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Three-dimensional Image Analysis of Plugging at the Septal Pore by Woronin Body in *Aspergillus oryzae* during Hyphal Lysis Induced by Hypotonic Shock

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Woronin bodies are unique organelles restricted to filamentous ascomycetes and deuteromycetes. In micrographs they are typically identified as membrane bound proteinaceous granules in very close association with the septa, and spherical or ovoid with a diameter (100 to 750 nm) slightly greater than that of septal pores (Fig. 1). Hyphal compartments serially separated by septa comprise long straight mycelia that are interconnected through septal pores enabling passage of cytoplasm and organelles such as nuclei between them. When hyphae are lysed, Woronin bodies appear to plug the septal pores and prevent excessive loss of cytoplasm. Although several analyses employing transmission electron and light microscopies supported the role of Woronin body, its cellular origin and biochemical composition had remained unknown. Recently, *HEX1* gene encoding the most abundant 19 kDa protein in Woronin bodies was identified from *Neurospora crassa*. Deletion of *HEX1* gene caused the disappearance of Woronin body and gave rise to hyphae that bled more cytoplasm when cells were lysed, implicating Hex1 in plugging of septal pores.

Aspergillus oryzae has been used in the manufacture of Japanese traditional fermented foods such as sake, *miso*, and soy sauce, and commercial enzyme production. 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*. Recently, a number of genes transcribed specifically in solid-state culture were identified and differences of post-translational modification affecting enzyme localization were investigated between

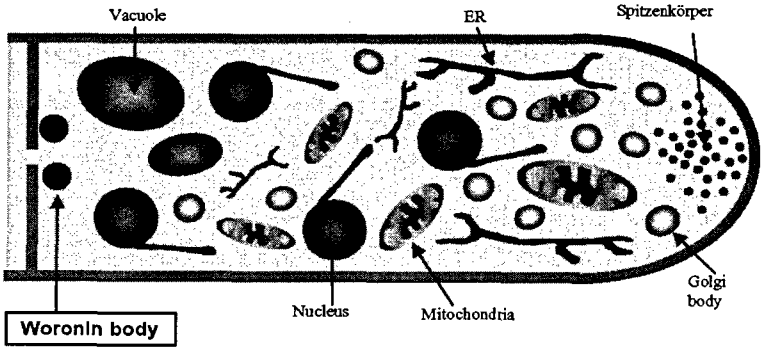

Fig. 1. Cytological aspects in hyphae of filamentous fungi.

submerged and solid-state cultures. In solid-state culture, after cultivation of mycelia on solid substrate such as wheat bran, the enzymes are extracted by addition of water. In *A. oryzae* cellular enzymes are known to disperse into the exterior during enzyme extraction from solid-state culture possibly as a result of hyphal lysis. Although hyphal lysis is possibly one of the aspects affecting enzyme production in solid-state culture, its mechanism is poorly understood at a cellular and molecular level.

In this study, we found hyphal tip bursting in *A. oryzae* when mycelia grown on agar media encountered water, but excessive loss of cytoplasm was prevented at the adjacent septum. In order to spatially characterize Woronin body during hypotonic shock, we utilized confocal microscopy and three-dimensional image reconstruction and clearly visualized plugging of Woronin bodies at septal pores by utilizing the two fluorescent proteins, EGFP and DsRed2, for the first time, in living hyphae.

Results

Hyphal tip bursting under hypotonic shock and defense against excessive loss of cytoplasm in adjacent compartments

In order to mimic enzyme extraction from solid-state culture at a cellular level, we designed an observation system with inverted microscopy. Mycelia of the *A. oryzae* strain grown on agar medium in the glass base dish were flooded with water and time-lapse microscopic observation of the hyphal tips at the edge of colonies was performed. Before flooding with water, no burst tips were found. Within a few minutes after flooding, most of the hyphal tips burst and their cytoplasm leaked out of the mycelia. Addition of 1 M NaCl solution to the culture did not result in such a lysis. This indicated that the hyphal tip bursting occurred due to hypotonic shock. On the contrary, hyphal tip bursting was scarcely found when mycelia grown in submerged culture were transferred into water, suggesting the less sensitive response of mycelia grown in submerged culture to hypotonic shock.

