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Review

Cell wall structure and biogenesis in Aspergillus species

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Aspergillus species are among the most important filamentous fungi from the viewpoints of industry, pathogenesis, and mycotoxin production. Fungal cells are exposed to a variety of environmental stimuli, including changes in osmolality, temperature, and pH, which create stresses that primarily act on fungal cell walls. In addition, fungal cell walls are the first interactions with host cells in either human or plants. Thus, understanding cell wall structure and the mechanism of their biogenesis is important for the industrial, medical, and agricultural fields. Here, we provide a systematic review of fungal cell wall structure and recent findings regarding the cell wall integrity signaling pathways in aspergilli. This accumulated knowledge will be useful for understanding and improving the use of industrial aspergilli fermentation processes as well as treatments for some fungal infections.

Key words: Aspergillus species; cell wall; polysaccharides; signal transduction; glucans

Fungi are found ubiquitously throughout the world and play an important role in ecosystems as decomposers of plant biomass. In particular, filamentous fungi excel in the ability to secrete a wide variety of hydrolytic enzymes, thereby contributing greatly to terrestrial carbon and nitrogen circulation. Thus, filamentous fungi are indispensable to the maintenance of ecosystems and material circulation on our planet. Nevertheless, they include many phytopathogens that inflict damage on crops. Certain pathogenic species can also infect humans and cause fatal conditions. The risks posed by filamentous fungi are rising in particular as a result of the increasing number of elderly people, HIV sufferers, and others with immunodeficiencies. Whether filamentous fungi bring benefits or harm to our everyday lives depends on human needs and actions, but these fungi produce many different kinds of enzymes, organic acids, and other chemical substances in their own living environments, including many substances of value as raw materials for food processing and pharmaceuticals. A great many fermented foods actually make use of these characteristics of filamentous fungi. The Aspergillus genus includes the koji mold Aspergillus oryzae, A. niger, and other species used in fermentation industries; human pathogens such as A. fumigatus; aflatoxin-producing species such as A. flavus and A. parasiticus; model filamentous fungus A. nidulans, and many other species that exhibit various characteristics. Owing to their importance in industry, medicine, and scientific research, Aspergillus species were among the first filamentous fungi to have had their genomes sequenced and compared.¹–⁴) The sequencing of Aspergillus genomes was a key factor spurring the advances that have been made in research on the cell biology of filamentous fungi over the past decade.

Aspergillus species such as A. oryzae, A. sojae, and A. luchuensis are commonly known in Japan as koji molds for the role they have long played in manufacturing traditional Japanese fermented products such as saké (rice wine), soy sauce (soy sauce), miso (soybean paste), and shochu (distilled beverage).⁵–⁶) A. oryzae is renowned for its outstanding ability to produce industrially important enzymes such as amylases and proteases.⁵,⁷) Koji molds are also noted for their ability to secrete enzymes in higher quantities in solid-state rather than liquid cultures.⁹) Their long history of utilization in the food industry has, moreover, earned them recognition from the US government’s Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) organisms⁹, and from the World Health Organization (WHO) for their safety.¹⁰) The outstanding safety and productivity displayed by A. oryzae makes it an ideal host organism for the production not only of fermented foods, but also of various enzymes and chemicals with pharmacological effects that could be applied to medical treatments in the future.⁵,⁷,¹¹–¹²) In general, the cells of such filamentous fungi need to adapt to changes in temperature and pH, oxidative and osmotic stress, and other changes in the culture environment so as to be able to produce these enzymes and compounds. Such stresses first affect the surface of the fungi. Fungal cells have a cell wall on their surface; for
this reason, it is effectively the cell wall that senses changes in the extracellular environment. The stresses detected by the cell wall are transmitted to the cytoplasm and elicit an appropriate cellular response. Also, because filamentous fungi develop filamentous networks of cells known as mycelia as they grow, the cell walls are being constantly synthesized and regenerated during the growth process. As such, the mechanisms regulating cell wall biogenesis, including cell wall stress responses, are the most important and sophisticated mechanisms that affect the survival of filamentous fungi.13–18

