Putative cell wall integrity sensor proteins in *Aspergillus nidulans*

Taiki Futagami and Masatoshi Goto*
Department of Bioscience and Biotechnology; Faculty of Agriculture; Kyushu University; Hakozaki, Japan

The cell wall integrity (CWI) signal transduction pathway, which has been well-studied in the yeast *Saccharomyces cerevisiae*, plays an important role in the regulation of cell wall biogenesis. Recently, we characterized the CWI stress sensor orthologs WscA and WscB in the filamentous fungus *Aspergillus nidulans*. Disruption of the *wscA* and *wscB* genes causes a change in the transcriptional levels of *agsA* and *agsB*, which encode α-1,3-glucan synthase, resulting in an increase in alkaline soluble cell wall glucan. However, the contribution of these putative sensors to downstream CWI pathway signaling remains unclear because MpkA-RlmA signaling remains active in *wscA*-*wscB* double disruptants exposed to cell wall stress associated with exposure to micafungin, a potent inhibitor of β-1,3-glucan synthase. In this addendum, we report the results of further studies involving hypo-osmotic shock as a stressor that suggest WscA and WscB are not essential for MpkA-RlmA signaling. Finally, we describe for the first time other *Aspergillus* CWI stress sensor candidate Mid2-like protein.

The cell wall integrity (CWI) signal transduction pathway plays an important role in the regulation of cell wall biogenesis in the yeast *Saccharomyces cerevisiae*. Recent progress in genomic studies indicates that the CWI signaling components, including sensor and response regulator proteins, are conserved in fungal species. While *S. cerevisiae* is a monocellular fungus that commonly lives on the surface of fruits and flowers in nature, *Aspergillus* species are multicellular filamentous fungi distributed widely throughout the soil, plant, and indoor environments. Included within this genus are the opportunistic human pathogen *A. fumigatus*, the industrial citric acid producer *A. niger*, and Koji molds such as *A. oryzae* and *A. kawachii*. Differences in habitat and biology associated with evolutionary history are believed to direct the responses of these species to environmental stimuli.

In *S. cerevisiae*, five plasma membrane-spanning CWI sensors (Wsc1, Wsc2, Wsc3, Mid2 and Mid1) that transmit environmental stimuli to downstream signaling pathways to initiate gene expression responses have been identified. At least two putative CWI sensors belonging to the Wsc family have been identified in *A. nidulans*. We recently characterized the putative CWI sensors WscA and WscB in *A. nidulans* and determined that they are both N- and O-glycosylated and localized on the cell surface. We found that *wsc*-disruptants are characterized by reduced colony size, the formation of fewer conidia, and a high frequency of swollen hyphae in hypo-osmotic YG medium, while osmotic stabilization with KCl restores the normal phenotype. Moreover, transcription of the α-1,3-glucan synthase encoding genes (*agsA* and *agsB*) is significantly altered in *wsc*-disruptant strains, resulting in an increase in the amount of alkaline-soluble cell wall glucan, including soluble α-1,3-glucan.

In *S. cerevisiae*, the activated sensors initiate the signaling cascade that eventually activates a mitogen-activated protein kinase (MAPK, Slt2). Activation of Slt2 triggers the phosphorylation of the transcriptional regulators Rlm1 and Swi4/ Swi6, which regulate the transcription of cell wall synthesis-related genes. On the other hand, MpkA-RlmA signaling, which corresponds to Slt2-Rlm1, induces
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 transient transcriptional upregulation of the agxB gene in response to exposure to the β-1,3-glucan synthase inhibitor micafungin in A. nidulans.8 We therefore compared the transcriptional response of wild type and wsc-disruptant strains to obtain direct evidence that WscA and WscB are located upstream of MpkA-RlmA signaling. However, transient transcriptional upregulation of the agxB gene was still observed in the ΔwscA ΔwscB strain, indicating that WscA and WscB are not essential for MpkA-RlmA signaling, at least with respect to stress associated with micafungin exposure. In S. cerevisiae however, the transcriptional response to caspofungin (an echinocandin class anti-fungal drug similar to micafungin) is mediated almost exclusively by Wsc1.8 Phosphorylation of Slt2 is reduced in the wsc1-disruptant after a 2 h caspofungin treatment. Our results therefore confirmed that the stress-sensing spectrum of A. nidulans Wsc proteins differs from that of S. cerevisiae.

