論文の内容の要旨

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論文題目

Studies on the mechanism of Woronin body tethering and septal plugging function in *Aspergillus oryzae*

（麹菌 *Aspergillus oryzae* における Woronin body の隔壁への繋留と隔壁孔をふさぐ機能の解析）

Introduction

Filamentous fungi grow through polarized tip extension, which forms tubular cells called hyphae. The presence of a septum divides the hyphae into distinct cells, and therefore filamentous fungi are classified as multicellular organisms. However, septa do not completely separate adjacent cells in the hyphae due to the presence of a septal pore, which allows the passage of cytoplasm and organelles between adjacent cells as well as includes the risk of uncontrolled cytoplasmic bleeding when hyphae are wounded. Woronin body is a Pezizomycotina-specific organelle typically tethered to the septum, and it plugs the septal pore to prevent the excessive loss of cytoplasm upon hyphal wounding.

Hex1 was first found as a major protein of Woronin body in *Neurospora crassa*, and the protein is conserved in other Pezizomycotina species. Its self-assembly confers a mechanically solid core to Woronin bodies, causing the resistance to the cytoplasmic streaming pressure arisen from hyphal wounding. Deletion of the *hex1* gene leads to the absence of Woronin bodies and severe cytoplasmic bleeding upon hyphal wounding. In the majority of Pezizomycotina species including *Aspergillus oryzae*, Woronin bodies are tethered to the septum at a distance of 100–200 nm, while in a small group defined by *N. crassa* have evolved cell cortex association of Woronin bodies. *leashin* (*lah*) locus was identified in *N. crassa* to be required for the cell cortex association of Woronin bodies. The locus consists of two split genes aligned in the same direction, *lah-1* and *lah-2*. LAH-1 binds to Woronin bodies via the membrane protein WSC through its N-terminal region, while the C-terminal region functions in the cell cortex association. *lah-2* gene at the downstream of *lah-1* is not functionally related to Woronin bodies, but LAH-2 is localized to the septum. In contrast, most of Pezizomycotina species possess a predicted LAH protein as a single polypeptide with over 5,000 amino acids, and its involvement in the tethering of Woronin bodies to the septum was suggested in *Aspergillus fumigatus*. However, the role of the LAH protein in the process for septal plugging has not been characterized, and it is not known whether the tethering to the septum is sufficient to fulfill the Woronin body function.

In *A. oryzae*, our laboratory found the hyphal tip bursting after adding water onto the colonies

grown on the agar medium. This enabled us to establish an assay (hypotonic shock experiment) for quantitatively evaluating the Woronin body function to prevent the excessive loss of cytoplasm upon hyphal wounding. The ability was severely impaired by the absence of Woronin bodies or moderately reduced by the deficiency in Woronin body differentiation from peroxisomes. Here, by employing this assay and microscopy, I analyzed the tethering and septal plugging function of Woronin bodies by investigating the roles of *A. oryzae* LAH protein.

Chapter 1. Disruption of the *Aolah* gene and phenotypic analysis of the disruptant

A gene (AO090011000895) encoding a protein consisting of 5,727 amino acids with a similarity to *N. crassa* LAH proteins was found in the *A. oryzae* genome database, and it was designated as *Aolah*. To investigate its function, the *Aolah* gene was disrupted. Transmission electron microscopic analysis revealed that no Woronin bodies were found near the septum in the *Aolah* disruptant, while Woronin bodies were tethered to the septum in the wild-type strain. Thus, it was concluded that AoLAH is required for the tethering of Woronin bodies to the septum. Next, Woronin body function was analyzed by the hypotonic shock experiment. In the *Aolah* disruptant, the ability of preventing the excessive loss of cytoplasm was reduced (63%) as compared with the wild-type strain (81%) but not as low as the *Aohex1* disruptant (14%). This result indicates that AoLAH is involved in the Woronin body function to prevent the excessive loss of cytoplasm upon hyphal wounding.

Chapter 2. Localization analysis of AoLAH N-terminal and C-terminal regions

According to homology comparison among *Aspergillus* species, LAH protein consists of N- (~2,000 amino acids) and C- (~1,000 amino acids) terminal conserved regions and a long-stretched (~2,500 amino acids) non-conserved region in the middle. To analyze the roles of individual parts, I divided the AoLAH protein into three: N-terminal (1-2039), middle (2040-4709), and C-terminal (4710-5727) regions.

First, localization analysis of the N-terminal region (AoLAH[1-2039]) was performed by EGFP fusion. The fluorescence of AoLAH[1-2039]-EGFP expressed in the wild-type strain was intensively localized at both sides of the septum, and also observed as dot structures in the cytoplasm, which is reminiscent of the Woronin body localization. Upon hyphal wounding, the fluorescence dot was detected at the septal pore, which is a behavior similar to septal plugging of Woronin bodies. AoLAH[1-2039]-EGFP was mainly localized independently of peroxisomes but occasionally found to associate with them, consistent with the fact that Woronin bodies differentiate by budding from peroxisomes. In the disruptant of *Aowsc* gene putatively encoding the Woronin body membrane protein, AoLAH[1-2039]-EGFP did not localize near the septum, and it was completely dispersed in the cytoplasm without any dot structures. This result suggested AoWSC-dependent recruitment of the AoLAH N-terminal region to Woronin bodies, which is in agreement with the report of *N. crassa*. When AoLAH[1-2039]-EGFP was expressed in the *Aolah* disruptant, the fluorescence was not observed near the septum but dot structures were found in the cytoplasm. This demonstrated that the

presence of the endogenous AoLAH protein is required for the localization of AoLAH[1-2039]-EGFP near the septum, which is a behavior similar to the tethering of Woronin bodies as observed by electron microscopy. Taken together, the AoLAH N-terminal region exhibited localization patterns typical to those of Woronin bodies, hereafter, I decided to use AoLAH[1-2039]-EGFP as a marker of Woronin bodies.