In addition to species that are useful to industry, the Aspergillus genus includes species that produce aflatoxins, which are among the most carcinogenic substances in the natural world, and aflatrem, a tremorgenic mycotoxin; still other Aspergillus species infect humans, often with dire consequences.19,20 A. fumigatus, for example, is the major cause of invasive aspergillosis (IA). It infects and causes severe symptoms in immunocompromised patients, and mortality rate for IA is very high as a result.20 Making contact with host cells is the first decisive step in a pathogen’s attempt to infect a host. The surface structure of A. fumigatus cells, including that of the cell wall surface layer, accordingly plays a critical role in the pathogenic expression of IA. A. fumigatus also produces an extracellular matrix (ECM) composed of polysaccharides, mainly α-1,3-glucan and galactosaminogalactan (GAG). The cell walls of A. fumigatus are coated in this ECM, creating a cell surface structure that is thought to be crucial to pathogenic expression.21,30

Cell wall biogenesis and the mechanisms regulating it were first studied in the budding yeast Saccharomyces cerevisiae.22–24 In contrast, our knowledge of filamentous fungi cell wall structure, mechanisms regulating cell wall biogenesis, and cell wall stress responses is limited compared with that of S. cerevisiae. The usefulness and virulence of filamentous fungi as described above are, however, stimulating growing interest in their cell walls. A deeper understanding of the functions of cell walls in filamentous fungi would likely lead not only to the development of new fermented food industry applications, but also to improvements in the microbial production of useful enzymes and other compounds, as well as greater control over pathogenic filamentous fungi. In this article, we will focus on Aspergillus cell wall structure and the mechanisms regulating cell wall biogenesis, introducing recent research findings and reviewing the latest knowledge on the cell walls of filamentous fungi.

I. Cell wall architecture of Aspergillus species

1.1. Overview of cell wall structure

The cell walls of fungi not only play a role in maintaining cell morphology, but also help to protect the cells from various kinds of extracellular environmental stress. As such, cell walls are indispensable to fungi. In their life cycles, fungi are exposed to changes in osmotic pressure, temperature, pH, and various other environmental factors. These environmental changes put stress on the fungi living in the environment concerned, acting first on the cell wall. Fungal cells adapt to the stress of environmental changes by responding in various appropriate ways, including rebuilding and repairing their cell walls. Regarding the mechanisms by which filamentous fungi respond to osmotic and oxidative stress, we refer the reader to the review by Hagiwara et al.25 For both animal and plant pathogens, the cell wall is the first body part to make contact with the host cells. Understanding the structure of the cell wall is accordingly crucial to devise methods of controlling pathogens. The cell walls of filamentous fungi are complex structures composed mainly of polysaccharides (Fig. 1). The main polysaccharides involved include α-glucans (mainly α-1,3-glucan (Fig. 2(A)), but also small quantities of α-1,4-glucan), β-glucans (β-1,3-glucan with β-1,6-branches (Fig. 2(B)), galactomannan, and chitin (Fig. 2(C)).26–29 Some fungi also equip themselves with an ECM composed of polysaccharides synthesized de novo in the outer layers of the cell wall (Fig. 1).21,30 Galactomannoproteins, GPI-anchored proteins, surface proteins, and other proteins are also found in the cell wall (Fig. 1).27–30,31,32 In this section, we review recent findings on the structure, biosynthesis, and biofunctions of the polysaccharides that make up the cell walls of Aspergillus species.
I.ii. Cell wall α-1,3-glucan

Components of the cell walls of *Aspergillus* species can be fractionated on the basis of their solubility in alkali. Alkali-soluble fractions are composed mainly of linear-chain α-1,3-glucan (Fig. 2(A)). Much of the research conducted so far on the biofunctions of α-1,3-glucan has been carried out on the pathogenic fungi *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Histoplasma capsulatum* [34–36], and the important rice pathogen *Magnaporthe grisea* [37], rather than on *Aspergillus* spp. This research has, moreover, focused largely on functions related to the pathogenic expression of these species. Research on the functions of α-1,3-glucan in *Aspergillus* spp. initially focused on analysis of the functions of α-1,3-glucan synthase (*AGS*) genes [33,38–43].

