博士論文（要約）

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）

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General introduction

The Kingdom Fungi

All organisms in the living world are grouped into three domains as the Eubacteria, Archaea and Eukaryotes according to their differences in cellular structure and physiology. There are two basic types of cells: prokaryotes and eukaryotes. Prokaryotes refer to the simplest type of cells without a true nucleus and no membrane-bound organelles, which comprise Eubacteria and Archaea domains. Eukaryotes in eukaryote domain are more complex in structure with nuclei and membrane-bound organelles. Plants, animals and fungi are the main kindom under the eukaryote domain.

All fungi have a range of features that clearly separate them from other organism that serve to define the fungal kingdom (Deacon, 2005). Fungi are eukaryotic but typically have haploid nuclei, which is an important difference from almost all other eukaryotes. They typically grow as filaments called hyphae, with apical growth, but sometimes as yeasts. They are heterotrophic, which means that they need preformed organic compounds as energy sources and carbon skeletons for cellular synthesis. Fungal cells have cell walls that typically contain chitin and glucans, unlike the cell walls of plants and some protists, which contain cellulose, and unlike the cell walls of bacteria. Fungi reproduce by both sexual and asexual ways, and typically produce spores.

Filamentous fungi as multicellular organisms

Fungi are divided into two big groups: yeasts and molds. Yeasts are single rounded forms that reproduce through mechanisms such as budding or fission. Molds are a group of fungi that grow in the form of multicellular filaments called

hyphae (Moore, 2011; Madigan, 2005). Thus, molds are also known as filamentous fungi. The filamentous cells grow in a branching way forming a network called mycelium (Madigan, 2005).

Fungi are classified (Fig. 0-1) and hyphae of the lower fungi as the Glomeromycota, and Chytridiomycota are barely septated. In contrast, the hyphae of the filamentous Ascomycota and Basidiomycota are regularly septated at regular intervals along their length but with different types of septal structure (Fig. 0-2) (Markham *et al*,.1994).

Pezizomycotina is the largest subphylum of the Ascomycota, the hyphae of Pezizomycotina are regularly septate with a simple septum that possesses a single pore (septal pore) dividing the hyphae into hyphal compartments. Septal pores in Pezizomycotina (50-500 nm diameter) in the center of septum allow the passage of cytoplasm and organelles between adjacent cells (Bracker and Butler, 1964 ; Markham, 1994; Freitag *et al*., 2004; Lew *et al*., 2005; Tey *et al*., 2005; Ng *et al*., 2009). This cytoplasmic continuity between the individual cell compartments through the septal pore provokes cooperation and coordination of activities but also poses a problem, uncontrolled cytoplasmic bleeding when hyphae are wounded.

Woronin body, an organelle function to plug the septal pore upon hyphal wouding

Pezizomycotina has evolved a specialized vesicle that functions to seal the septal pore in response to cellular damage. This organelle was firstly described as refractive particles associated with septa based on light microscope observations by

Michail Stepanowitsch Woronin (Woronin, 1864), and it was subsequently named as Woronin body by Buller (Buller, 1933) in 1933. Their small size usually is close to the resolution limit of light microscopes except for in *Nurospora crassa* the Woronin body can be observed by light microscopy as a short hexagonal rod up to 1 μm in diameter (Jedd *et al*., 2000), and with transmission electron micrographs they were identified as 150-500 nm electron-dense organelles bound by a single membrane(Momany *et al*., 2002). The organelle is classified into two morphologically distinct subclasses; spherical in most of Pezizomycotina including *Aspergillus oryzae* and hexagonal in a small member of the species such as *Nurospora crassa.* Septal pore occluded by a Woronin body after hyphal wounding had been observed under light microscope and electron microscopy in *N.crassa* and other species(Brenner and Carroll 1968; Trinci and Collinge, 1974; Collinge and Markham, 1985). However, no molecular knowledge of Woronin body has been investigated for many years.

Woronin body was first purified from *Neurospora crassa* (Jedd and Chua *et al*., 2000, and HEX-1 (hexagonal 1) was identified as a major protein of Woronin bodies (Jedd and Chua *et al*., 2000; Tenney *et al*., 2000). Proteins homologous to *N. crassa* Hex1 are well conserved in Pezizomycotina species and characterization has been performed such as in *A.oyzae*, *A.fumigatus* and *Trichoderma atroviride* (Asiegbu *et al*., 2004; Curach *et al*., 2004; Soundararajan *et al*., 2004; Maruyama *et al*., 2005; Beck *et al*., 2013; Son *et al*., 2013; Tang *et al*., 2014). In *N.crassa*, Woronin bodies can not be observed under light microscopy in the Hex1-deletion strain, moreover, by quantifying the protein released upon hyhal tip lysis the deletion strain bled more cytoplasm and droplets of cytoplasm are seen at hyphal tips of the Hex1-deletion

strain but not in wild-type hyphae and (Jedd *et al*., 2000); Electron microscopy and immunoelectron microscopy analyses in the plant pathogen *Magnaporthe grisea* revealed that Woronin bodies were absent in the *hex1* disruptant (Soundararajan *et al*., 2004); In *Aspergillus oryzae*, 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 the wild type strain, extensive loss of cytoplasm from the second compartments was observed during hyphal tip bursting induced by hypotonic shock(Maruyama *et al*., 2005); the absence of Woronin body was also found in *Fusarium graminearum* (Son *et al*., 2013) and *Trichoderma atroviride* (Tang *et al*., 2014).

N. crassa Hex1 self-assembles into a hexagonal crystal lattice, resulting in the formation of a solid hexagonal core of the Woronin body (Jedd and Chua, 2000; Yuan *et al*., 2003), which confers a mechanically resistance to the cytoplasmic streaming pressure arisen from hyphal wounding. Phosphorylation of Hex1 is suggested to contribute to the formation of the multimeric core of the organelle (Tenney *et al*., 2000; Juvvadi *et al*., 2007).

Hex1 encode a conserved consensus PTS1 (Peroxisome Targeting Signal Type 1) at the C terminus, which could target to peroxisomes (Jedd *et al*., 2000). In *N.crassa* Woronin body biogenesis from peroxisomes was reported to occur at the hyphal apex, a process determined in part by apically biased *hex-1* gene expression (Tey *et al*., 2005). By performing long time-lapse sequences that encompass tip-growth and septation it was observed that at early time points apical Woronin bodies are associated with large peroxisomes, and these vesicles

generally move forward in a tip-directed manner. However, other Woronin bodies slow and begin to tumble at the cell cortex. These were observed undergoing fission events and they later achieved a stationary position at the cell cortex approximately at septation time points (Tey *et al*., 2005) This fission process of separating Woronin body from peroxisome was reported as Pex11 (peroxisomal biogenesis factor 11)-dependent in *A.oryzae* (Escaño *et al*., 2009).

Woronin sorting complex (WSC) is Woronin body specific four-pass transmembrane protein (Liu *et al*., 2008). Without WSC, Hex1 assemblies are observed in the matrix of apical peroxisome without associating with the matrix face of the peroxisome membrane, from which they can bud off to produce the WB organelle. Furthermore, these structures accumulated in the apical cell because they failed to associate with the cell cortex as they do in the wild-type strain. These results demonstrated that WSC is required for Woronin body biogenesis.

Leashin, a Woronin body tethering protein

Woronin bodies in most Pezizomycotina species are found in the vicinity of the septal pore at a distance of 100–200 nm (Momany *et al*., 2002; Maruyama *et al*., 2005), whereas in a small group of species that includes members of the genera *Neurospora* and *Sordaria*, Woronin bodies exhibit a delocalized pattern of cortex association (Ng *et al*., 2009) (Fig. 0-2).

Previously, optical trapping experiments in *Nectria haematococca* (Berns *et al*., 1992) where Woronin bodies were pulled away from the septum with laser tweezers but went back to their original position, suggested that there is an elastic

filament tether Woronin bodies to the septum. In *N. crassa*, Woronin body is associated with cell cortex instead of septal pore and *leashin* (*lah*) locus was originally identified to be required for the cell cortex association of Woronin bodies (Ng *et al*., 2009). This *leashin* locus consists of two adjacent genes *leashin1* (*lah1*) and *2* (*lah2*) (Fig. 0-3); LAH-1 functions as a tether that binds to Woronin bodies through its N-terminal region via WSC, while the C-terminal region of LAH-1 mediates the cell cortex association of Woronin bodies. In contrast, LAH-2 is localized to the hyphal tip and septum, and is not functionally related to Woronin bodies.

In species with Woronin bodies tethered to the septum, it is speculated that the *lah* gene locus expresses a single polypeptide with binding capacity to both Woronin bodies and the septum (Ng *et al.,* 2009; Beck *et al.,* 2013). Hex1 localization studies in *Aspergillus fumigatus* demonstrated that deleting the C-terminal region of LAH impairs the tethering of Woronin bodies to the septum (Beck *et al.,* 2013). However, the role of the LAH protein in septal plugging has not been characterized, and it is not known whether septal tethering by LAH protein alone is sufficient for Woronin body to prevent excessive loss of cytoplasm. Recently it was found that plugging of septal pore by Woronin body is reversible to regulate the hayphal heterogeneity (Bleichrodt *et al.,* 2012).

Septal pore associated proteins

A number of proteins were found to localize to the septal pore to execute different funcitons. The SO (SOFT) protein is a cytoplasmic protein that accumulates at the septal pore in response to various stresses, and it is required for efficient

plugging of septal pores (Fleissner *et al*., 2007; Maruyama *et al*., 2010), which localize at the septal pore upon hyphal wounding, and the NIMA kinase (Shen *et al*., 2014) that locates to the mature septa during interphase but removed during mitosis. Recently, a group of septal pore associated proteins controlling diverse aspects of septal organization and intercellular communication has been identified in *N.crassa* (Lai *et al*., 2012), they possess long, intrinsically disordered domains able to form aggregates at septal pores and loss-of-function of these proteins caused excessive septation, septal pore degeneration, and uncontrolled Woronin body activation.

Aspergillus oryzae: a target for study of Woronin body function

Aspergillus oryzae has been used in the manufacture of Japanese traditional fermented foods such as sake, *miso* and soy sauce, and commercial enzyme production (Kitamoto 2002). Genome sequencing and analysis of *A. oryzae* has been performed (Machida *et al.,* 2005). Disruption of *genes* encoding Ku70, Ku80 and LigD that play a role in nonhomologous chromosomal integration resulted in highly efficient gene-targeting in *A.oryzae* (Takahashi *et al.,* 2006; Mizutani *et al.,* 2008).Development of auxotrophic host transformation system (Jin *et al.,* 2004) has facilitated the genomic manipulation of *A. oryzae.* Visualization of nuclei (Maruyama *et al.,* 2001), vacuoles (Ohneda *et al.,* 2002), mitochondria (Mabashi *et al.,* 2006) and endoplasmic reticulum (Maruyama et al., 2006; Kimura *et al.,* 2010) has strengthened the research foundation of cell biology in *A. oryzae*.

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*

(Tsuchiya *et al.,* 1994). 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 was poorly understood at a cellular and molecular level. Previously, in our laboratory, microscopic analysis of hyphal lysis induced by hypotonic (hypo-osmotic) shock has provided a three-dimensional view of septal plugging by Woronin body in *A. oryzae* (Maruyama *et al*., 2005). Our laboratory microscopically 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) (Fig. 0-4) for quantitatively evaluating the Woronin body function to prevent the excessive loss of cytoplasm upon hyphal wounding.

Objective

In the filamentous fungus *A. oryzae*, our laboratory established 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 attempted to study on the tethering and septal plugging function of Woronin bodies by investigating the roles of *A. oryzae* LAH protein.

