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Putative cell wall integrity sensor proteins in *Aspergillus nidulans*

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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.

Keywords: Aspergillus, cell wall integrity, cell wall biogenesis, micafungin, hypo-osmotic stress, sensor protein

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The cell wall integrity (CWI) signal transduction pathway plays an important role in the regulation of cell wall biogenesis in the yeast *Saccharomyces cerevisiae*.[1,2] Recent progress in genomic studies indicates that the CWI signaling components, including sensor and response regulator proteins, are conserved in fungal species.[3,4] 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 Mtl1) that transmit environmental stimuli to downstream signaling pathways to initiate gene expression responses have been identified.[1,2,5] At least two putative CWI sensors belonging to the Wsc family have been identified in *A. nidulans*.[3,4,6,7] 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.[7] 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 alkali-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).[1,2] 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

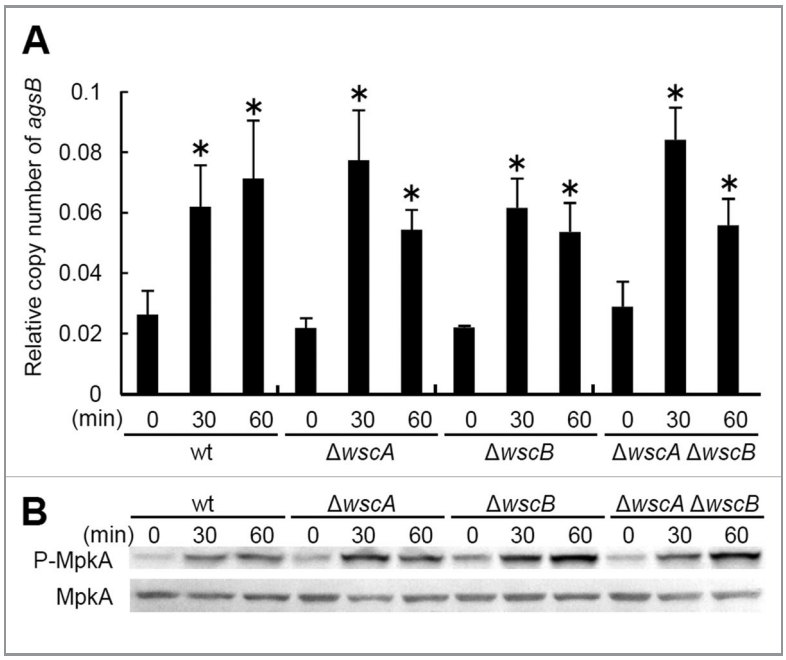

Figure 1. Response to hypo-osmotic shock in *Aspergillus nidulans*. (A) Relative transcription of the *agsB* gene, and (B) phosphorylation of MpkA in the wild type and *wsc*-disruptant strains. *, statistically significant difference ($p < 0.05$) relative to the result at 0 min.

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transient transcriptional upregulation of the *agsB* gene in response to exposure to the β-1,3-glucan synthase inhibitor micafungin in *A. nidulans*.[6] 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.[7] However, transient transcriptional upregulation of the *agsB* gene was still observed in the Δ*wscA* Δ*wscB* 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 Δ*wscA* Δ*wscB* 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 *agsB* 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 *agsB* occur even in the Δ*wscA* Δ*wscB* strain (**Fig. 1A and B**). This result supported our previous observation that MpkA-RlmA signaling is functional in the Δ*wscA* Δ*wscB* 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. nidulnans*,[9-11] but the Δ*wscA* Δ*wscB* 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**).[12-14] 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/Mtl1 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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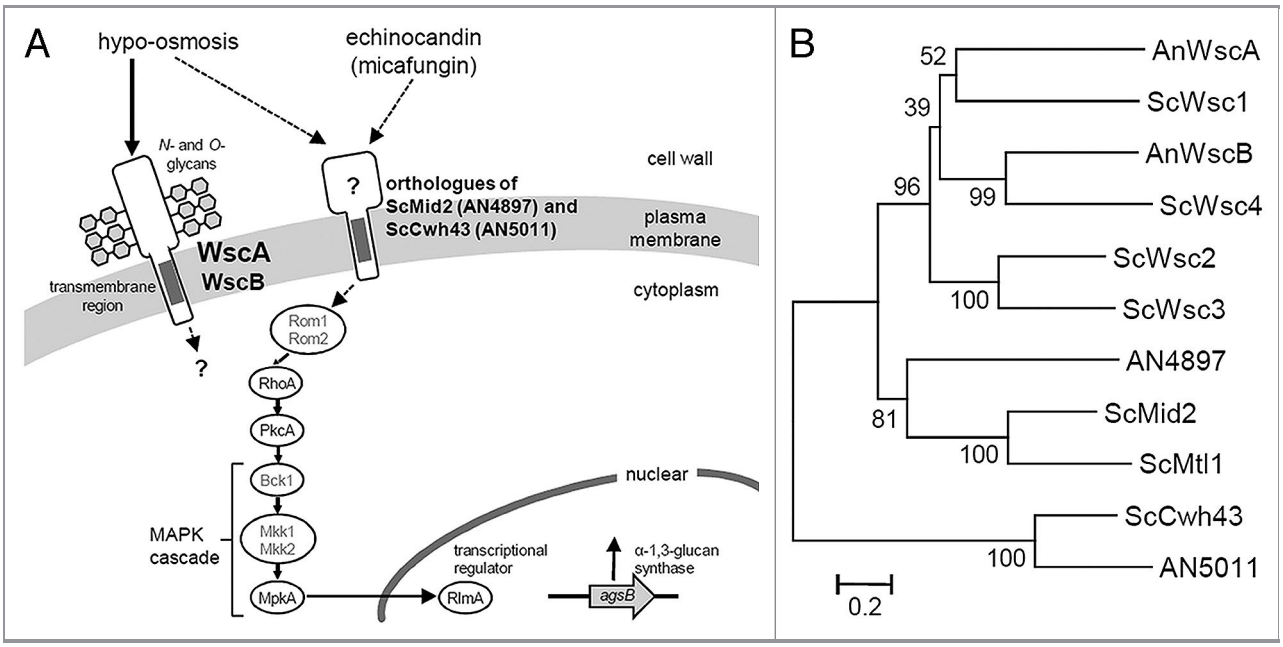

Figure 2. (A) A model for CWI signaling in *A. nidulans* in response to stress associated with micafungin and hypo-osmosis. Dotted lines indicate the unclear relationship derived from the results of this and our previous study.[7] *S. cerevisiae* orthologs that have not been functionally characterized 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. The tree was constructed using the neighbor-joining method based on alignment of the amino acid sequences. Bootstrap values are indicated at the tree roots (percentage of 1,000 bootstrap replicates that support the branch). The scale bar represents 0.2 substitutions per amino acid position. An, *Aspergillus nidulans*; Sc, *Saccharomyces cerevisiae*.

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