*A. fumigatus* has three *AGS* genes, namely *ags1*, *ags2*, and *ags3* (Fig. 3(A)). In the *A. fumigatus ags1* (orthologous with *A. nidulans agsB*) disruptant, the cell wall α-1,3-glucan content decreased by 50%, but no such change in the cell wall α-1,3-glucan content occurred in the *A. fumigatus ags2* (orthologous with *A. nidulans agsA*) disruptant. Furthermore, neither disruptant showed any abnormal hyphal apices or abnormal conidiation. The remaining *A. fumigatus AGS* gene, *ags3*, is a type with no *A. nidulans* ortholog (Fig. 3(A)). The *ags3* disruptant showed a rise in the expression of *ags1*, likely owing to overexpression to compensate for the *ags3* deficiency. This kind of complementary regulation between *AGS* genes presumably results in maintaining cell wall integrity. The *ags1* and *ags2* disruptants showed no changes in virulence, but the *ags3* disruptant displayed an increase in virulence. This change in virulence is probably due to an excessive increase in melanin content in the conidia of the *ags3* disruptant, rather than a change in cell wall composition. In recent years, multiple disruptants of *ags1*, *ags2*, and *ags3* have been constructed. *A. fumigatus* triple *AGS* disruptants showed no noticeable growth abnormalities when plated, but their cell walls were devoid of α-1,3-glucan. They also showed the same kind of decrease in conidiation shown in...
by single *agsI* or *ags2* disruptants.\textsuperscript{41) The mycelia of triple *AGS* disruptants moreover showed increases in β-1,3-glucan and chitin complementary to the disappearance of α-1,3-glucan.\textsuperscript{41) Whereas germinating conidia of *A. Fumigatus* wild-type aggregate, those of the triple *AGS* disruptants did not.\textsuperscript{41) Evaluation of the virulence of triple *AGS* disruptants using an experimental murine aspergillosis model showed them to be less virulent than their parental strains.\textsuperscript{42) *A. niger* has five *AGS* genes, namely *agsA*, *agsB*, *agsC*, *agsD*, and *agsE* (Fig. 3(A)).\textsuperscript{39) Expression of *A. niger* *agsA* (orthologous to *A. fumigatus* *ags3*) and *agsE* (orthologous to *A. fumigatus* *ags1*, and *A. nidulans* *agsB*) is induced in the presence of cell wall stress-inducing compounds such as calcofluor white (CFW), sodium dodecyl sulfate (SDS), and caspofungin, an inhibitor for β-1,3-glucan synthase.\textsuperscript{39) Several strains of *A. nidulans* with mutations in the *AGS* genes *agsA* and *agsB* have been created.\textsuperscript{33,43) The *A. nidulans* *agsA* disruptant has so far shown no conspicuous phenotypical differences with the wild type.\textsuperscript{33) However, *agsB* disruptants display high sensitivity to congo red (CR) and some types of cell wall degrading enzymes.\textsuperscript{33) Furthermore, hyphal cells of *agsB* disruptants dispersed evenly in liquid medium.\textsuperscript{33) The double *agsA/*agsB disruptant was also phenotypically identical to the *agsB* disruptant. Biochemical analysis of the cell wall polysaccharides of these gene disruptants has shown that *agsB* disruption causes almost total loss of cell wall α-1,3-glucan.\textsuperscript{33) This α-1,3-glucan is believed to have the same linear chain structure as mutan
(which is found in biofilm produced by tooth decay bacteria *Streptococcus mutans*), because structural analysis showed its structure to match that of mutan enzymatically synthesized using glucanocyl transferase. Recently, He et al. have reported that the *A. nidulans* *agsB* gene is expressed mainly during conidiation, with two amylose-like proteins (AmyD and AmyG) performing different roles in α-1,3-glucan synthesis. Genes encoding AmyD and AmyG form a gene cluster with the gene encoding AgsB, with a gene sequence that is common to some *Aspergillus* spp. The *agsB* gene is highly similar to *A. nidulans*, with a gene sequence that is typical in fungal cell walls. However, no enzymes catalyzing such crosslinking activity have been identified in aspergilli.