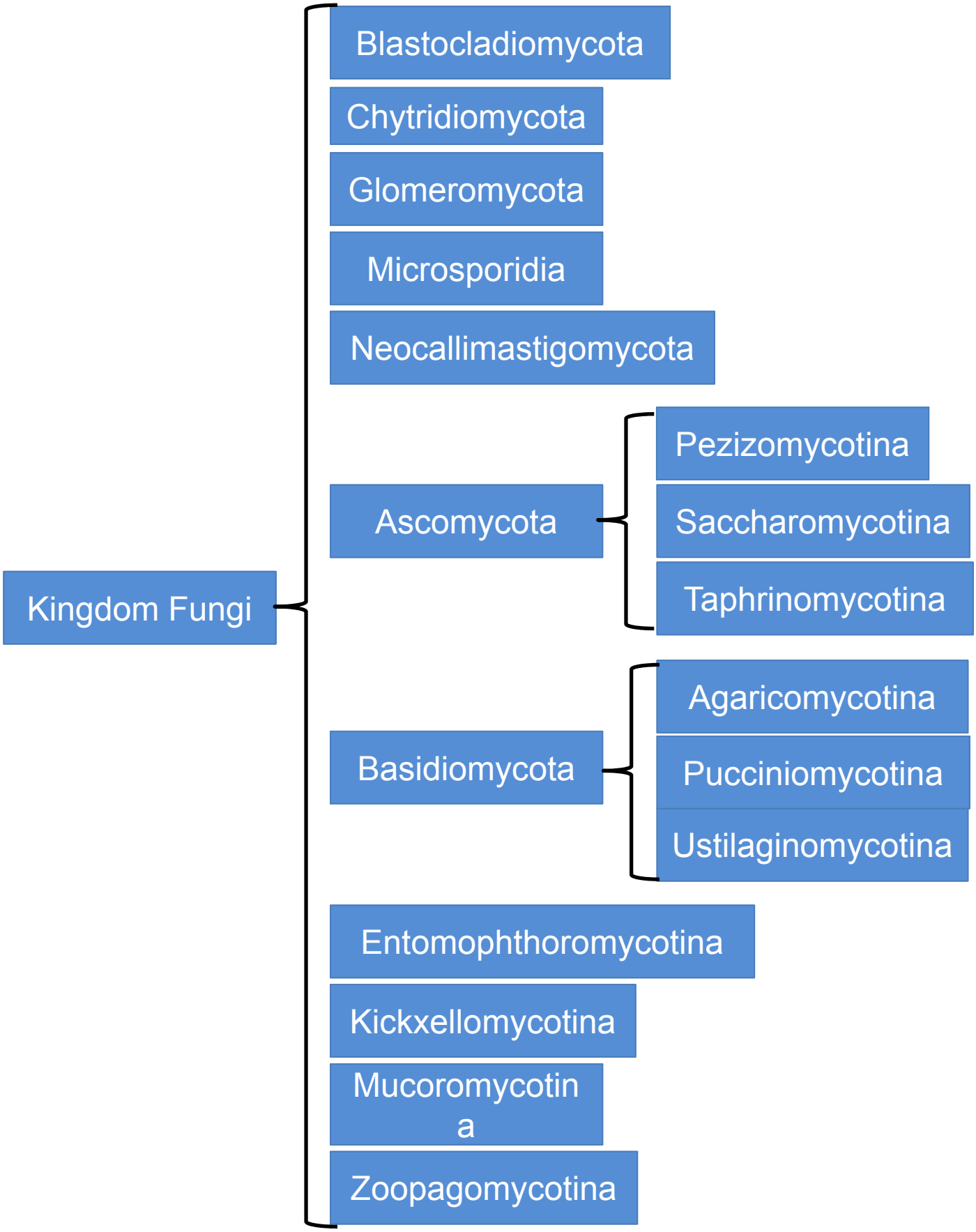


FIG. 0-1.Scientific classification of Kindom Fungi

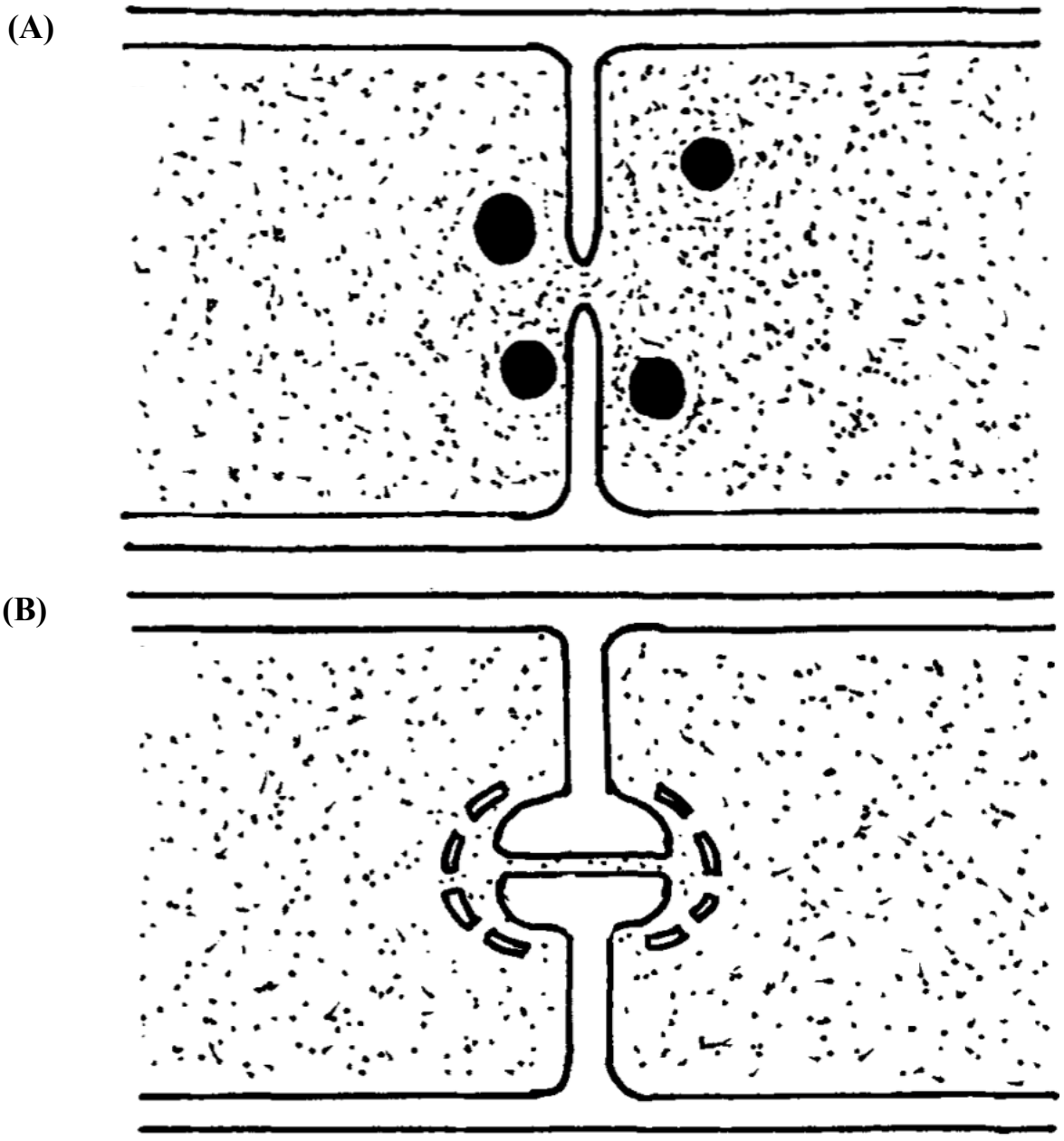


FIG. 0-2. Models illustrating septal structure in ascomycota (A) and basidiomycota(B).

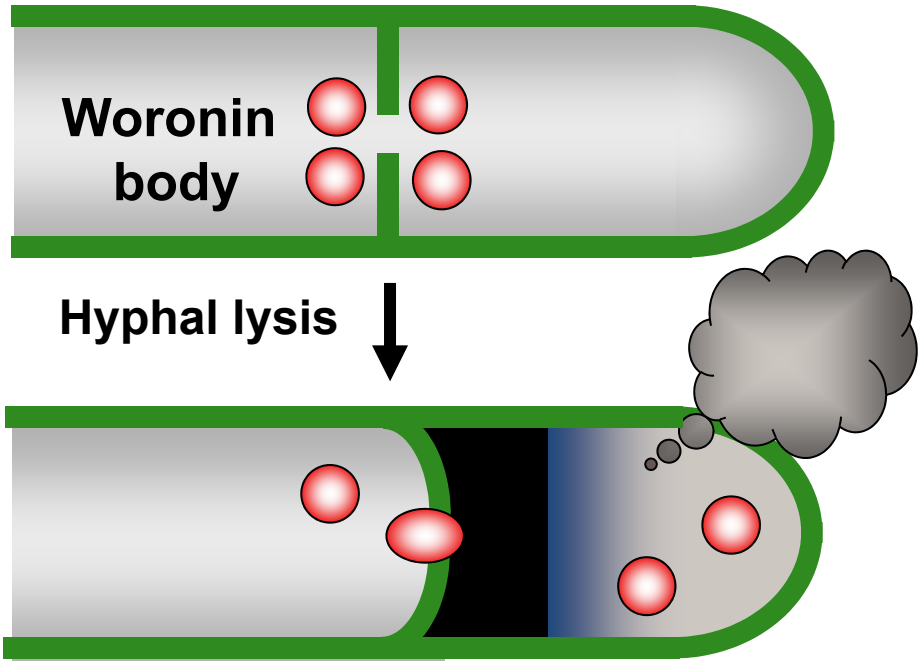


FIG. 0-3. Schematic model of septal plugging by Woronin body unpon hyphal wounding.

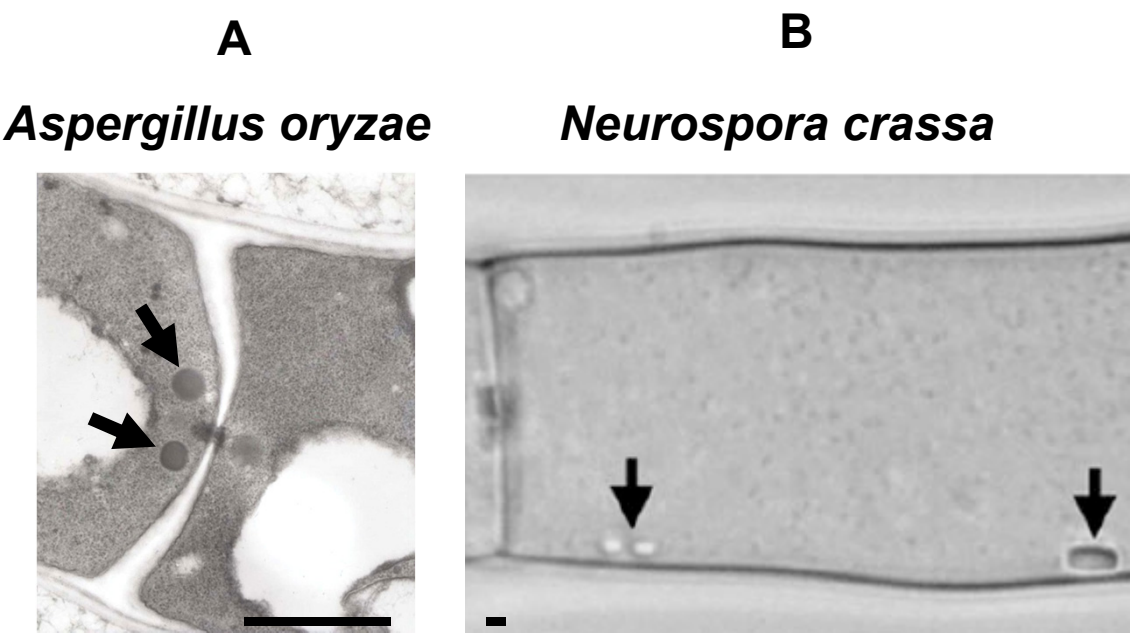


FIG. 0-4.(A) Transmission electron microscopic observation of Woronin bodies found near the septum in *Aspergillus oryzae*. (B) Differential Interference Contrast(DIC) image of Woronin body residing on cell cortex in *Neurospora crassa*. Woronin bodies are pointed by arrows. Bars:1μm