Because the ΔwscA ΔwscB strain exhibits growth defects under hypo-osmotic conditions,7 hypo-osmotic shock was considered an appropriately stressful condition for investigating the relationship between WscA/WscB- and MpkA-RlmA-signaling. For this experiment, we first cultivated each strain in YG liquid medium with 0.6 M KCl, then transferred the cells to YG liquid medium without KCl. We collected the mycelia after 0, 30 and 60 min and quantified the transcriptional levels of the agxB and histone H2B genes using real-time RT-PCR. We also examined the level of MpkA phosphorylation as described previously.7 Our results indicate that phosphorylation of MpkA and transient upregulation of agxB occur even in the ΔwscA ΔwscB strain (Fig. 1A and B). This result supported our previous observation that MpkA-RlmA signaling is functional in the ΔwscA ΔwscB strain. Together with our previous results,7 this suggests that WscA and WscB participate in the tolerance to hypo-osmotic stress in A. nidulans, although their precise roles are unknown, as is the physiological importance of transient MpkA-RlmA signaling in hypo-osmotic stress tolerance.

The CWI pathway appears to be different in S. cerevisiae and A. nidulans (Fig. 2A). For example, a protein kinase C (PkcA) is essential for the viability of A. nidulans,9,11 but the ΔwscA ΔwscB strain was not lethal. This suggests that another CWI stress sensor exists or that there is cross-talk between signaling pathways upstream of PkcA in A. nidulans. This line of reasoning is supported by a report indicating that the central signaling component is well-conserved, whereas the sensors and transcriptional regulators of these modules have diverged significantly.4

In S. cerevisiae, Wsc1 and Mid2 act as the primary sensor proteins in the CWI pathway.1,2,5 Although Wsc family proteins have been identified in A. nidulans, no Mid2 ortholog has been reported.3,4,6,7 Recently, we found a Mid2-like protein (systematic name: AN4897) in the genome of A. nidulans during a global analysis of putative O-glycosylated serine/threonine-rich proteins (Figs. 2A and B).12,13 This protein shows a significant Pfam-A match to the Mid2 domain (E-value, 1.9e-05) and it has structural features similar to S. cerevisiae Mid2, including the N-terminal signal sequence, Mid2 domain, a transmembrane region and a putative C-terminal cytoplasmic tail. The homolog of AN4897 is also conserved among Aspergillus species, including A. fumigatus, A. flavus, A. terreus, A. clavatus, A. oryzae, A. niger and A. kawachii.

A phylogenetic tree of putative CWI sensor proteins shows AN4897 located in an intermediate position between the Wsc2/ Wsc3 branches and Mid2/Mid1 branches (Fig. 2B). The conserved Mid2 domain is not reflected well in the phylogenetic tree due to the large number of serine/threonine residues. The Aspergillus genome also possesses a homolog of S. cerevisiae putative CWI sensor Cwh43 (systematic name: AN5011) (Fig. 2A and B).6,15 Determining the roles played by potential sensor proteins such as Mid2 and Cwh43 homologs will increase our understanding of CWI signaling in Aspergillus species.

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Figure 2. (A) A model for CWI signaling in *A. nidulans* in response to stress associated with micafungin and hypo-osmosis. Dotted lines indicate the unclear relationship derived from the results of this and our previous study. S. cerevisiae orthologs that have not been functionally characterized in *A. nidulans* (Rom1, Rom2, Bck1, Mkk1 and Mkk2) are indicated in gray. (B) Phylogenetic tree of putative *A. nidulans* and *S. cerevisiae* CWI sensor proteins.