I expressed EGFP fusion of AoLAH C-terminal region (AoLAH[4710-5727]-EGFP) in the wild-type strain and *Aolah* disruptant. AoLAH[4710-5727]-EGFP localized closely to the septal pore in both the strains, indicating that the AoLAH C-terminal region itself is capable of being associated to the septal pore.

Chapter 3. Functional analysis of AoLAH middle region

The long-stretched middle region is non-conserved among the LAH proteins of *Aspergillus* species, and its involvement in the Woronin body function has not yet been investigated. In this study, I functionally characterized the middle region in *A. oryzae*. The N- and C-terminal fusion construct (AoLAH[(1-2039)+(4710-5727)]) without the middle region and full-length AoLAH were expressed in the *Aolah* dirsuptant. Transmission electron microscopy revealed that Woronin bodies were found near the septal pore in both the strains. However, the distance of Woronin bodies to the septum was shorter in the strain expressing the middle-region deleted AoLAH (average distance 56.2 nm) than that with the full-length AoLAH (average distance 111.2 nm). This suggests that the middle region of AoLAH has a role in regulating the distance of Woronin bodies to the septum.

Elasticity in the tethering of Woronin bodies to the septum was previously suggested in *Nectria haematococca*, in which Woronin bodies quickly moved back to the original position after pulled away with laser. However, elastic movement of Woronin bodies had not been naturally observed. The mammalian muscle protein titin exhibits elasticity like a molecular spring via its intrinsically disordered region, and the middle region of AoLAH protein is predicted to be disordered. Hence, it was hypothesized that the middle region might confer the elastic characteristics to the tethering linker of Woronin bodies. To test this, I sought to observe the Woronin bodies visualized with AoLAH[1-2039]-EGFP in living cells. When the first septum of hyphae was observed, at least one of the tethered Woronin bodies showed a quick back-and-forth movement within ~5 seconds per a movement in the full-length AoLAH expressing strain, demonstrating that some of the tethered Woronin bodies are capable of displaying an elastic movement. In contrast, such a movement was not observed for the Woronin bodies tethered to the septum by the middle-region deleted AoLAH. This indicates that the middle region of AoLAH is required for the elastic movement of Woronin bodies.

Hypotonic shock experiment inducing hyphal wounding was performed in the strain expressing the middle-region deleted AoLAH. Expression of the middle-region deleted AoLAH did not restore the ability to prevent the excessive loss of cytoplasm in the *Aolah* disruptant (62%), whereas the full-length AoLAH fully complemented the disruptant (81%), which indicates that the middle region of AoLAH is involved in the Woronin body function. Upon the hyphal wounding, the Woronin

bodies visualized with AoLAH[1-2039]-EGFP plugged the septal pore in the strain complemented with the full-length AoLAH. In the strain expressing the middle-region deleted AoLAH, besides the septal plugging, the Woronin bodies tethered to the septum were still frequently observed, which is in contrast to the full-length AoLAH expressing strain where tethered Woronin bodies were hardly observed near the septum upon hyphal wounding. This is attributed to the loss of movement activity toward the septal pore by deletion of the middle region in AoLAH. Collectively, it is indicated that the efficient septal plugging requires not only the tethering of Woronin bodies to the septum but also their movement activity rendered by the AoLAH middle region.

Chapter 4. Investigation of mechanisms for septal tethering of Woronin bodies

Although AoLAH protein tethers Woronin bodies to the septum, mechanisms of its contribution to the septal plugging remains unknown. Our laboratory has identified a number of proteins (AoSO, AoFus3 and their putative interacting proteins SoiA, SoiB, FipA and FipB) that are involved in the ability of preventing the excessive loss of cytoplasm. It is unknown how Woronin body function is related to these phenomena. In Chapter 2, AoLAH[1-2039]-EGFP was established as a reliable marker for Woronin body, and I analyzed Woronin body localization in the strains lacking the septum-related proteins, I expressed AoLAH[1-2039]-EGFP in these disruptants. Under fluorescence microscopy, in Δ*soiA*, Δ*soiB*, Δ*fipA* strains, Woronin bodies were localized at both sides of the septum, which is similar to that of the wild-type strain. Woronin body localization in strains lacking other septum-related proteins is being analyzed.

Conclusion

In this study, I investigated the mechanism of Woronin body tethering and septal plugging function in *A. oryzae* by characterizing individual regions of the tethering protein AoLAH. It was revealed that only tethering to the septum is not sufficient for Woronin bodies to fulfill their function of preventing the excessive loss of cytoplasm. I unexpectedly found that the long-stretched non-conserved middle region of AoLAH is involved in the elastic movement and septal plugging function of the tethered Woronin bodies. Since the middle region of AoLAH is predicted to be intrinsically disordered, the elastic movement activity may be attributed to characteristics typical of the disordered proteins. This study provides new evidence for physiological importance of such an intrinsically disordered protein, and further analysis of the protein would give an insight into more detailed machinery of the septal plugging by the tethered Woronin bodies.

Publication

Pei Han, Feng Jie Jin, Jun-ichi Maruyama, Katsuhiko Kitamoto. A large non-conserved region of the tethering protein Leashin is involved in regulating the position, movement and function of Woronin bodies in *Aspergillus oryzae*. Eukaryotic Cell. in press.