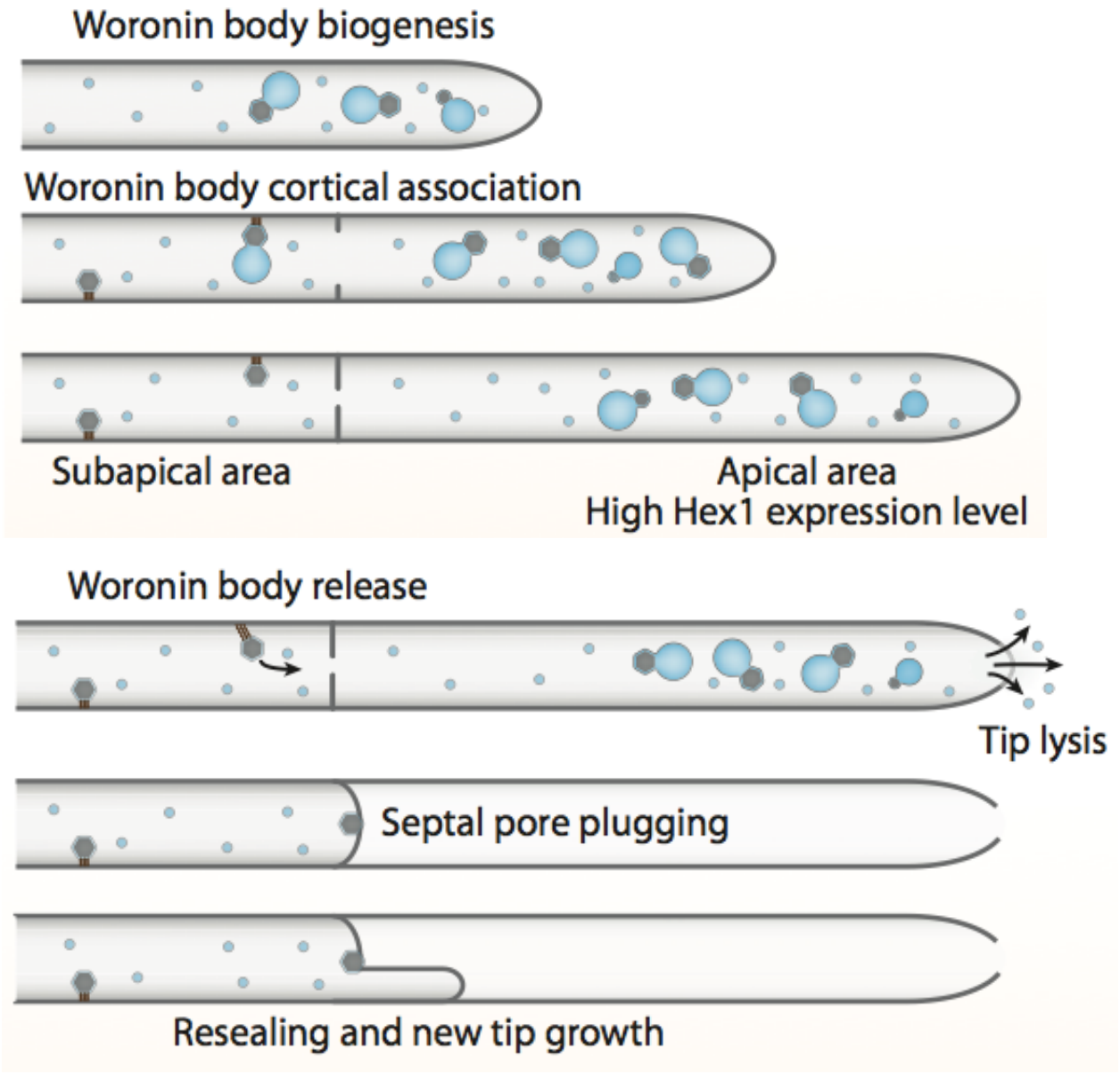


FIG. 0-5 Schematic model of Woronin body biogenesis in *N. crassa.*

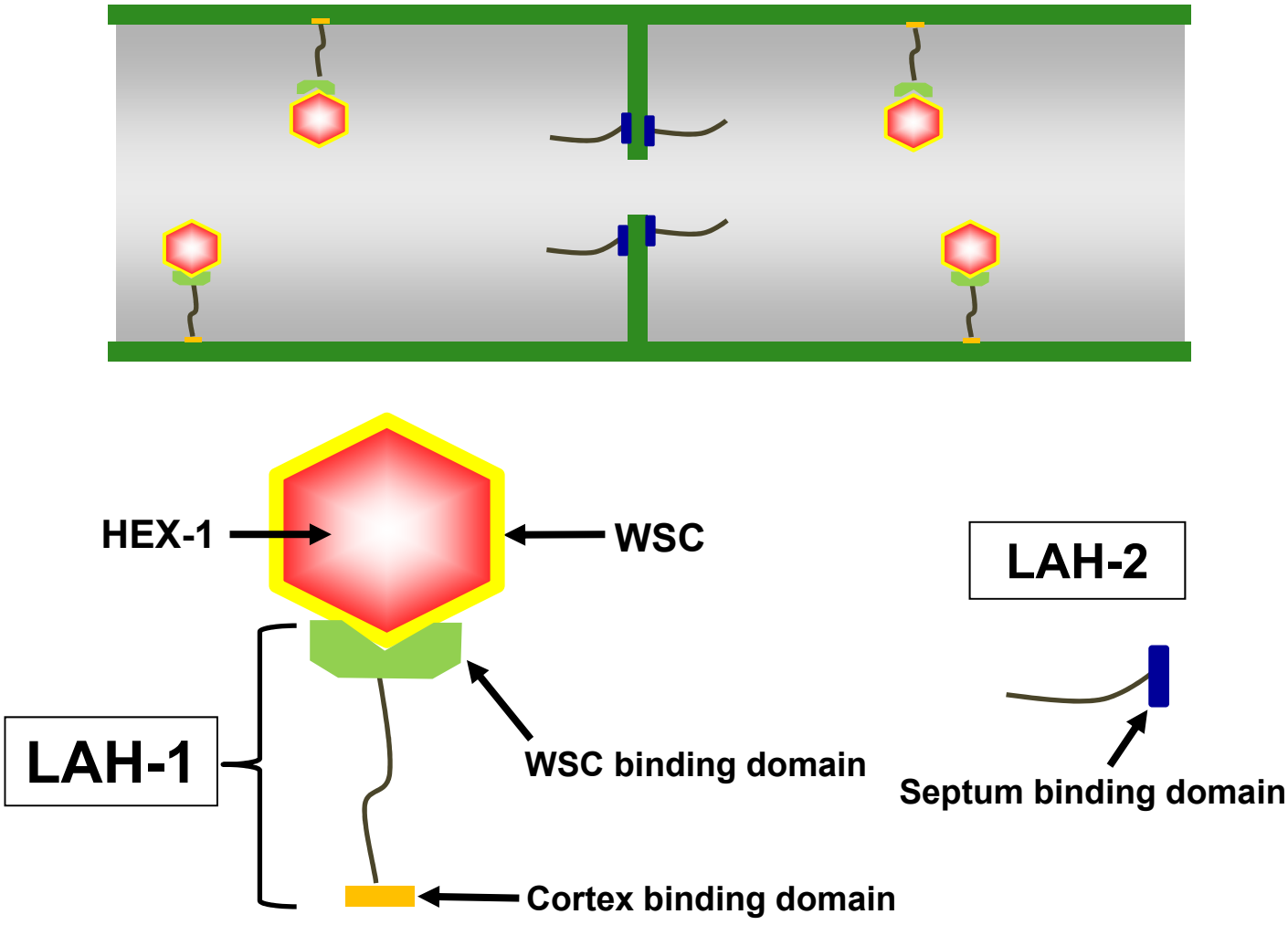


FIG. 0-6. Schematic model of Woronin body tethering in *Nuerosora crassa*.

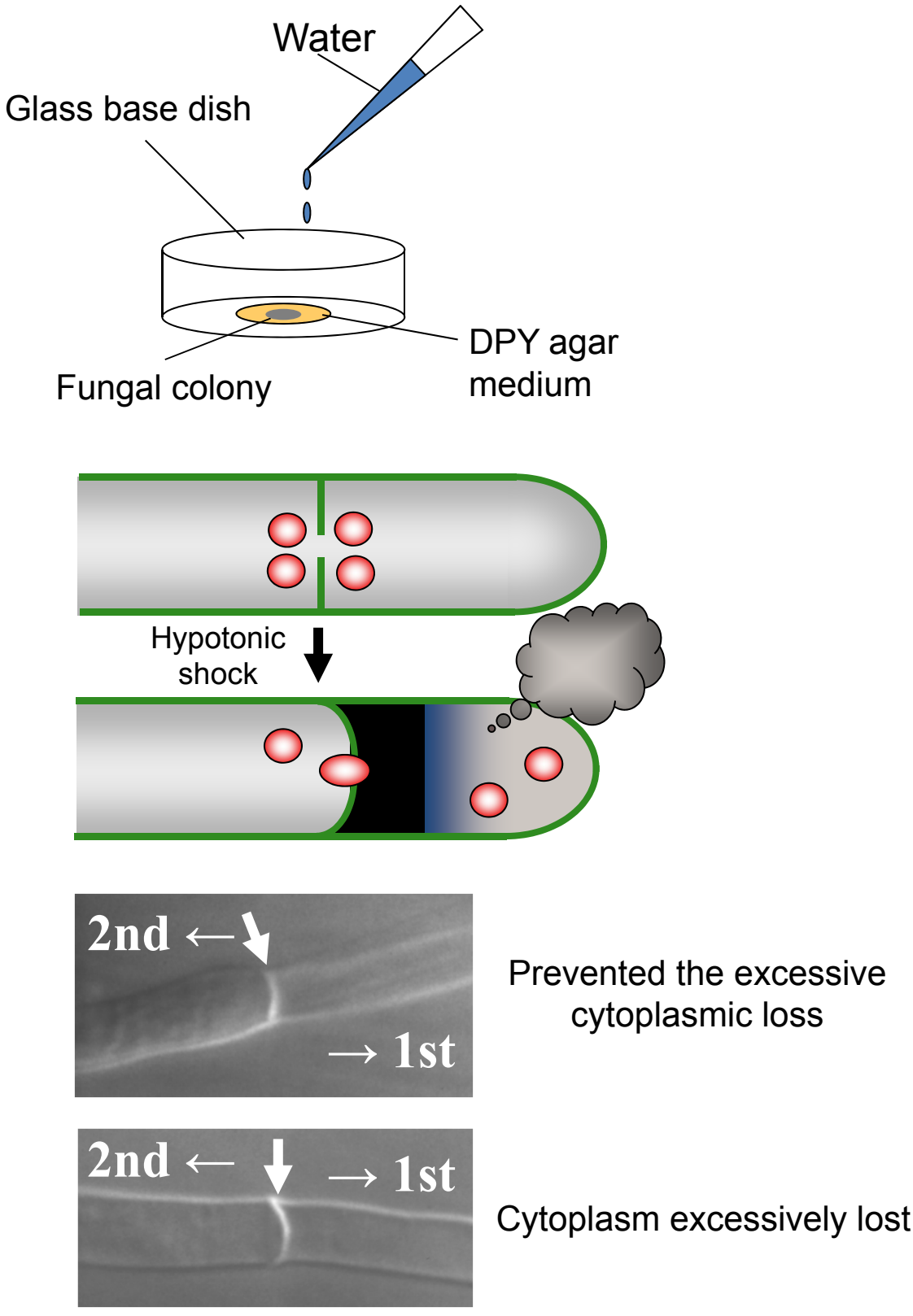


FIG. 0-7. Schematic model of hypotonic shock experiment. 1 ml water was added to *A. oryzae* colonies cultured on a thin layer of DPY agar medium at 30° C for 18 h. Randomly selected 50 hyphae showing hyphal tip bursting in each culture was observed by differential interference contrast (DIC) microscopy.

Chapter 1

Disruption of the *Aolah* gene and phenotypic analysis of the disruptant

Introduction

Septal pore of filamentous fungi allows the transport of cytoplasm and organelles between cells (Bracker and Butler, 1964; Freitag *et al*., 2004; Lew *et al*., 2005; Tey *et al*., 2005; Ng *et al*., 2009), which include risks of excessive cytoplasmic loss when hyphal wounding occurs. In *A. oryzae*, this risk was clearly demonstrated by exposing a colony grown on agar medium to hypotonic shock, where most of the hyphal tip burst and lose the cytoplasm (Maruyama *et al*., 2005).

Woronin body is a Pezizomycotina-specific organelle that functions to plug the septal pore in the event of hyphal damage and prevents excessive cytoplasmic loss from the cell adjacent to the lysed cell (Markham and Collinge, 1987). Woronin bodies are typically observed in close proximity to the septal pore (Momany *et al.*, 2002; Maruyama *et al.*, 2005), however, in a small group defined by *Neurospora crassa,* Woronin bodies are associated with cell cortex rather than the septum (Ng *et al.*, 2009).

Hex1 was identified as a major protein of the Woronin body in *N. crassa* (Jedd and Chua, 2000; Tenney *et al.*, 2000), and homologous proteins have been characterized in other species (Asiegbu *et al.*, 2004; Curach *et al.*, 2004; Soundararajan *et al.*, 2004; Maruyama *et al.*, 2005; Beck *et al.*, 2013; Son *et al.*, 2013; Tang *et al.*, 2014). Deletion of the *hex1* gene from *N. crassa* leads to absence of Woronin body at the cell cortex (Jedd et al., 2000). Electron microscopy analysis revealed that Woronin bodies were not found in the vicinity of the septal pore after deleting the *hex1* gene in other Pezizomycotina species including *A. oryzae* (Soundararajan *et al*., 2004; Maruyama *et al*., 2005; Son *et al*., 2013; Tang *et al*., 2014).

Presence of an elastic filament has been suggested between Woronin body and the

septum based on the fact that Woronin body recoils to the original positions after pulled away from the septum with laser tweezers (Berns *et al*., 1992). Although the machinery of Woronin body tethering had not been uncovered, LAH-1 was first identified as a protein that tether Woronin body to the cell cortex in *N. crassa* (Ng *et al*., 2009). The *lah* locus consists of two adjacent genes *lah1* and *lah2* (Fig. 0-3); LAH-1 N-terminal region binds to Woronin bodies via the Woronin body membrane protein WSC, while the C-terminal region of LAH-1 mediates the cell cortex association. In contrast, LAH-2 is localized to the septum, and is not functionally related to Woronin bodies. To examine the protoplasmic bleeding, *N. crassa* colony, which induces tip lysis, bled protoplasm from hyphal tip the visualized by phyloxin dye as red drop. and the volume of bleeds from colony was calculated. Δ*lah1* strain but not Δ*lah2* strain display a significant increase in hyphal tip lysis induced protoplasmic bleeding compared to the wild-type strain (Ng *et al*., 2009).

In contrast to the two LAH proteins in *N. crassa*, most of Pezizomycotina species possess a predicted LAH tether protein as a single polypeptide (Ng *et al.,* 2009; Beck *et al.,* 2013). During the preparation of this PhD thesis, it was reported that partial deletion of the C-terminal region of LAH leads to the absence of Hex1 protein localization near the septum in *Aspergillus fumigatus* (Beck *et al.,* 2013). However, no direct evidence was providing for the function of LAH protein in Woronin body tethering to the septum, and the role of the LAH protein in the process for septal plugging has not been characterized.

Here, in this chapter, I sought to disrupt the *lah* gene in *A. oryzae* for studying its roles in the tethering and septal plugging function of Woronin bodies. For comparison of the phenotypes, genes encoding the Woronin body matrix protein AoHex1 and

membrane protein AoWSC in *A. oryzae* was also disrupted in the same genetic background. Electron microscopic analysis was performed to directly prove the involvement of AoLAH in Woronin body tethering. Hypotonic shock experiment inducing hyphal wounding was performed to quantitatively evaluate Woronin body function in these disruptants.

Results

1. Identification of the *Aolah* gene

I searched the *A. oryzae* genome database (DOGAN: Database of the Genomes Analyzed at NITE [National Institute of Technology and Evaluation, Japan]; http://www.bio.nite.go.jp/dogan/Top) for a gene homologous to *N. crassa lah* genes. In this organism, a gene (AO090011000895) encoding a protein with similarity to *N. crassa* proteins LAH-1 and LAH-2 were found and designated as *Aolah*. The predicted amino acid sequence of AoLAH was aligned and compared with other *Aspergillus* species (*A. fumigatus* and *A. nidulans*) LAH proteins using the CLUSTALW (http://www.genome.jp/tools/clustalw/). Amino acid sequence data for LAH proteins of *A. fumigatus* (Afu2g08060) and *A. nidulans* (AN12480) were taken from the *Aspergillus* Genome Database (AspGD) (http://www.aspgd.org/). The *Aolah* was re-predicted to contain an additional 1,198 amino acids extending from the original predicted N-terminus by comparing with other *Aspergillus* LAH proteins (Fig. 1-1).

In contrast to the *N. crassa leashin* (*lah*) locus, which consists of two unidirectionally aligned genes for LAH-1 and LAH-2 (Ng *et al*., 2009), the *Aolah* gene was predicted to encode a single polypeptide of 5,727 amino acids (Fig. 1-2). The N-terminal region of AoLAH shared 12% identity with that of *N. crassa* LAH-1, whereas the C-terminal region displayed 26% identity with that of LAH-2, and few conserved sequences were found in the middle region of AoLAH (Fig. 1-3).