Chitin is a β-1,4-linked homopolymer of N-acetylglucosamine (GlcNAc) whose synthesis is important for hyphal development (Fig. 2(C)). Chitin synthase (CHS) is an integral plasma membrane protein that catalyzes GlcNAc polymerization from UDP-GlcNAc. To date, fungal CHS has been classified into seven classes according to their structural characteristics (Fig. 3(C)). In this review, the classification proposed by Chigira et al. and Choquer et al. has been accepted. Class I, II, and IV genes are present in all fungi, whereas classes III, V, VI, and VII are specific to filamentous fungi and certain dimorphic yeasts. Among aspergilli, CHS genes have been comparatively characterized in *A. nidulans* and *A. fumigatus*. Both these species contain two class III genes. *A. nidulans* protein ChsB appears to play important roles in chitin synthesis in hyphal tips and conidia, as well as in polarized hyphal growth. Deletion of the *A. fumigatus* class III chsB gene results in similar but less marked defective phenotype, suggesting that its function might be related to those proposed in *A. nidulans*. Class V and VI CHSs, CsmA and CsmB, respectively, consist of a C-terminal chitin synthase domain and an N-terminal myosin motor-like domain. Myosins are motor proteins that move along actin filaments. Both csmA- and csmB-deletion mutations cause similar growth defects, such as the formation of balloons and intrahyphal hyphae and in hyphal lysis especially under low osmotic conditions, and these mutations are synthetically lethal. Mutants of the *A. fumigatus* chsE gene (encoding class V CHS) also show reduced hyphal growth and periodic swellings along hyphal lengths.

The orthologous genes encoding classes III, V, and VI CHSs have been isolated from other filamentous fungal species and their functions have been investigated. Results from these observations indicate that CHSs belonging to classes III, V, and VI play crucial roles in hyphal tip growth and maintenance of cell wall integrity. CHSs belonging to classes I, II, and IV tend to make no or relatively small contributions toward chitin biosynthesis in filamentous fungi. In addition, the genomes of the yeasts *S. cerevisiae*, *S. pombe*, and a dimorphic yeast *Candida albicans* do not possess CHSs in classes III, V, and VI. These observations suggest that the CHSs of these three classes likely play critical roles in polarized hyphal growth, especially in filamentous fungi. Taken together, β-1,3-glucan and chitin biosynthesis are fungal specific, and thus, the enzymes related to biosynthesis of these polymers are potential drug targets for therapeutic intervention in fungal diseases affecting humans.

**I.v. Other polysaccharides and proteins**

Recent studies have suggested that biofilm formation by *A. fumigatus* might be one of the most important virulence factors in IA and aspergilloma.
a biofilm characterized by mycelia embedded in an ECM, referred to as a mycetoma. In both chronic and invasive aspergillosis, hyphae produce an ECM, which is mainly composed of polysaccharides, including GM, GAG, and α,1,3-glucon. It is known that the same polysaccharides are present in ECM found in vivo in chronic and invasive aspergillosis.21) GAG and α,1,3-glucon play major roles in interhyphal adhesion.21,33,41,72) Gravelat et al. have reported that the gene uge3, encoding a fungal epimerase, is required for adherence by mediating the synthesis of GAG, which functions as the dominant adhesive on A. fumigatus and mediates adherence to plastic, fibrinectin, and epithelial cells.72) In addition, GAG suppresses host inflammatory responses in vitro and in vivo, in part through masking cell wall β-glucans from recognition by dectin-1. In addition, GAG is essential for full virulence in two murine models of invasive aspergillosis. These findings indicate that GAG plays a pivotal role as a bifunctional virulence factor in the pathogenesis of invasive aspergillosis.72) More recently, a coregulated five-gene cluster has been identified and proposed to encode proteins required for GAG biosynthesis.73) One of these genes, sph3, has been predicted to encode a protein belonging to the spherulin 4 family, whose function is unknown. Analysis of an sph3-deficient mutant in A. fumigatus has demonstrated that the gene is necessary for GAG production. In addition, biochemical analysis using recombinant protein has revealed that Spf3 possesses hydrolytic activity against both purified and cell wall-associated GAG and that the protein is a glycoside hydrolase essential for GAG production, thus defining a new glycoside hydrolase family, GH113.73) The putative GAG biosynthetic gene cluster has been found not only in A. fumigatus but also in other Aspergillus species, such as A. nidulans, niger and oryzae (Miyazawa et al. unpublished results). Thus, common mechanisms for GAG biosynthesis and for subsequent ECM formation are likely employed in Aspergillus species. In addition to polysaccharides, ECM also contains hydrophobins and melanin, both of which are known to be involved in virulence. Better understanding of the biological roles of ECM in filamentous fungi requires further studies, which should include comparative functional analysis of ECM among Aspergillus species.