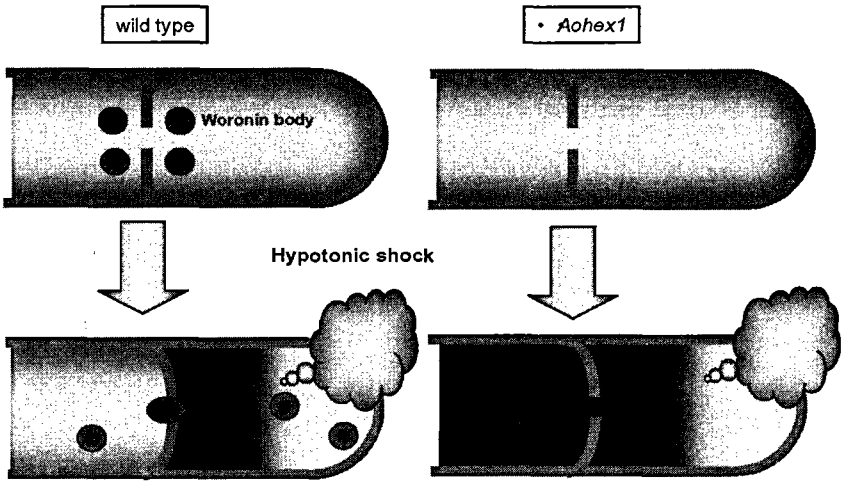

Fig. 2. Schematic model of hyphal tip bursting induced by hypotonic shock. When the *A. oryzae* colony grown on agar media is flooded with water, most of the hyphal tips at the edge of a colony burst and their cytoplasm leaks out of the mycelia. The second compartment prevents extensive loss of cytoplasm by Woronin body plugging the septal pore. Disruption of *Aohex1* gene caused disappearance of Woronin body and extensive loss of cytoplasm upon hypotonic shock.

Since hyphal compartments are interconnected through septal pores, some defense system was presumed to protect excessive loss of cytoplasm during hypotonic shock. Hence, cytoplasmic status and extent of leakage in the second compartments adjacent to the burst hyphal tip were checked by expressing EGFP in the cytoplasm. Most of the second compartments adjacent to lysed apical compartments retained the cytoplasm as evidenced by the presence of EGFP-fluorescence. This result revealed that some defense system in *A. oryzae* prevented excessive loss of cytoplasm in response to hyphal tip bursting induced by hypotonic shock (Fig. 2).

Cloning of Aohex1 gene encoding Woronin body protein

Since Woronin body is known to plug the septal pore adjacent to the damaged compartment, this organelle was supposed to play an essential role in preventing excessive loss of cytoplasm during hypotonic shock. The gene with homology to *N. crassa HEX1* gene encoding a major protein of Woronin body was cloned from *A. oryzae* and designated as *Aohex1*. The *Aohex1* ORF consisted of 681 bp and included an intron of 150 bp which showed up in some of the sequences in *A. oryzae* EST database, indicating an alternative splicing phenomenon as verified by RT-PCR analysis. The spliced transcript was deduced to encode a polypeptide (AoHex1) of 176 aa and the non-splicing in the alternative intron of the ORF resulted in an additional gene product (nsAoHex1) of 226 aa. Both the forms possessed a PTS1 (peroxisome targeting signal, -SRL) sequence at their C-termini, suggesting that some of the components required for peroxisome biogenesis could function similarly in the formation of Woronin body.

Confocal microscopy and three-dimensional image analysis for visualizing the plugging of septal pore by Woronin body using DsRed2 and EGFP fluorescent proteins

In order to spatially characterize Woronin body at the septal pore during hypotonic shock, dual fluorescence labeling strategy was adopted. Although septal pores can be found by transmission electron microscopy, no reports on their visualization by GFP are available. For visualization of 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. When the middle plane of septa was observed by confocal microscopy, septal pores were marked as a discontinuity at the center of linear green fluorescence of RNase T1-EGFP (Fig. 3A). 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 on the septum (Fig. 3B).