A. fumigatus and *A. nidulans lah* gene were also predicted to encode one polypeptide instead of two proteins. Sequence similarity comparisons of LAHs among Aspergilli were performed with Dotter software (Sonnhammer *et al*., 1995) (Fig. 1-3). When the residues of compared sequences match, a dot is plotted at the corresponding

position. According to dot plot analysis result, diagonal lines were seen in N- and C-terminal regions, while in middle regions, rectangular patterns were found. Diagonal lines revealed that the N- (~2000 amino acids) and C- (~1000 amino acids) terminal regions of AoLAH shared homologies with those of *A. fumigatus* and *A. nidulans* (Fig. 1-3). The AoLAH N-terminal region had 42% and 30% identities with those of the LAH proteins from *A. fumigatus* and *A. nidulans*, respectively, whereas the C-terminal region had 60% and 55% identities with the respective LAH proteins. However, the long-stretched middle region of AoLAH (~2700 amino acids) had low identities with the LAH proteins of *A. fumigatus* and *A. nidulans*. Rectangular patterns between the middle regions in dot-plot analysis (Fig. 1-3) indicate that several poly-lysine stretches are commonly present in these proteins (Fig. 1-4).

2. Disruption of the *Aolah* gene

To investigate the function of AoLAH, I sought to perform a deletion analysis of this gene. Fig. 1-5 shows a schematic model of the *Aolah* gene disruption in *A. oryzae.* The 1.5-kb upstream and downstream regions of *Aolah* gene were amplified and fused with the *adeA* marker gene (Jin *et al*., 2007). This disruption fragment was introduced into the *A. oryzae* NSRKu70-1-1 strain (Escaño *et al*., 2009), and the *Aolah* deletion strains were obtained by replacing the *Aolah* 17.4-kb open reading frame (ORF) with the *adeA* marker gene. Successful homologous recombination was confirmed by Southern blot analysis (Fig.1-5). All the disputants obtained showed nearly identical phenotypes, and a strain NSK-Δlah2 was used as the representative *Aolah* disruptant.

3. Electron microscopy of Woronin bodies in Δ*Aolah* strain

In *N. crassa* studies, Hex1 assemblies nucleate and associate with the matrix face of the peroxisomal membrane to produce budding intermediates (Brenner and Carrol *et al*., 1968; Wergin *et al*., 1973), and these structures associate with the cell cortex coincide with septation (Momany *et al*., 2002; Tey *et al*., 2005), which allows partitioning and inheritance of newly formed Woronin bodies to the basal region. The *lah* mutant accumulates Hex1 crystal assemblies in the apical region, which means that *lah* mutant is defective in Woronin body inheritance (Ng *et al.*, 2009).

To investigate how Woronin bodies located in the absence of AoLAH, I examined the localization of Woronin body in the *Aolah* disruptant (NSK-Δlah2) by transmission electron microscopy (TEM) (Fig. 1-6). The NSRKu70-1-1A strain that was constructed by introducing the *adeA* marker gene into NSRKu70-1-1 (Escaño *et al*., 2009) was used as a wild-type strain. In the wild-type strain, Woronin bodies were observed as electron-dense spherical structures (Fig. 1-6 left, arrows) located in close vicinity of the septum. In contrast, in the *Aolah* disruptant, no Woronin bodies were seen near the septum, only untethered Woronin bodies located away from the septum were found (Fig. 1-6 right, arrows). Based on these imaging findings, it was concluded that AoLAH is required for the tethering of Woronin bodies to the septum.

4. Identification and disruption of *Aowsc* gene

In *N. crassa*, LAH protein functions downstream of WSC: N-terminal region of LAH associate with Woronin bodies via the C-terminus of WSC (Ng *et al.*, 2009). Here, in this study a gene (AO090102000111) encoding a protein with identity to *N. crassa* WSC was found in *A. oryzae* genome database and named as *Aowsc*.

The predicted amino acid sequence of AoWSC was aligned and compared with WSC proteins by CLUSTALW. Amino acid sequence data of WSC proteins from *A. fumigatu*s and *A. nidulans* were taken from the *Aspergillus* Genome Database. AoWSC shared 50% identity with that of *N. crassa* WSC, 80% identity with *A. fumigatus* WSC and 71% identity with *A. nidulans* WSC. SMART (http://smart.embl-heidelberg.de/) was used for prediction of domains. Transmembrane region and Mpv17/PMP22 family domain were found conserved among the WSC proteins (Fig. 1-7).

To investigate the role of AoWSC in *A. oryzae*, I disrupted the *Aowsc* gene as the schematic model shows (Fig. 1-8). Disruption of the *Aowsc* gene was confirmed by Southern blot analysis (Fig. 1-8). All the disputants obtained showed nearly identical phenotypes and strain NSK-Δwsc1 was used as the representative *Aolah* disruptant.

5. Susceptibility of Δ*Aolah* and Δ*Aowsc* strains to cell wall destabilizing agents

Previously, in *A. fumigatus*, the deletion of *hexA* (*hex1* homolog) resulted in an increased sensitivity to stressors that affect the integrity of the cell wall and membrane such as SDS, the antifungal agent fludioxonil, the acyclic sesquiterpene alcohol farnesol, the cell wall perturbing agents Calcofluor White and Congo Red (Beck *et al*., 2013), probably due to a higher sensitivity to physical damage as a result of absence of Woronin bodies by *hexA* disruption.

In this study, *Aohex1* disruptant was also constructed with the same genetic background, and I treated the Δ*Aohex1,* Δ*Aolah* and Δ*Aowsc* strains with micafungin, a fungistatic against Aspergillus spp. (Chandrasekar and Sobel *,* 2006; Roncero and Duran *et al*. 1985). Δ*Aohex1* strain displayed the most severe sensitivity while Δ*Aolah* and Δ*Aowsc* were mildly sensitive to the agent (Fig. 1-9).

6. Ability to prevent the excessive loss of cytoplasm upon hyphal wounding in Δ*Aolah* and Δ*Aowsc* strains

Hyphal tip bursting can be induced by the hypotonic shock of *A. oryzae* colonies grown on agar medium (Fig. 0-7) (Maruyama *et al*., 2005; Escaño *et al*., 2009). Woronin body visualized by the AoHex1 fluorescent fusion protein was previously observed to plug the septal pore adjacent to the lysed apical cell upon hypotonic shock (Maruyama *et al*., 2005). Woronin body function was examined by the ability to prevent excessive cytoplasmic loss in the cells adjacent to the burst tip cell under differential interference microscopic (DIC) observation. In this assay, it was considered that the septal pore was effectively plugged by the Woronin bodies if the cytoplasm was retained by the cells, and conversely, the excessive loss of cytoplasm was attributed to Woronin body dysfunction. In previous reports (Maruyama *et al*., 2005), Δ*Aohex1* displayed increased cytoplasmic leakage from the hyphae due to the absence of Woronin bodies. As Woronin body was found at the septal pore adjacent to the lysed hyphal cell (Maruyama *et al*., 2005), such an assay could reveal the role of the *Aolah* and *Aowsc* genes, if any, in the function of the Woronin body.

In order to investigate this, hyphal tip bursting was induced by hypotonic shock in Δ*Aolah* and Δ*Aowsc* strains, by flooding the colony grown on agar medium with water. For comparison, *Aohex1* disruptant was also subjected to hypotonic shock experiment. A few minutes after adding water, the cytoplasmic constituents leaked out from the lysed apical compartment in the wild-type, Δ*Aohex1,* Δ *Aowsc,* Δ *Aolah* strains (Fig.1-10). Although 81% of the wild-type cells retained their cytoplasmic constituents upon hypotonic shock, only 63% of cells of the *Aolah* disruptant retained their

cytoplasmic constituents (Fig. 1-11). As expected, the *Aohex1* disruptant, which lacks Woronin bodies, showed a significantly impaired ability (14%) to prevent the excessive loss of cytoplasm (Fig. 1-10). The *Aowsc* disruptant showed a reduced ability (55%) to prevent the excessive loss of cytoplasm compared to wild-type cells. These data suggest that AoLAH and AoWSC is involved in Woronin body function to prevent the excessive loss of cytoplasm.

Discussion

The septal pore gating in the Pezizomycotina species depends mainly on a specific organelle named Woronin body, and this organelle plugs the septal pore as an emergency aid when hyphal wounding occurs. Study of the Woronin body positioning has been carried out in *N. crassa* and LAH-1 protein was firstly reported as a tethering protein to associate Woronin body with the cell cortex (Ng *et al.*, 2009). However, *N. crassa* is a special case since in most of Pezizomycotina species Woronin bodies are localized near the septum rather than cell cortex. A recent report in *A. fumigatus* revealed the absence of Hex1 localization near the septum when deleting the LAH C-terminal region (Beck *et al.*, 2013), however, not much information was obtained about the LAH protein and how it affects the septal plugging function of Woronin body.

In this Chapter, *Aolah* gene in *A. oryzae* was identified and disrupted, and the role of AoLAH protein in the tethering and septal plugging of Woronin bodies was investigated.

A gene homologous to *N. crassa lah* genes was found in *A. oryzae* genome database and predicted to encode a large single polypeptide (Fig. 1-2). Shared homologies between AoLAH and *N. crassa* LAH-1/LAH-2 were found (Fig. 1-2), although it was described that the *A. fumigatus* LAH shared no homology with *N. crassa* LAH-1 (Beck, *et al*., 2013). Sequence similarity comparisons of LAHs among Aspergilli (Fig. 1-3) revealed that the N- (~2000 amino acids) and C- (~1000 amino acids) terminal regions of AoLAH shared homologies with those of *A. fumigatus* and *A. nidulans* except that the long-stretched middle region of AoLAH (~2700 amino acids) was not conserved.

The *Aolah* disruptant was obtained, and electron microscopic analysis was performed to directly examine the involvement of AoLAH in Woronin body tethering. It was clearly found that no Woronin bodies were seen near the septum except for the untethered Woronin bodies located away (Fig. 1-6). This result firmly supported that AoLAH is necessary for Woronin body tethering to the septum.

In *A. fumigatus*, deletion of the *hex1* gene leads to increased sensitivity to the stressors that affect the integrity of the cell wall, but this was not found in *A. fumigatus lah* disruptant (Beck *et al*., 2013). Here, in this study, severe sensitivity of Δ*Aohex1* to the cell wall perturbing agent micafungin was observed, but Δ*Aolah* and Δ*Aowsc* also displayed a lower degree of sensitivity (Fig. 1-9). These results indicate that without Woronin body tethering to the septum may lead to a higher sensitivity to physical damage.

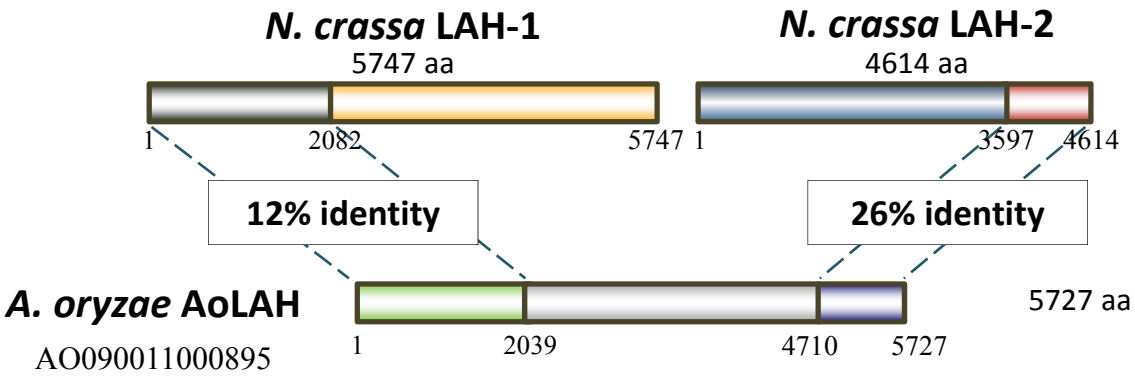
Moreover, the excessive loss of cytoplasm was examined in an *Aolah* disruptant upon hyphal wounding induced by the hypotonic shock (Maruyama *et al*., 2005). *Aohex1* gene disruption causes the loss of Woronin bodies and leads to a significant defect in the ability of cells to prevent the loss of cytoplasm (Fig. 1-11) (Maruyama *et al*., 2005). Disruption of the *Aolah* gene also reduced this protective ability, but to a lesser extent than that observed in the *Aohex1* gene disruptant (Fig. 1-11). This finding indicates that the tethering of Woronin bodies to the septum by AoLAH is important for their efficiency to plug the septal pore upon hyphal wounding, thereby preventing the excessive loss of cytoplasm.

Δ*Aowsc* strain also displayed more excessive loss of cytoplasm compared with wild-type strain (Fig. 1-11), but not as severe as in Δ*Aohex1* disruptant. It is possible because that Indicating without mature Woronin body there

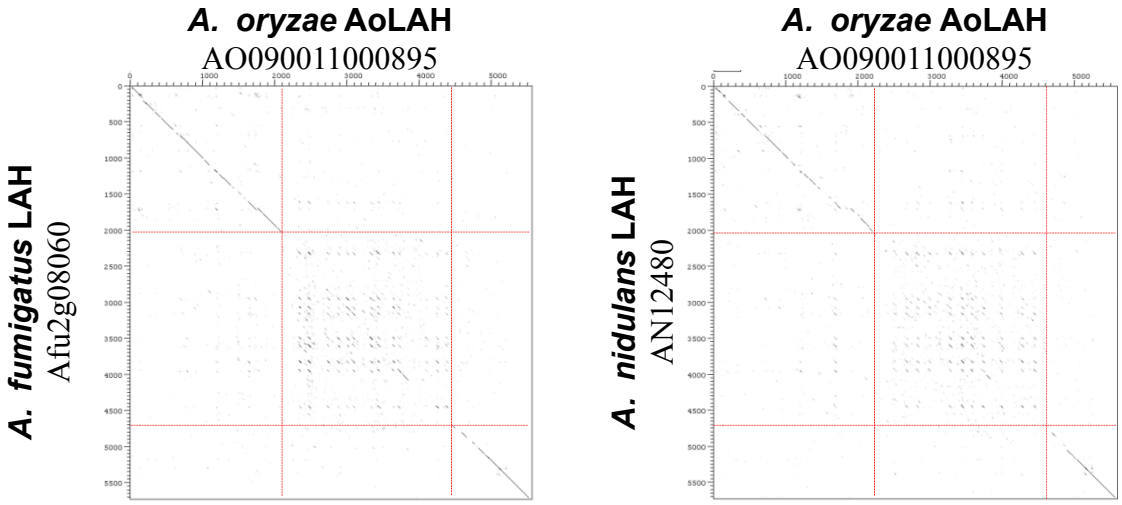
Taken together, these results in this Chapter indicate that AoLAH-mediated tethering of Woronin bodies to the septum is involved in the ability to prevent the excessive loss of cytoplasm upon hyphal wounding.