II. Cell wall integrity signaling system of Aspergillus species

II.i Overview of cell wall integrity signaling

The cell wall integrity (CWI) signaling system that senses cell wall abnormalities and regulates gene responses required to maintain the integrity of cell walls has been studied in detail in the budding yeast S. cerevisiae.22–24,74,87) Cell wall abnormalities are detected by sensor proteins on the membrane. These proteins, which have been labeled WSC after the term “cell wall integrity and stress-response component,” are positioned furthest upstream in the CWI signaling pathway. Sensor proteins Wsc1p, Wsc2p, and Wsc3p are glycosylated transmembrane proteins characterized by their possession of an extracellular cysteine-rich domain, a Ser/Thr-rich region equipped with glycosylation sites, and an intracellular region in the strongly charged C-terminus.23,75–77) Mid2p and Mtl1p, which are also considered to be cell wall stress sensors, are membrane proteins with partially overlapping functions. These are sensors for sensing physical stimulation of the cell wall. They sense morphological changes that accompany growth or pheromone reception as well as cell wall stress caused by exposure to high temperature or high osmotic pressure, and transmit the signals to downstream signaling pathways.75,78,80) The detected cell wall stimuli are transmitted downstream through activation of the CWI signaling pathway. Cell wall stimuli are first transmitted to Rho1p, which is a small G protein.22,80) Rho1p is a low molecular weight GTPase that is activated by the guanosine nucleotide exchange factors (GEFs) Rom1p, Rom2p, and Tus1p.25,81) Its activity is regulated by Bem2p and Sac7p.25) Activated Rho1p binds to protein kinase C and activates Pkc1p, thereby inducing activation of the downstream MAP kinase (MAPK) cascade.82) This MAPK cascade is composed of the MAPK kinase Bck1p,84) two functionally overlapping MAPK kinases Mkk1p and Mkk2p,85) and the MAPK Mpk1p/Slt2p.86,87) Mpk1p phosphorylates and activates the transcription factor Rim1p, which regulates the expression of at least 25 cell wall biogenesis-related genes involved in the synthesis of β-1,3-glucan and chitin.87) The genome sequences of Aspergillus spp. have been published2–4), and the genes encoding proteins homologous to the constituent factors of the CWI pathway in yeast have been identified in Aspergillus spp. as well.7,15) In this section, we will review the latest information on the mechanisms of CWI pathway regulation in Aspergillus spp. (Fig. 4), taking into account the differences in the modes of CWI pathway regulation that have been discovered since genome sequencing.

II.ii Cell-surface sensors for CWI signaling in Aspergillus species

Progress has been made on analyzing the cell wall stress sensor proteins in Aspergillus species as well.17,18,88–90) Goto et al. have identified the gene that encodes the cell wall stress sensor WscA in A. nidulans (homologous to Wsc1p in S. cerevisiae) (Fig. 4).4) A. nidulans WscA contains a Wsc motif rich in cysteine residues, a Ser/Thr-rich region that carries three putative N-glycosylation sites, a transmembrane region, and a cytoplasmic region in the C-terminus. They also discovered that A. nidulans WscA is O-mannosylated in the Ser/Thr-rich region, and that A. nidulans PmtA and PmtC are involved in this reaction.89) PmtA and PmtC are O-mannosyltransferases that are strongly involved in the stability of WscA.91) Glycosylation of proteins also plays an important role in filamentous fungi, as demonstrated by the fact that the pmtA disruptant exhibits abnormal cell morphology and alterations in cell wall composition.92,93) Futagami et al. showed both WscA and WscB sensor proteins to be N- and O-glycosylated and localized in the plasma membrane.4) They also found that wsc gene disruptants (ΔwscA and ΔwscB) showed poor growth on a medium, a high
frequency of swollen hyphae under hypo-osmotic conditions, and reduced conidiation. Interestingly, this abnormal phenotype recovered under osmoregulation with potassium chloride. Furthermore, transcription levels of two α-1,3-glucan synthase genes (agsA and agsB) were altered in the wscA disruptant, resulting in an increase in the amount of alkali-soluble cell wall glucan (i.e. α-1,3-glucan). In contrast, treatment with micafungin, which is a β-1,3-glucan synthase inhibitor, was found to upregulate agsB expression in the wild-type strain and both the ΔwscA and ΔwscB strains. This expression response was dependent on A. nidulans MpkA (orthologous to S. cerevisiae Mpk1p MAPK), indicating that cell wall stress is detected and transmitted to the MpkA pathway even without WscA and WscB. A gene encoding a Mid2-like protein with a structure similar to that of the S. cerevisiae cell wall stress sensor Mid2p has also been found in the A. nidulans genome. Recently, the functions of this A. nidulans Mid2-like protein A (MtlA) were analyzed. MtlA protein was found to be involved not only in conidiation, but also in cell wall synthesis and tolerance to stress from CFW, CR, micafungin, and other cell wall synthesis inhibitors. These findings suggest that MtlA protein functions as a cell wall stress sensor in A. nidulans.

