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Functional Characterization of AoHex1 Involved in Woronin Body Formation in *Aspergillus oryzae*

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Woronin bodies are unique organelles restricted to filamentous ascomycetes and deuteromycetes. They are observed in very close association with the septa. When hyphae are lysed, Woronin bodies appear to plug the septal pores within a few seconds and prevent excessive loss of cytoplasm. However, its cellular origins and biochemical composition had remained unknown since the first report of its discovery by Woronin (1864).

Aspergillus oryzae is one of the favored hosts for commercial enzyme production owing to its safety and ability to secrete a large amount of proteins into media. Solid-state culture is a unique culturing system known to provide higher-level of enzyme production when compared to the submerged culture in *A. oryzae*, in which cellular proteins are known to disperse into media as a result of hyphal lysis. While gene transcription and post-translational protein modification occurring specifically in solid-state culture were recently investigated, the mechanisms of lysis in solid-state culture are poorly understood at cellular and molecular levels. This study describes the functional relevance of *A. oryzae* AoHex1 required for Woronin body formation during lysis in solid-state culture.

1. Excessive loss of cytoplasm in the *Aohex1* disruptant under hypotonic shock

Since after cultivation of mycelia on solid-state culture the enzymes are extracted by adding water, I designed an observation system with inverted microscopy for flooding the colony grown on agar media with water, mimicking enzyme extraction in solid-state culture. Whereas few burst tips were found before flooding with water, most of the hyphal tips burst and their cytoplasm leaked out of the mycelia within several minutes after flooding. Addition of 1 M NaCl solution to the culture did not result in such a lysis, indicating that the hyphal tip bursting occurred due to hypotonic shock.

In order to observe the difference between the control strain and the *Aohex1* disruptant with regard to their cytoplasmic status and extent of leakage during hypotonic shock, the second compartments adjacent to lysed apical compartments were checked. In the control strain most of the second compartments adjacent to lysed tips retained the cytoplasm. On the contrary, cytoplasm leaked out of the second compartment in most of the lysed hyphae in the *Aohex1* disruptant. Therefore, it may be noted that AoHex1 was indeed required for preventing extensive loss of cytoplasm in the second compartment of bursting hyphae under hypotonic shock.

2. Three-dimensional image analysis for visualizing the plugging of the septal pore by Woronin body using DsRed2 and EGFP fluorescent proteins

To clearly visualize the septal pore and septal plugging by Woronin body, dual fluorescence labeling strategy was adopted. In order to visualize septa, a secretory protein, RNase T1, from *A. oryzae* was fused with EGFP and expressed. Previous studies from our laboratory have also detected green fluorescence of RNase T1-EGFP fusion protein at septa. In order to mark septal pores, I utilized confocal microscopy and observed green fluorescence of RNaseT1-EGFP fusion protein. On the middle plane of septa, septal pores were marked as a discontinuity at the center of linear green fluorescence of RNase T1-EGFP. In order to more spatially demonstrate septal pores, three-dimensional images were reconstructed. The septal pores were successfully marked as dark regions surrounded by green fluorescence of RNase T1-EGFP fusion protein, on the septum. For visualization of Woronin bodies along with septa, DsRed2-AoHex1 fusion protein was also expressed. The *A. oryzae* strain expressing both the two fusion constructs was observed by fluorescence microscopy. Red fluorescence of DsRed2-AoHex1 was detected as small circular spots and some of them were found in the vicinity of septa labeled by green fluorescence of RNase T1-EGFP fusion protein. The red fluorescent spots were also observed at the center of the green fluorescent septa adjacent to lysed compartments. In order to confirm the localization of Woronin bodies at the septal pores during hyphal lysis, the strain expressing both RNase T1-EGFP and DsRed2-AoHex1 fusion proteins were observed by confocal microscopy. Unlysed compartments showed straight septa and were accompanied with a number of red fluorescent spots of Woronin bodies in their vicinity but not at the septal pores. On the contrary the septa next to lysed compartments were curved probably due to cytoplasmic pressure during hyphal lysis and contained red fluorescent spots of Woronin bodies at their pores. Three-dimensional reconstruction demonstrated that the red fluorescence of DsRed2-AoHex1 located at the septal pore next to the lysed compartment and sometimes protruded from the septa, strongly supporting the location of Woronin bodies at septal pores. Hence, dual fluorescent labeling revealed that the Woronin bodies formed by

AoHex1 protein plugged the septal pore adjacent to lysed apical compartments upon hypotonic shock.

Conclusion

I established a novel observation system mimicking enzyme extraction in solid-state culture and demonstrated that *A. oryzae Aohex1* gene involved in Woronin body formation prevented excessive loss of cytoplasm during hyphal lysis upon hypotonic shock by plugging the septal pore. This is the first study to investigate environmental responses such as lysis during solid-state culture at a cellular level, which could help understand the physiology of *A. oryzae* and be beneficial for industrial uses.

References

Maruyama, J., Juvvadi, P. R., Ishi, K., and Kitamoto, K., *Aspergillus oryzae* AoHex1 is required for Woronin body formation and septal plugging during hyphal lysis induced by hypotonic shock. Microbiology (Submitted)