FIG. 1-1 Amino acid sequence alignment of N-terminal regions from *Aspergillus* LAH proteins. Based on comparison with other LAH proteins, the *Aolah* gene was re-predicted to contain additional 1,198 amino acids at N-terminus (indicated by an arrow) of the original ORF AO090011000895 from the *A. oryzae* genome database (DOGAN: Database of the Genomes Analyzed at NITE [National Institute of Technology and Evaluation, Japan]; http://www.bio.nite.go.jp/dogan/Top). Amino acid sequence data of LAH proteins from *A. fumigatus* (AfLAH) and *A. nidulans* (AnLAH) were taken from the *Aspergillus* Genome Database (AspGD; http://www.aspgd.org/).

(A)



(B)



	N-terminal vs N-terminal	Middle vs Middle	C-terminal vs C-terminal
A. oryzae vs ***A. fumigatus***	42%	16%	60%
A. oryzae vs ***A. nidulans***	30%	14%	55%

FIG. 1-2. Comparison between AoLAH and LAH proteins from other fungal species (*Neurospora crassa*, *Aspergillus fumigatus*, and *Aspergillus nidulans*). **(A) Identities between AoLAH and *N. crassa* LAH proteins**. ClustalW program (http://www.genome.jp/tools/clustalw/) was used for the sequence identity analysis.(B) **Dot plot analysis and identities between AoLAH (vertical axis) and LAH proteins from *A. fumigatus* and *A. nidulans*** (horizontal axis). Amino acid sequence data of LAH proteins from *A. fumigatus* and *A. nidulans* were taken from the *Aspergillus* Genome Database (AspGD; http://www.aspgd.org/). Sequence similarity comparisons were performed with Dotter software (33). When the residues of compared sequences match, a dot is plotted at the corresponding position. Note that diagonal lines are seen in N- and C-terminal regions while in middle regions rectangular patterns were found. Scale is in amino acid residues. Red lines indicate borders between the N-terminal, middle, and C-terminal regions.

A. oryzae AoLAH	*A. fumigatus* LAH	*A. nidulans* LAH
2316 KKKKKDKKKK 2325	2345 KKKKKKNKKK 2354	2670 SKKAKKKKKK 2679
2945 KKGKKKKKNR 2954	2473 KKSKKKKKKK 2482	2823 KASKKKKKNK 2832
3067 KKNKKKKKKK 3076	2738 KKNKKKNKRK 2747	2988 KKSKKKNKKK 2997
3155 KKKTKKEQKK 3164	2874 KKKAKKKKNR 2883	3272 KNAKKKKKKK 3281
3491 KKDKKKKKKQ 3500	3003 KKSKKNKKKK 3012	3445 KKKNKKKKKK 3454
3593 KKKAKKDKKK 3602	3089 KKTKKEKKKK 3098	3575 KKKVKKDKKK 3584
3819 KDKKKKKKRK 3828	3330 KKKDKKKKKK 3339	3734 KKRAKKEKKR 3743
3942 KKAKKKERKR 3951	3438 KKKAKKDKKK 3447	4273 KKKNKKAKKQ 4282
4443 KKQKKKAKKQ 4452	3642 KSKKNKKKKK 3651	4443 SKKEKKKLKK 4452
	3718 KKKAKKKDKK 3727	
	4246 SKKKSKKAKK 4255	

FIG. 1 -3. Amino acid sequences of poly-lysine regions in the middle region of LAH proteins.

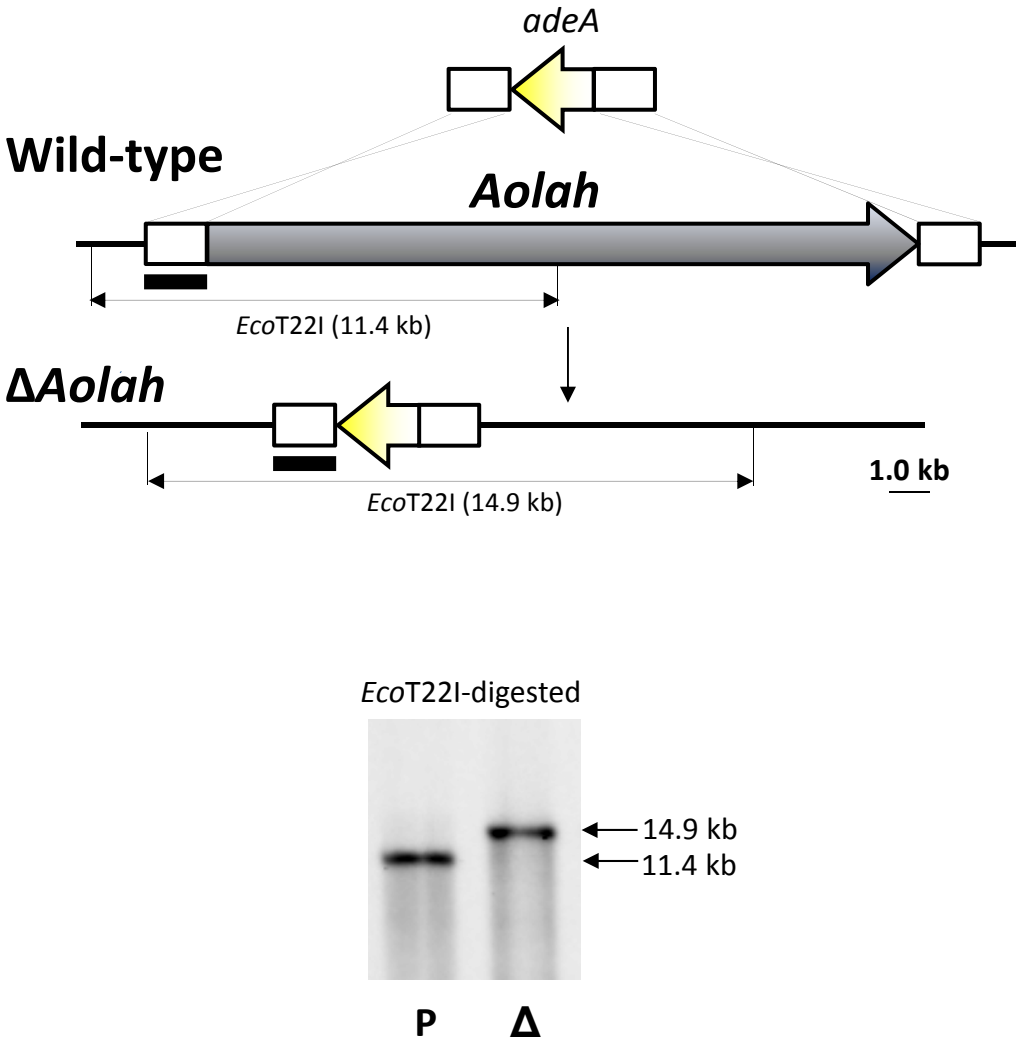


FIG. 1-4. Schematic model of the *Aolah* gene disruption and Southern blot analysis of the *Aolah* disruptant. Genomic DNAs of the parent strain (NSRKu70-1-1; P) and *Aolah* disruptant (NSK-Δlah2; Δ) were digested with *Eco*T22I, and then subjected to Southern blot analysis. Filled bars indicate the used probe.

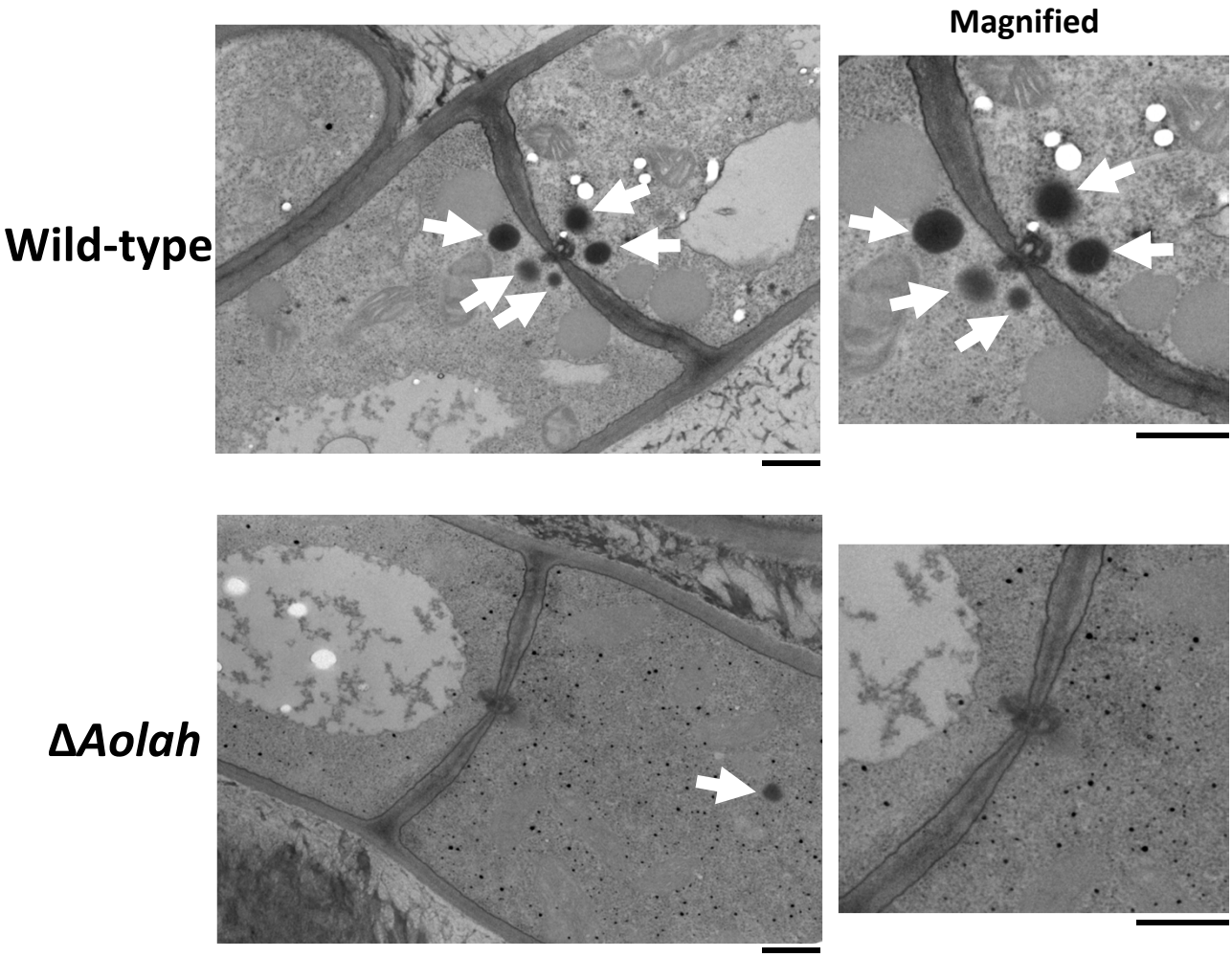


FIG. 1-5. Transmission electron microscopic images of the wild-type *A. oryzae* and *Aolah* disruptant strains. Images around the septal pore were magnified. Arrows indicate Woronin bodies. Bars: 500 nm.

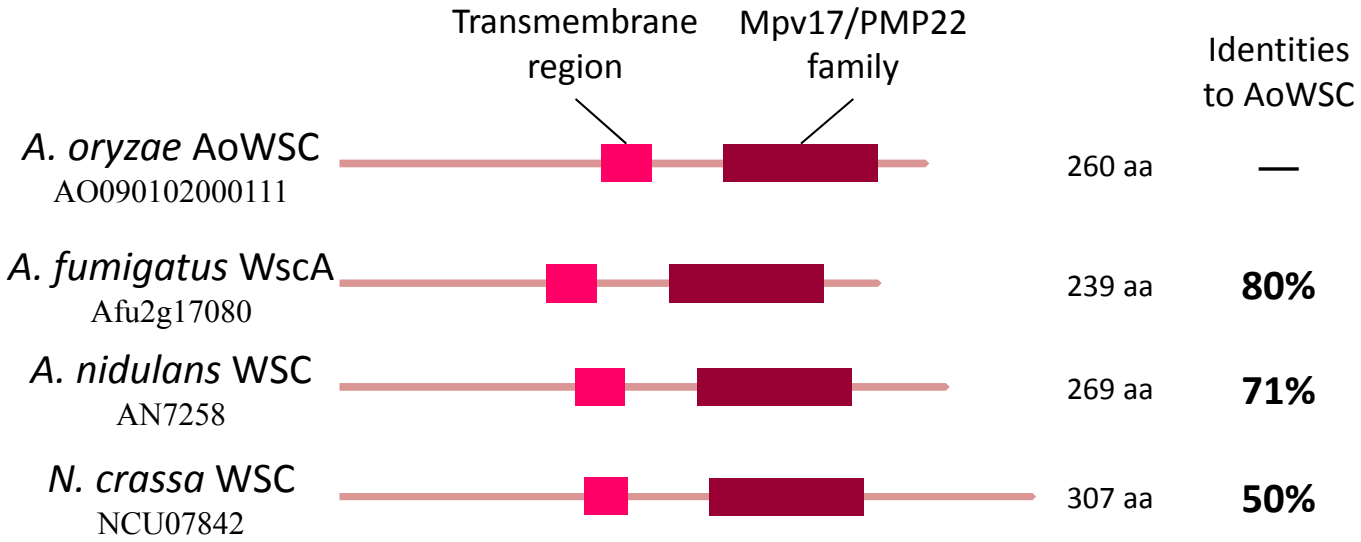


FIG. 1-6. Domain prediction of WSC proteins. Amino acid sequence data of WSC proteins from *A. fumigatus* and *A. nidulans* were taken from the *Aspergillus* Genome Database (AspGD; http://www.aspgd.org/). SMART (http://smart.embl-heidelberg.de/) was used for prediction of domains. ClustalW program (http://www.genome.jp/tools/clustalw/) was used for the sequence identity analysis.