With regard to the A. fumigatus CWI signaling pathway, Dicht et al. have analyzed the functions of putative cell wall stress sensor proteins Wsc1, Wsc2, Wsc3, and MidA (corresponding to Wsc1p, Wsc2p, Wsc3p, and Mid2p in S. cerevisiae) and Rho GTPases Rho1, Rho2, and Rho4. Based on an analysis of the phenotypes of mutants for the genes encoding these CWI sensors and Rho GTPases, they have proposed a model for the A. fumigatus CWI signaling pathway. According to the model, cell wall stress caused by CFW, CR, or heat stress is detected by MidA or some other unknown sensor protein. Their signals then probably activate the MpkA pathway through Rho1 and PkcA. Wsc1 functions specifically in response to caspofungin, an echinocandin antifungal agent that inhibits the activity of β-1,3-glucan synthase. A. fumigatus Wsc1, Wsc3, and MidA have overlapping functions, and abnormalities in these proteins affect colony growth and conidiation. Signals from these sensors are transmitted to the MpkA pathway. A. fumigatus Rho2 and Rho4 do not directly affect the activation of MpkA, but are essential to CWI pathway regulation. Rho4 has a role in regulating septum formation, and is also involved in tolerance to β-1,3-glucan synthase inhibitors. These findings suggest that the functions of Wsc and Mid proteins differ between A. fumigatus and A. nidulans.

II.iii Protein kinase C pathway in Aspergillus nidulans

In addition to cell wall stress sensors and Rho GTPases, the functions of protein kinase C (PKC) have been analyzed in several species of filamentous fungi. For example, the PKC of Neurospora crassa, a red bread mold, is essential for growth and reportedly involved in the light response signaling
pathway. In Aspergillus spp., loss of the A. nidulans PKC-encoding gene pkcA (corresponding to pkc1 in yeast) is lethal.1,6 Yeast in which pkc1 has been deleted is viable in the presence of an osmotic stabilizer, whereas pkcA loss in A. nidulans is lethal regardless of the presence or absence of an osmotic stabilizer.93-97 In contrast, suppression of pkcA gene expression in A. nidulans results in a phenotype showing hypersensitivity to caspofungin, CFW, and other cell wall stress agents, as well as abnormalities in cell wall structure.97,98 These findings suggest that in A. nidulans as well, PkcA is involved in the CWI signaling pathway (Fig. 4). Furthermore, A. nidulans PkcA is localized to hyphal apices and growing septa, indicating a role in the formation of septa and phialide apices.97 PkcA is known to have other functions as well, being involved in the regulation of various cellular responses including conidiation and germination, secondary metabolism, and farnesol-induced cell death.95,96,98 Katayama et al. constructed a temperature-sensitive pkcA mutant of A. nidulans that exhibited apoptotic cell death at 42 °C, but showed almost normal growth at 30 °C.99 They further found that PkcA suppresses the induction of apoptosis through the MpkA pathway.99 Additionally, they revealed that although PkcA is also involved in establishing hyphal polarity under conditions of heat stress, MpkA plays no role in this regulation.99 Recently, Katayama et al. constructed a mutant strain of A. nidulans that encodes constitutively activated PkcA.100 Expression of this PkcA-activation mutation in A. nidulans results in increased levels of expression of multiple chitin synthase genes (chsB, chsC, chsD, csmA, and csmB) and the agsB gene.100 This finding indicates that A. nidulans PkcA regulates the transcription of cell wall synthase genes. Whether these transcriptional responses are dependent on the MpkA pathway is a matter that begs further discussion (see the next section), but at least in A. nidulans, PkcA appears to play a central role in the CWI pathway (Fig. 4). Moreover, PKC of filamentous fungi is involved in the regulation of multiple cellular responses, and likely plays a key role in cell wall maintenance.

