greatly advanced by sequenced genome information and the development of genome-wide analysis tools. In this chapter, we described two major stress response signaling systems that seem to be crucial in solid-state fermentation processes. The CWI and osmotic stress signaling systems of aspergilli are largely similar to those of *S. cerevisiae*, whereas some components are missing or added in the pathways (i.e., the Sho1 shunt is missing in osmotic stress signaling; *ags* genes and the NikA HK are added in the CWI and osmotic stress signaling pathways, respectively). *Aspergillus* fungi evolutionally must have remediated the stress response mechanisms in adapting to their harsh environmental niches. Because fungal hyphae invade the substrates in solid-state fermentation, this condition somehow mimics the process of infection of pathogenic fungi for plants and animals. Therefore, further studies of solid-state cultivation and the fungal infection process may mutually accelerate the understanding of stress biology in filamentous fungi.

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