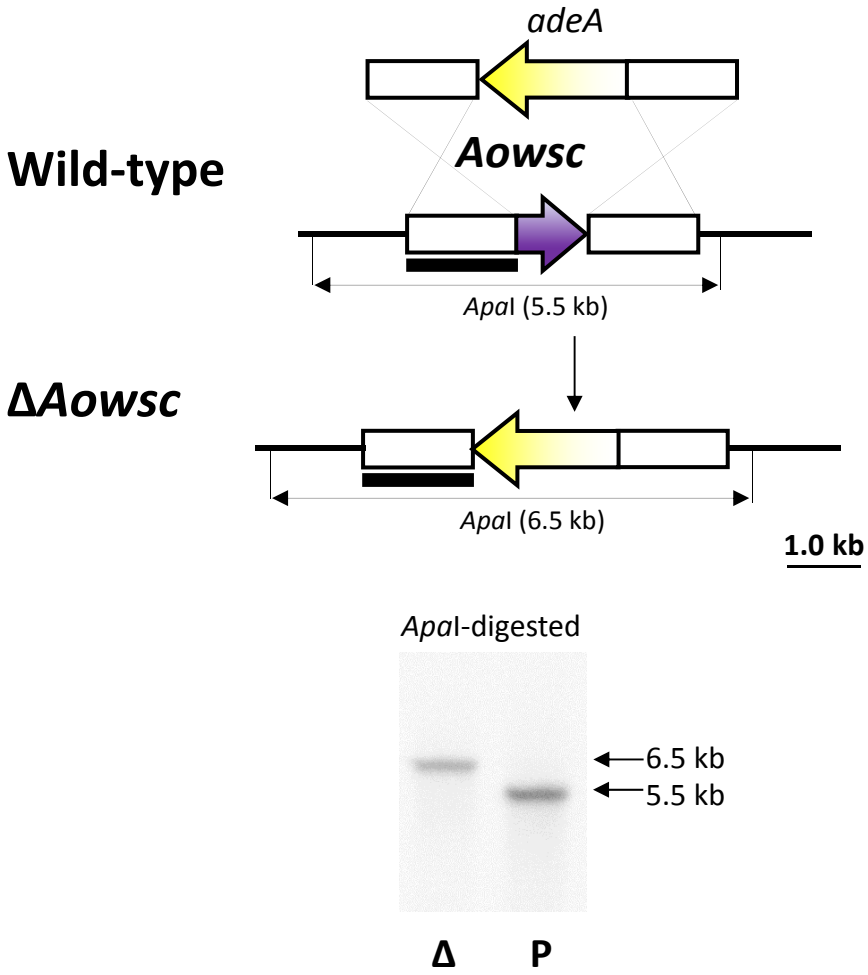


FIG. 1-7. Southern blot analysis of the *Aowsc* disruptant. Genomic DNAs of the parent strain (NSRKu70-1-1; P) and *Aowsc* disruptant (NSK-Δwsc1; Δ) were digested with *Apa*I, and then subjected to Southern blot analysis (lower). Filled bars indicate the used probe.

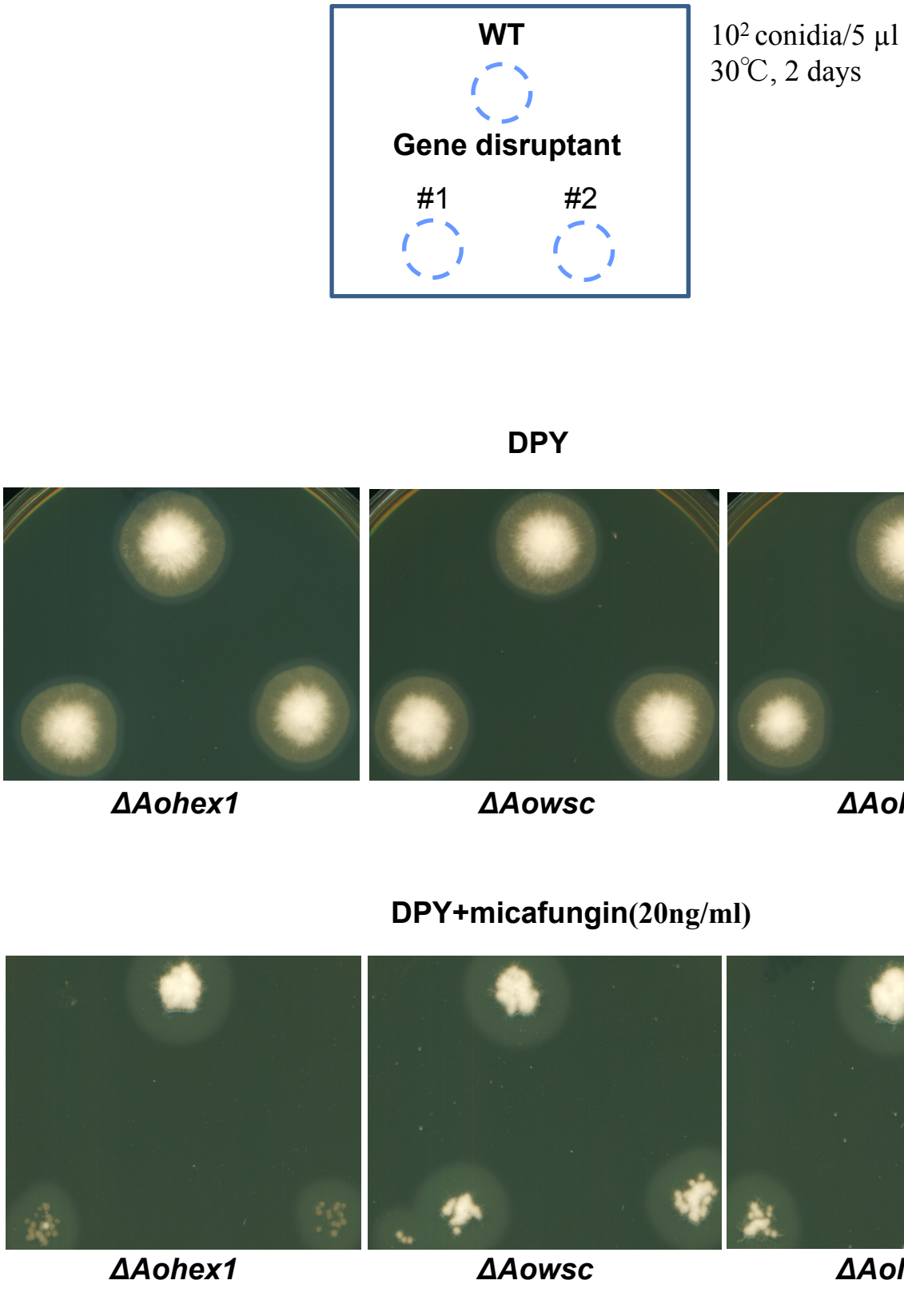


FIG. 1-8. Δ*Aolah* **strains were slightly sensitive to Micafungin.** 10^2 conidia/5 µl 30 ℃, 2 days

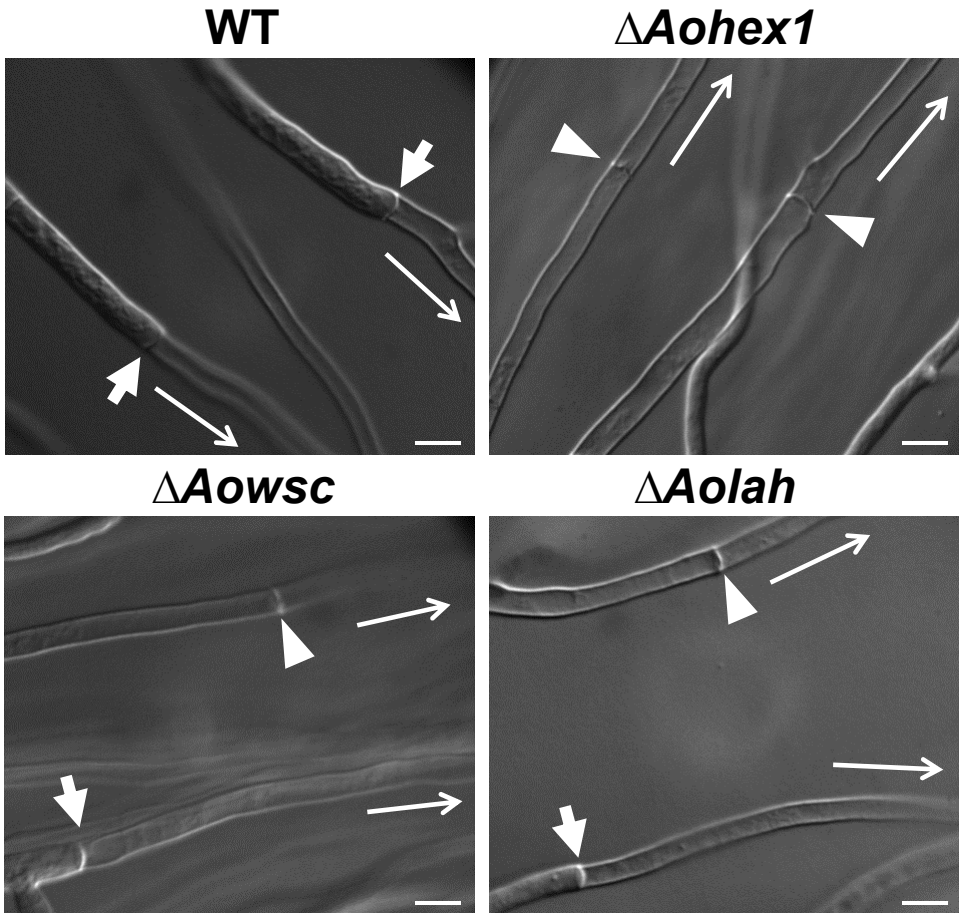


FIG. 1-9. Ability of Aolah disruptant cells to prevent the excessive loss of cytoplasm upon hyphal wounding. **(A)** Differential interference contrast images of wounded hyphae capable/incapable of preventing the excessive loss of cytoplasm from the second cell upon hyphal tip bursting. A colony grown on agar medium was subjected to hypotonic shock by flooding with water to induce hyphal tip bursting, and the first septum adjacent to the burst tip cell was observed by differential interference contrast microscopy. Arrows indicate the first septa in the hyphae preventing the excessive loss of cytoplasm, and arrowheads show the septa in the hyphae not preventing excessive cytoplasmic loss. Long arrows indicate the directions towards the burst hyphal tips. Bars: 10 μm.

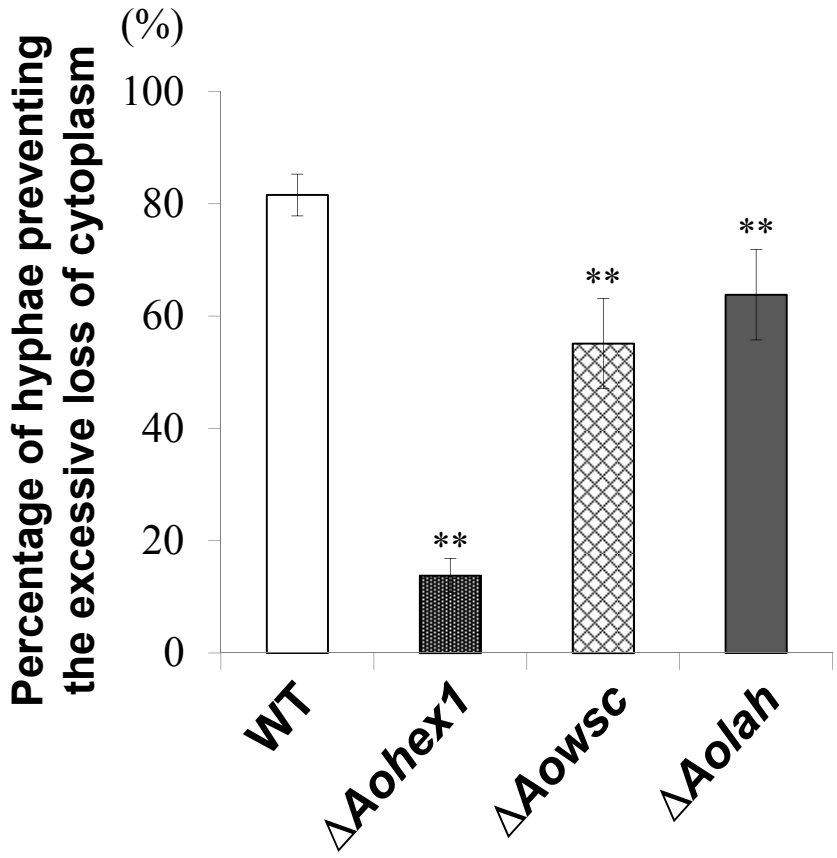


FIG. 1-10. Ability of Aolah disruptant cells to prevent the excessive loss of cytoplasm upon hyphal wounding. **(B)** Assay of the ability of cells to prevent the excessive loss of cytoplasm upon hyphal wounding. The wild-type strain and disruptants of the Aohex1, Aowsc, and Aolah genes were subjected to hypotonic shock, and hyphae with cells adjacent to the burst tip cell that retained the cytoplasm were counted by differential interference contrast microscopy. Average percentages of hyphae preventing the excessive loss of cytoplasm are shown in the graph. Fifty randomly selected hyphae with burst hyphal tips were observed for each culture. The error bars indicate standard deviations. **, p <0.01 (One-way ANOVA, Dunnett's post hoc test. Comparison with the wild-type strain. n=9).