II.iv. MAP kinase pathway for CWI signaling in Aspergillus species

In Aspergillus spp., the genes for encoding MAPK (which corresponds to Mpk1p (Slt2p) in S. cerevisiae) have been isolated.15,101,102 In A. nidulans, analysis of mpkA disruptant phenotypes indicated that this MAP kinase is involved in germination of conidial spores and polarized growth.101 In A. fumigatus, MpkA is reported to be involved in oxidative stress responses, siderophore formation under iron depletion, and regulation of the production of secondary metabolites.102 In addition to these physiological functions of MpkA, the roles played by MpkA in the CWI pathway of Aspergillus spp. have also been analyzed.

Fujioka et al. constructed A. nidulans mpkA, rlmA (orthologous to S. cerevisiae RLM1), and Answ16/Answ16 (orthologous to S. cerevisiae SWI4/SWI6, encoding the Mpk1p-activating TF complex Swi4p-Swi6p in yeast) disruptants that they then used to investigate the cell wall stress response to a β-1,3-glucan synthase inhibitor (micafungin) by analyzing cell wall synthase and mpkA gene transcripts.15 The results showed that the expression levels of almost all cell wall synthase genes were transiently elevated by cell wall stress. This transient elevation in expression was also observed in ΔmpkA and ΔrlmA strains with the exception of two α-1,3-glucan synthase genes (agsA and agsB). This finding suggests that transcription of the β-1,3-glucan synthase gene fksA and several chitin synthase genes (chsA-D, csmA, and csmB) is regulated independently of MpkA-RlmA (Fig. 4).15 In A. nidulans, transcription of the agsB gene encoding AgsB, which plays a major role in α-1,3-glucan synthesis, is dependent on MpkA regulation.15 The expression level of agsA is extremely low under normal wild-type growth conditions, but was upregulated slightly in ΔmpkA and ΔrlmA strains.15 Fujioka et al. further investigated the regulation mode of the mpkA promoter by constructing an mpkA promoter fused with a GUS reporter. Whereas activity of the GUS reporter system under control of the mpkA promoter was high in the wild-type strain, that of the ΔmpkA strain is significantly low.15 This result indicates that the transcription of mpkA itself is autoregulated via MpkA. Fujioka et al. also confirmed that mpkA expression is independent of RlmA and AnSwi4-AnSwi6.15 Therefore, the expression of the mpkA gene is autoregulated via an unidentified transcription factor regulated by MpkA.15 According to a recent report, MpkA-dependent expression of A. nidulans fksA and csmB was induced by micafungin treatment, and the ΔmpkA strain showed reduced expression of some cell wall genes.15,100 The contradictory nature of these results suggests that there is some room for debate on whether MpkA and RlmA are involved in controlling transcription of fksA and chitin synthase genes in A. nidulans. Recently, Yoshimi et al. discovered that MpkB, a different MAP kinase with the same phosphorylation motif as MpkA, additively contributes to micafungin activity in A. nidulans, but also revealed that MpkB does not directly regulate the CWI pathway.103 This suggests that in A. nidulans, the transcription of chitin synthase and many other cell wall-related genes seems to be regulated not by MpkA or MpkB, but rather by unidentified signaling pathways.