Subsequently, we attempted to visualize Woronin bodies by expressing AoHex1 protein fused with DsRed2 in the strain in which septa were labeled by RNase T1-EGFP fusion protein. Since AoHex1 protein has PTS1 at the C-terminus, 5'-side of the *Aohex1* cDNA was fused with *DsRed2* gene in frame. The red fluorescent small spots of DsRed2-AoHex1 were found in the vicinity of septa labeled by green fluorescence of RNase T1-EGFP fusion protein (Fig. 3C). Localization of DsRed2-AoHex1 fusion protein at septa was hardly recognized in the unlysed hyphae but noticed at the center of the green fluorescent septa adjacent to the compartments lysed naturally in the stationary culture of minimal liquid media, suggesting that expression of DsRed2-AoHex1 fusion protein successfully visualized Woronin bodies.

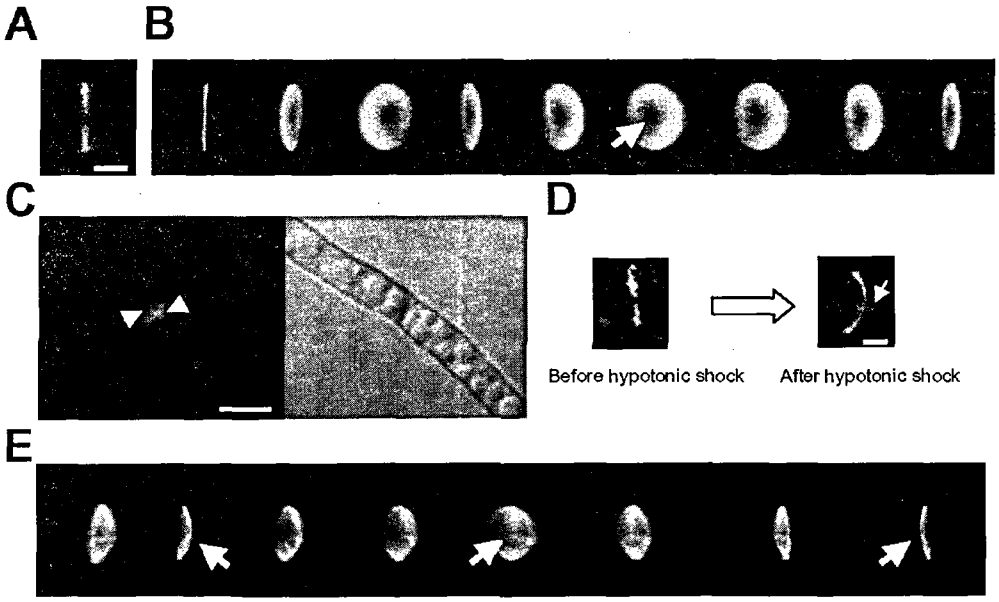

Fig. 3. Dual fluorescent images of the strain expressing DsRed2-AoHex1 and RNase T1-EGFP fusion proteins. (A) Confocal image of the septum visualized by expression of RNase T1-EGFP fusion protein. Bar: 2 μm (B) Three-dimensional images of the septum showing the septal pore [indicated by an arrow]. (C) Overlaid images by dual staining of Woronin body in unlysed hypha. Red-fluorescent Woronin body resided in the vicinity of septa fluorescing green as indicated arrows. Bar: 10 μm (D) Confocal images of unlysed (left) and lysed (right) septa. The red fluorescent spot of DsRed2-AoHex1 at the septal pore adjacent to the lysed apical compartment upon hypotonic shock is pointed by an arrow. Bar: 2 μm (E) Three-dimensionally reconstructed images of septal plugging by Woronin body. Red fluorescence of DsRed2-AoHex1 at the septal pore adjacent to the lysed apical compartment upon hypotonic shock is indicated by arrows.

In order to confirm the localization of Woronin bodies at the septal pores during hyphal lysis induced by hypotonic shock, the strain expressing both RNase T1-EGFP and DsRed2-AoHex1 fusion proteins was observed by confocal microscopy (Fig. 3D). 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 (Fig. 3E), strongly supporting the location of Woronin bodies at septal pores. Hence, dual fluorescent labeling revealed that the Woronin bodies labeled by DsRed2-AoHex1 fusion protein plugged the septal pore adjacent to lysed apical compartments upon hypotonic shock.