Chapter 2

Localization analysis of AoLAH N-terminal and C-terminal regions

Introduction

AoLAH is required for the tethering of Woronin bodies to the septum based on the fact that after deleting *Aolah* gene Woronin bodies were absent near the septal pore (Chapter 1, Fig. 1-6). Moreover, *Aolah* disruptant exhibited reduced ability to prevent the excessive loss of cytoplasm, confirming the involvement of AoLAH with Woronin body plugging (Chapter 1, Fig. 1-10). AoLAH was predicted to be a large protein consisting of 5,727 amino acid residues. According to the homology comparison with other LAHs in Chapter 1, it consists of conserved N- and C-terminal regions and a non-conserved middle region (Fig. 1-3). To analyze the contribution of individual regions of AoLAH to Woronin body function, I decided to divide the AoLAH protein into N-terminal (1-2039), middle (2040-4709), and C-terminal (4710-5727) regions based on the results of dot plot analysis (Fig. 1-3).

In *Neurospora crassa*, Woronin body biogenesis occurs via a budding process from peroxisome in the apical cell (Fig. 0-5). Subsequently, Woronin bodies associate to the cell cortex by LAH, leaving Woronin bodies to the sub-apical cell. It was reported that in Δ*lah1* strain, free Woronin bodies accumulate in the apical compartment but are not supplied to the sub-apical compartment due to the defect in cell cortex association (Ng *et al.*, 2009). The *lah* gene locus consists of two adjacent genes *lah1* and *lah2*. By fusion with fluorescent proteins, N-terminal region of LAH-1 was found to co-localize with WSC on the Woronin body surface. However, when the WSC C-terminus was deleted, the localization pattern of N-terminal region of LAH-1 was abolished, and Woronin bodies were not supplied to the sub-apical cell. These results support the conclusion that N-terminal region of

LAH-1 associates with Woronin body via WSC C-terminus (Ng *et al*., 2009). *N. crassa* LAH-2 does not localized to the Woronin body, but it is found at the septal pore and hyphal tip region.

The LAH protein in *A. fumigatus* was predicted to consist of 5538 amino acids. When the N-terminal 1,100 amino acids of LAH was fused to GFP, the fusion protein displayed Woronin body-like localization near the septum (Beck *et al*., 2013). Meanwhile, the C-terminal region (approximately 1000 amino acids) of *A. fumigatus* LAH fused with GFP was targeted to the rim of septal pore. When the C-terminal region of *A. fumigatus* LAH was deleted, localization of Woronin body protein Hex1 was found in the cytoplasm but not near the septa (Beck *et al*., 2013).

The N-terminal region of AoLAH (AoLAH[1-2039]) showed homology to LAH-1, and the C-terminal region of AoLAH shares similarity with *N. crassa* LAH-2 (Fig. 1-3). In order to investigate the function of AoLAH N- and C-terminal, their localization were analyzed by EGFP fusion in this chapter.

Results

1. Localization of full-length AoLAH under native promoter

Transmission electron microscopic analysis in Chapter 1 revealed the absence of Woronin body near the septal pore when *Aolah* gene is disrupted. To further analyze the AoLAH protein, I examined the localization of this tethering protein under its native promoter. *Aolah-egfp* fusion gene was constructed as the schematic model shows (Fig. 2-1): The 1.5-kb C-terminal and downstream regions of *Aolah* gene were amplified and fused with the *adeA* marker gene. This fragment was introduced into the *A. oryzae* NSRKu70-1-1 strain (Escaño *et al*., 2009). AoLAH-EGFP expressing strains were obtained and successful homologous recombination was confirmed by Southern blot analysis (Fig. 2-1). All the transformants obtained showed nearly identical phenotypes and displayed similar growth as the control strain (data not shown). It was observed that the AoLAH-EGFP localized closely to the septal pore, confirming the function of AoLAH as a tether for Woronin body to the septal pore (Fig.2-2).

2. Localization analysis of AoLAH N-terminal region

In *N. crassa*, the tethering protein LAH-1 binds and tethers Woronin bodies to the cell cortex (Ng *et al.*, 2009). The N-terminal region of AoLAH (AoLAH[1-2039]) showed homology to LAH-1 (Fig. 1-2). To analyze the role of AoLAH N-terminal region in the association with Woronin body, I performed localization analysis of AoLAH N-terminal region by visualizing AoLAH[1-2039]-EGFP fusion protein under confocal fluorescence microscopy.

2.1 Construction of the fusion protein AoLAH[1-2039]-EGFP

In order to visualize the AoLAH[1-2039]-EGFP fusion in the hyphae, a cassette containing the *Aolah[1-2039]-egfp* fusion gene was constructed under the control of *amyB* promoter (pgAoLAH[1-2039]G) (Fig. 2-3). A study has shown that the *amyB* promoter is induced at an intermediate level of expression using glucose as carbon source (Tada *et al*., 1991). The expression plasmid was constucted (See Materials and methods for details). The generated plasmid pgAoLAH[1-2039]G was introduced into the wild-type strain (NSRKu70-1-1A) (Escaño *et al*., 2009), *Aolah* disruptant (NSK-Δlah2) and *Aowsc* disruptant (NSK-Δwsc1). The positive transformants were selected on M agar medium. The resulting transformants were named NSK-LAH[1-2039]G, NSK-Δlah-LAH[1-2039]G and NSK-Δwsc-LAH[1-2039]G, respectively. Strains expressing AoLAH[1-2039]-EGFP were grown for 18-24 hr at 30°C on glass bottom dish with CD+0.0015% methionine liquid medium using glucose as carbon source and observed under the confocal microscope (Fig.2- for schematic model).

2.2 Localization of AoLAH N-terminal region in wild-type strain

In the wild-type strain NSK-LAH[1-2039]G, the fluorescence of AoLAH[1-2039]-EGFP was intensely localized at both sides of the septum with a certain distance in between, and was also observed as punctate structures in the cytoplasm (Fig. 2-4 upper). The localization pattern is reminiscent of the localization of Woronin bodies as shown by transmission electron microscopy (Fig. 1-6).

To determine whether the N-terminal region of AoLAH was associated with Woronin bodies, I induced hyphal tip bursting by hypotonic shock to observe whether AoLAH[1-2039]-EGFP move together with Woronin bodies to the septal pore upon hyphal wounding. Upon hyphal tip bursting, the fluorescence of AoLAH[1-2039]-EGFP was detected at the septal pore (Fig. 2-4 lower). This localization behavior is similar to that observed during septal plugging by Woronin bodies, as previously demonstrated by the expression of AoHex1 fused with a fluorescent protein (Maruyama *et al*., 2005).

2.3 Co-localization of AoLAH N-terminal region and peroxisome

Hex1 protein was used to represent Woronin body by tagged with fluorescent proteins (Escaño *et al*., 2009; Beck *et al*., 2013); however, there was defect of using it as a Woronin body marker. Woronin bodies differentiate from the peroxisome via a budding process (Tey *et al*., 2005; Liu *et al*., 2008; Escaño *et al*., 2009). In previous reports for *A. oryzae* and *A. fumigatus*, Hex1 protein fused with a fluorescent protein

was found to localize near the septum, but the fusion protein often co-localized or associated with peroxisomes (Escaño *et al*., 2009; Beck *et al*., 2013), demonstrating that its localization does not always correspond to Woronin bodies. Since AoLAH[1-2039]-EGFP displayed a similar localization pattern as Woronin bodies at normal condition and upon hyphal wounding, it might be a more reliable marker for the Woronin body.

To examine whether the localization of AoLAH[1-2039]-EGFP was independent of peroxisomes, mDsRed fused with peroxisomal targeting signal 1 (PTS1) was co-expressed with AoLAH[1-2039]-EGFP in *A. oryzae*. A DNA fragment for mDsRed fused with PTS1 was amplified and the expression plasmid was constructed (Fig. 2-5) (See Materials and methods for details). The generated plasmid was named pgDPTS1N and introduced into the strain NSK-LAH[1-2039]G. One of the resulting transformants was named NSK-LAH[1-2039]G-DPTS1, and it was used in experiments for the simultaneous visualization of Woronin bodies and peroxisomes.

Fluorescence microscopic analysis revealed that AoLAH[1-2039]-EGFP was localized near the septum and in the cytoplasm independently of peroxisomes (Fig. 2-6). It was reported in *N. crassa* that hyphal apical cells contain undifferentiated Woronin bodies that associate with peroxisomes (Tey *et al*., 2005). Here, the peripheral association of AoLAH[1-2039]-EGFP with peroxisomes was also observed, in addition to numerous fluorescent punctate structures in the cytoplasm (Fig. 2-6). These findings demonstrated that AoLAH[1-2039]-EGFP was localized independently of peroxisomes, with the exception of the observed association in apical cells, which likely represented actively budding Woronin bodies.

2.4 Co-localization of AoLAH N-terminal region and AoWSC

In *N. crassa*, LAH-1 was reported to binds to Woronin bodies via WSC (Ng *et al*., 2009). As the N-terminal region of AoLAH (AoLAH[1-2039]) showed homology to LAH-1, AoWSC fused with mDsRed was co-expressed with AoLAH[1-2039]-EGFP in *A. oryzae* to investigate the spatial relation of AoLAH and AoWSC.

The predicted amino acid sequence of AoWSC was aligned and compared with other *Aspergillus* WSC proteins by CLUSTALW (Fig. 2-7). According to the comparison and mRNA analysis, AoWSC contained an additional 1,198 amino acids extending from the original predicted C-terminus. A cassette containing a fusion gene for the AoWSC-mDsRed was constructed under the control of *amyB* promoter. The expression plasmid was constructed (See Materials and methods for details). The generated plasmid pgAoWSCD was introduced into the strain NSK-LAH[1-2039]G. One of the resulting transformants was named NSK-LAH[1-2039]G-WSCD and was used in the subsequent experiments.

As shown in fluorescence microscopy, AoWSC-mDsRed was localized at both sides of the septum and also observed as dot-like structure in the cytoplasm, which is co-localized with AoLAH[1-2039]-EGFP (Fig. 2-7).

2.5 Localization of AoLAH N-terminal region in Δ*Aowsc* strain

Since the AoLAH N-terminal region co-localized with AoWSC and *N. crassa*, LAH-1 was reported to associate with Woronin bodies via WSC (Ng *et al*., 2009), I further investigated whether the localization patterns of the AoLAH N-terminal region were dependent on AoWSC. AoLAH[1-2039]-EGFP was expressed in Δ*Aowsc* strain.

In the *Aowsc* disruptant, no intense localization of AoLAH[1-2039]-EGFP was detected near the septum; rather, the fusion protein was completely dispersed in the cytoplasm, and no punctate structures were observed (Fig. 2-8). This result suggested that the recruitment of the AoLAH N-terminal region to Woronin bodies is dependent on AoWSC, a finding that is in agreement with the report of *N. crassa* (Ng *et al*., 2009).

2.6 Localization of AoLAH N-terminal region in Δ*Aolah* strain

If AoLAH[1-2039]-EGFP localization represents the septal tethering of Woronin bodies, such localization would not occur in the *Aolah*-disruptant background. To examine this, AoLAH[1-2039]-EGFP was expressed in the Δ*Aolah* strain.

As expected, no fluorescence was observed near the septum when AoLAH[1-2039]-EGFP was expressed in the *Aolah* disruptant, although punctate structures were found in the cytoplasm (Fig. 2-9). Time-lapse tracking revealed that the punctate structures found in the cytoplasm randomly moved in the wild-type and *Aolah* disruptant strains (data not shown), indicating that they were not attached to the septum. This result demonstrated that the presence of endogenous AoLAH protein is required for the localization of AoLAH[1-2039]-EGFP near the septum similarly to Woronin body. As the AoLAH N-terminal region exhibited localization patterns typical to those of Woronin bodies, the AoLAH[1-2039]-EGFP fusion protein was used as a marker of Woronin bodies in subsequent experiments.

3. Localization analysis of AoLAH C-terminal region

The C-terminal conserved region of AoLAH shares a similarity with *N. crassa* LAH-2 (Fig. 1-2), which is closely associated with the septal pore (Ng *et al*., 2009). Woronin body localized at the cell cortex in *N. crassa*, while in *A.oryzae* it is tethered at the septal pore. To examine the role of AoLAH C-terminal region in Woronin body localization at the septal pore, AoLAH C-terminal EGFP (AoLAH[4710-5727]-EGFP) fusion protein was constructed and expressed in the wild-type and Δ*Aolah* strains.

3.1 Construction of the fusion protein AoLAH[4710-5727]-EGFP

In order to visualize the AoLAH[4710-5727]-EGFP fusion in the hyphae, one cassette contained the *Aolah[4710-5727]-egfp* fusion was constructed under the control of *amyB* promoter. The expression plasmid was constructed (See Materials and methods for details). The generated plasmid pgAoLAH[4710-5727]G was introduced into the wild-type strain NSRKu70-1-1A (Escaño *et al*., 2009), NSK-Δlah2 and NSK-Δwsc1. The positive transformants were selected on M agar medium. The resulting transformants were named NSK-LAH[4710-5727]G, NSK-Δlah-LAH[4710-5727]G and NSK-Δwsc-LAH[4710-5727]G, respectively. Strains expressing AoLAH[4710-5727]-EGFP was grown for 18-24 hr at 30°C on glass bottom dish with CD+0.0015% methionine liquid medium using glucose as carbon source and observed under the confocal microscope.

3.2 Localization analysis of AoLAH C-terminal region in wild-type and Δ*Aolah* strains

The C-terminal conserved region of AoLAH shares similarity with *N. crassa*

LAH-2, which is closely associated with the septal pore (Ng *et al*., 2009). To examine its localization, AoLAH C-terminal EGFP (AoLAH[4710-5727]-EGFP) was expressed in the wild-type and *Aolah* disruptant strains. As shown in Fig. 2-11, AoLAH[4710-5727]-EGFP was localized to the septal pore in both the strains, indicating that the C-terminal region of AoLAH associates with the septal pore.