In A. niger, analysis of the transcriptional modes of the genes gfaA and agsA that encode glutamine: fructose-6-phosphate amidotransferase and α-1,3-glucan synthase, respectively, revealed that expression of both is induced in response to cell wall stress.14,104 In silico analysis suggests the presence of RlmA and MsnA (homologous to S. cerevisiae stress response transcription factors Rlm1p and Msn2p/Msn4p) target sites (DNA-binding domains) in the promoter regions of these genes. GUS reporter assays of these promoter regions indicate the presence of one RlmA binding site in the promoter region of A. niger’s agsA gene, indicating that the induction of this gene in response to cell wall stress is fully dependent on this site.14 In the A. niger rlma deletion strain, agsA induction is completely eliminated and gfaA expression is also reduced under cell wall stress conditions. In the rlma disruptant, CFW treatment resulted in a rise in cell wall chitin
content. The *rlmA* deletion strain also shows higher sensitivity than wild type toward cell wall stress agents. These results indicate that in *A. niger*, CWI is maintained through RlmA-dependent regulation of the transcription of *gfaA*, *agsA*, and other cell wall synthesis genes. A comparison of the above findings for *A. nidulans* and *A. niger* suggests that the two species differ in their MpkA-mediated cell wall stress response mechanisms.

In *A. oryzae*, the CWI signaling pathway has been studied by analyzing the functions of the subtilisin-like protease KexB (orthologous to *S. cerevisiae* Kex2p). S. cerevisiae Kex2p is a Ca²⁺-dependent transmembrane serine protease that specifically recognizes and cleaves KR and RR sequences of precursor proteins in the Golgi apparatus to produce mature secretory proteins. α-1,3-glucan, β-1,3-Glucan, chitin, and other polysaccharide synthases as well as Wsc and other sensor proteins are membrane proteins that are probably secreted through exocytosis and function on the membrane. The KexB recognition sequence is actually predicted to be present in the putative amino acid sequence of α-1,3-glucan synthase in *A. oryzae* (Mizutani et al. unpublished results). These cell wall-related proteins are accordingly thought to mature into normally functioning proteins after undergoing modification by KexB in the Golgi apparatus. The *A. oryzae* *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 these Δ*kexB* strains were restored under high osmotic pressure in both solid-state and liquid culture conditions. Comparison of the gene expression profiles of the Δ*kexB* strain and the wild type with *A. oryzae* cDNA microarray analysis showed increased transcription of *mpkA* (the gene that encodes MpkA, a protein thought to be involved in the CWI pathway of koji molds) in the Δ*kexB* strain compared with the wild-type. Persistent activation of MpkA on CD medium was also evident in the Δ*kexB* strain. In cells subjected to high osmotic stress, *mpkA* transcription was downregulated, and even in the Δ*kexB* strain, sustained phosphorylation of MpkA was not observed. This result does not conflict with the way in which the aforementioned abnormal phenotypes of Δ*kexB* strain were restored under high osmotic pressure. Higher levels of transcripts for genes encoding β-1,3-glucan synthases, β-1,3-glucan synthase, and chitin synthases were also found in the Δ*kexB* strain. The above findings suggest that *A. oryzae* KexB is indispensable to the maintenance of CWI. KexB deletion causes cell wall formation-related maturation abnormalities that are thought to disturb the CWI pathway. It is unclear whether the overexpression of cell wall-related genes observed in the *A. oryzae* Δ*kexB* strain is dependent on MpkA. Further research on an *A. oryzae* Δ*kexB*Δ*mpkA* strain will be required to clarify this point.

### III. Conclusion and prospective

In the past decade, studies of cell wall biogenesis and adaptation mechanisms to cell wall stress in filamentous fungi have advanced greatly because of sequenced genome information and the development of genome-wide analysis tools. In this review, we have described the current understanding of cell wall biogenesis and CWI signaling. Whereas components of CWI signaling in aspergilli are largely similar to those in *S. cerevisiae*, regulatory targets of CWI in aspergilli seem to differ from those in *S. cerevisiae* and some cell wall polysaccharides are missing or added among the aspergilli; for instance, α-1,3-glucan and ECM are added in *Aspergillus* species. *Aspergillus* fungi evolutionarily must have redesigned their stress response mechanisms in adapting to their harsh environmental niches. As fungal hyphae invade substrates in fermentation, this condition somehow mimics infection process of pathogenic fungi in plants and animals. Therefore, further studies of cell wall biogenesis might mutually accelerate the understanding of adaptation to cell wall stress in fermentation processes as well as infection processes of filamentous fungi.

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