Disruption of Aohex1 gene caused disappearance of Woronin bodies and excessive loss of cytoplasm under hypotonic shock

In order to investigate the relevance of *Aohex1* gene in septal plugging during hypotonic shock, the *Aohex1* disruptant was obtained. On agar media the *Aohex1* disruptant exhibited a fluffy and compact colony morphology with a complete block in conidiation. Western analysis using anti-AoHex1 antibody indicated the absence of AoHex1 proteins (approximately 19 kDa and 24 kDa) in the disruptant (Fig. 4A). Transmission electron microscopic analysis showed that no Woronin bodies were seen in the vicinity of septa in the *Aohex1* disruptant while they were noted as electron-dense spherical structures close to septa in

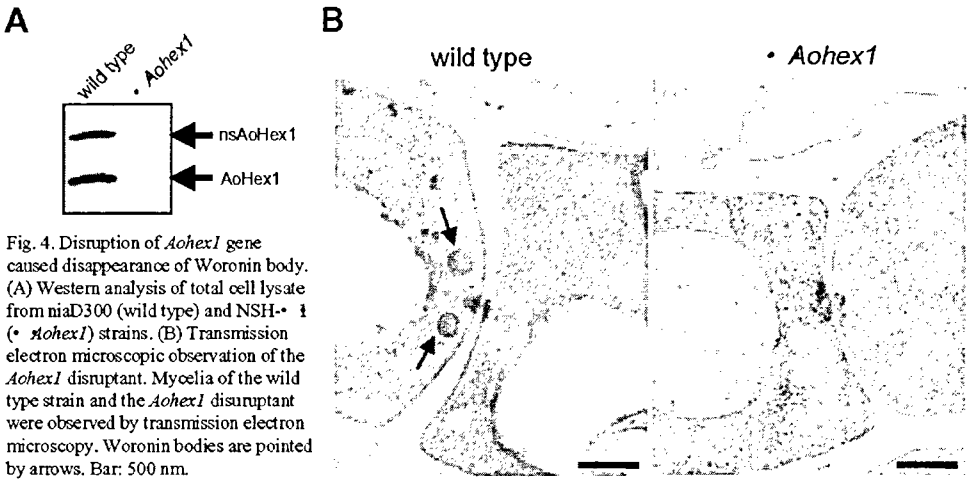

Fig. 4. Disruption of *Aohex1* gene caused disappearance of Woronin body. (A) Western analysis of total cell lysate from niaD300 (wild type) and NSH-• 1 (• *Aohex1*) strains. (B) Transmission electron microscopic observation of the *Aohex1* disruptant. Mycelia of the wild type strain and the *Aohex1* disruptant were observed by transmission electron microscopy. Woronin bodies are pointed by arrows. Bar: 500 nm.

the wild type strain (Fig. 4B). It was, therefore, concluded that *A. oryzae* AoHex1 is required for Woronin body formation.

In order to assess the cytoplasmic status in the *Aohex1* disruptant during hyphal lysis, the strain was subjected to hypotonic shock on the agar culture. On the contrary to the wild type strain, cytoplasm leaked out of the second compartment in most of the lysed hyphae in the *Aohex1* disruptant. Considering these results together, 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 (Fig. 2).

In this study we constructed a microscopic observation system of *A. oryzae* mimicking enzyme extraction during solid-state culture and found hyphal tip bursting by flooding mycelia grown on agar media with water. It is supposed that hyphal tip bursting induced by addition of water onto solid-state culture may lead to the dispersal of cellular enzymes into the exterior and AoHex1 prevented extensive loss of cytoplasm during enzyme extraction.

Our confocal microscopic observation is the first report to simultaneously visualize Woronin body and septal pores in living filamentous fungi, allowing us to precisely verify Woronin body plugging septal pores by three-dimensional reconstruction. This visualizing system could provide a powerful tool to investigate residues of AoHex1 required for targeting to Woronin body and plugging septal pores, leading to further understanding on molecular mechanisms of Woronin body function.

Reference

Maruyama, J., Juvvadi, P.R., Ishi, K., Kitamoto, K., 2005. Three-dimensional image analysis of plugging at the septal pore by Woronin body during hypotonic shock inducing hyphal tip bursting in the filamentous fungus *Aspergillus oryzae*. Biochem. Biophys. Res. Commun. 331, 1081-1088.