Discussion

In Chapter 1, AoLAH was found to function as a tether for associating Woronin bodies to the septal pore, and excessive loss of cytoplasm was found in *Aolah* disruptant when the hyphae encountered cell wounding. In this Chapter, to functionally characterize the contribution of individual regions of AoLAH to Woronin body function, this extremely large AoLAH protein, which is over 5,000 amino acids, was divided into N-terminal, middle, and C-terminal regions according to amino acid sequence homology with the LAH proteins from other *Aspergillus* species (Fig. 1-3).

The N-terminal conserved region of AoLAH (amino acids 1-2039) fused with EGFP was detected at a distance from both sides of the septal pore (Fig. 2-4 upper), which is similar to Woronin body localization as observed under transmission electron microscopy (Fig. 1-6). The fluorescence of AoLAH[1-2039]-EGFP was observed at the septal pore upon hyphal wounding (Fig. 2-4, lower). This is similar to the previous result of our laboratory that the DsRed2-AoHex1 fusion protein plugged the septal pore when subjected to hypotonic shock experiment (Maruyama *et al*., 2005).

In previous reports, Hex1 protein was used to visualize Woronin body by fusion with fluorescent proteins (Escaño *et al*., 2009; Beck *et al*., 2013). AoHex1 has been used as a Woronin body marker (Maruyama *et al*., 2005), however, fluorescent protein tagged-AoHex1 only partially complements an *Aohex1* disruptant and often co-localizes to the peroxisomes (Escaño *et al*., 2009). In *A. fumigatus*, Hex1 fused with a fluorescent protein was found to localize near the septum as Woronin body does, however, the fusion protein often co-localizes or associates with peroxisomes (Beck *et al*., 2013), and this means that Hex1 localization cannot always represent that of Woronin bodies.

Here, in this study, co-localization analysis of AoLAH[1-2039]-EGFP and peroxisome revealed that nearly all AoLAH[1-2039]-EGFP fluorescent dots observed in *A. oryzae* cells were independent of peroxisomes (Fig. 2-6), and expressing the AoLAH[1-2039]-EGFP fusion protein did not affect the ability of cells to prevent the excessive loss of cytoplasm (data not shown). Collectively, AoLAH[1-2039]-EGFP displayed a similar localization pattern to that of Woronin bodies at normal condition and upon hyphal wounding, indicating that the fusion protein is a more reliable marker for the Woronin body.

N. crassa LAH-1 is associated with Woronin bodies via binding to WSC protein (Ng *et al*., 2009). However, WSC homologue WscA in *A. fumigatus* was reported to lack the C-terminal domain, whereas *N. crassa* WSC C-terminus was shown to mediate the interaction between WSC and LAH-1 (Beck *et al*., 2013). In *A. oryzae*, AoLAH[1-2039]-EGFP co-localized with AoWSC as dot-like structures at both sides of the septum and also in the cytoplasm (Fig. 2-7). The fluorescence of AoLAH[1-2039]-EGFP was dispersed in the cytoplasm of the *Aowsc* disruptant (Fig. 2-8). This finding confirmed the AoWSC-dependent recruitment of the AoLAH N-terminal region to Woronin bodies similarly to *N. crassa*. Alternatively, a model in *A. fumigatus* was recently proposed that HexA (Hex1 homologous protein) might bind to LAH directly (Beck *et al*., 2013), which could be in the same line of our genetic data. However, it remains to be determined whether Hex1 or WSC directly bind to LAH. AoLAH[1-2039]-EGFP was not found near the septum in the Δ*Aolah* strain (Fig. 2-9), and this finding demonstrated that the presence of endogenous AoLAH protein is required for the localization of AoLAH[1-2039]-EGFP near the septum.

The C-terminal conserved region of AoLAH (amino acids 4710-5727) fused with

EGFP was closely associated with the septal pore in the wild-type strain (Fig. 2-11 upper). This result is in agreement with a recent report in *A. fumigatus* (Beck *et al.*, 2013), in which the C-terminal region of *A. fumigatus* LAH fused with GFP in the wild-type hyphae displayed fluorescent signals that were clearly focused to the central part of the septum (Beck *et al*., 2013). Similarly, *N. crassa* LAH-2 also localized to the septal pore (Ng *et al.*, 2009), and it seems that this conserved region at the C-terminus has a conserved function among the Pezizomycotina species to target to the septal pore. Interestingly, this targeting to the septal pore of AoLAH C-terminal region was still found when the endogenous LAH was disrupted (Fig. 2-11 lower). This observation indicates that the C-terminal region itself functions for tethering to the septal pore, which has not been reported in other species.

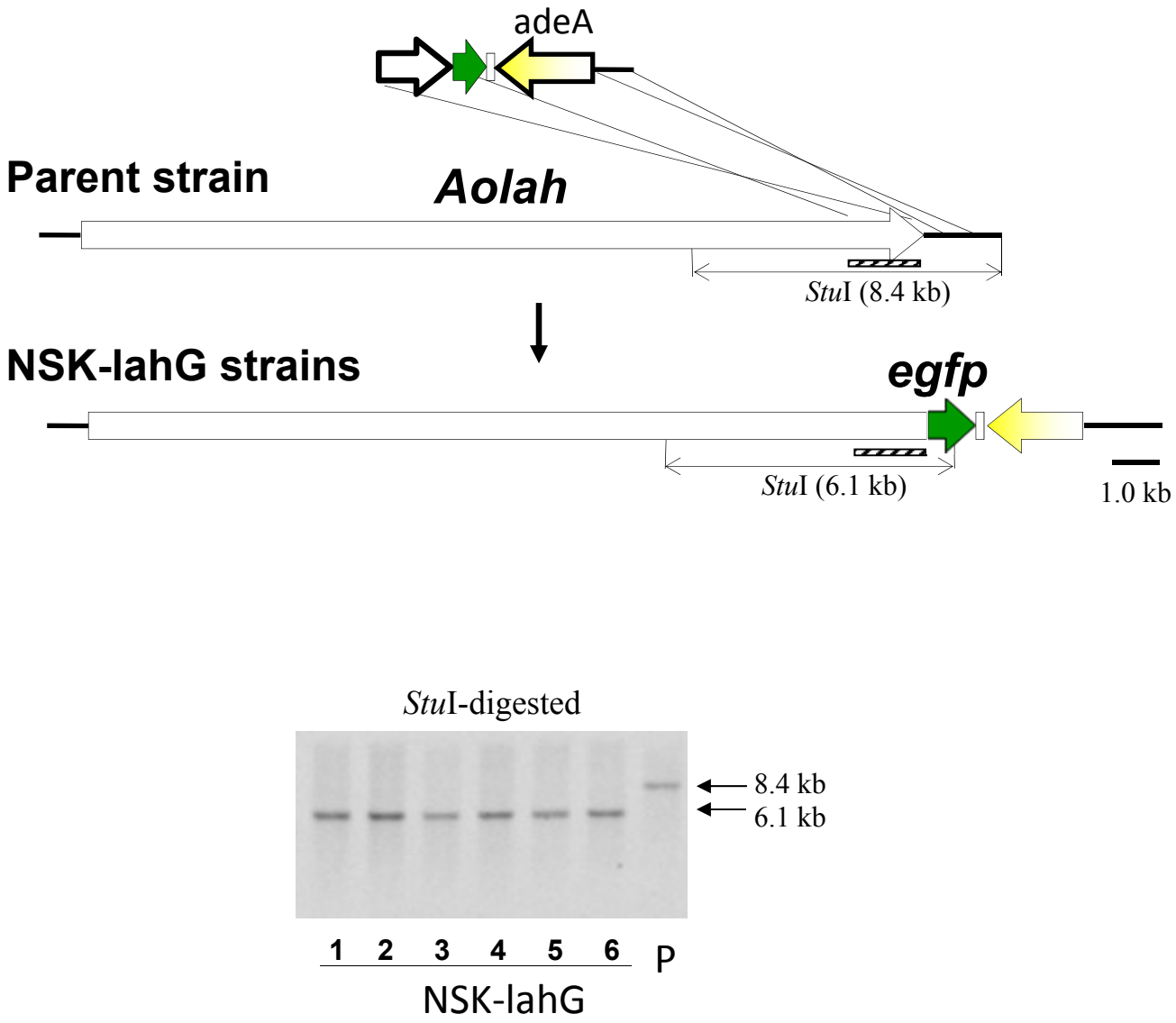
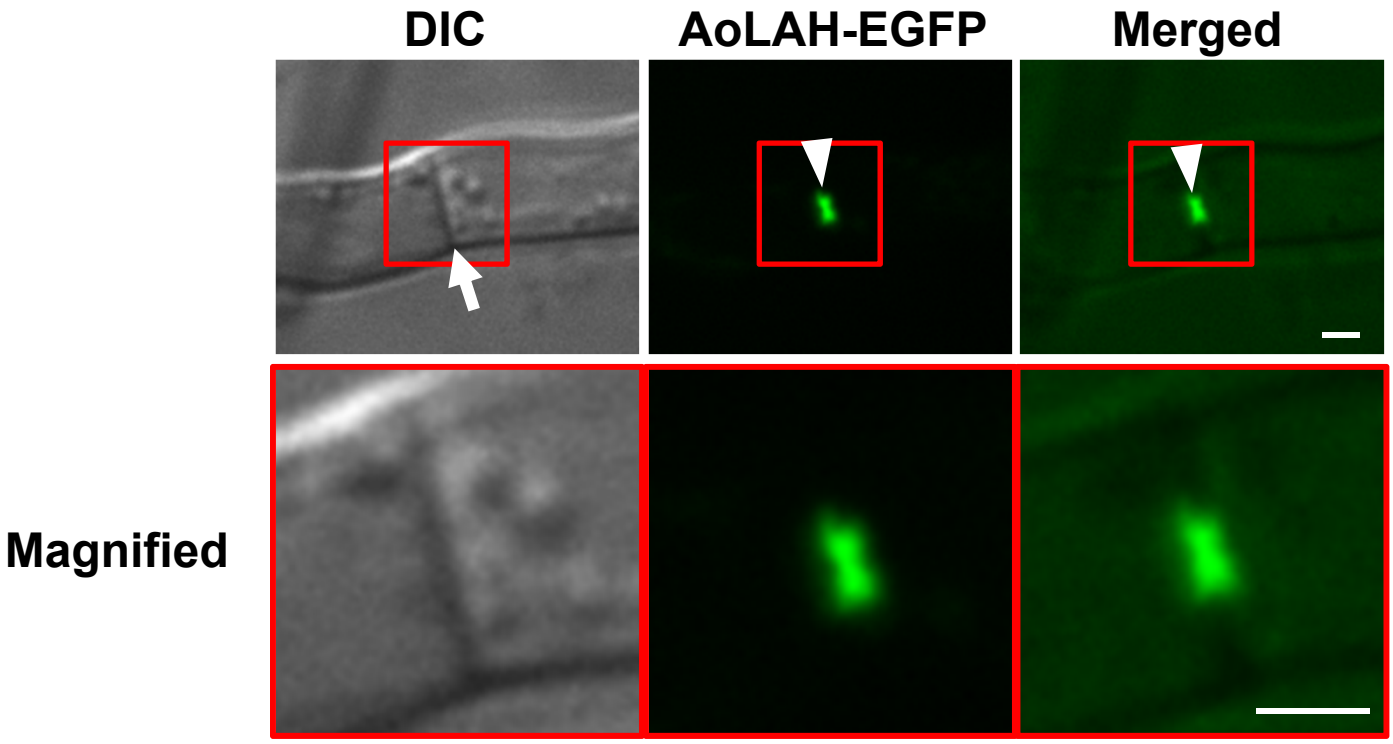


FIG. 2-1. **Schematic model of the construction of AoLAH-EGFP expression under native promoter.**



Bars = 1 μm

FIG. 2-2. Localization of AoLAH fused with EGFP.

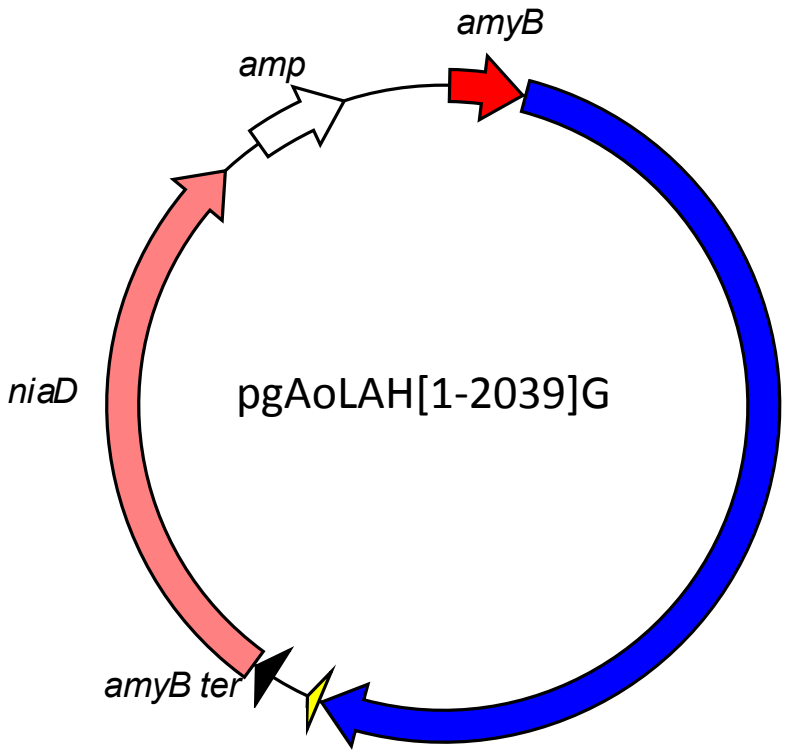


FIG. 2-3.Plasmid construct for expression of AoLAH N-terminal-EGFP fusion protein. Plasmid pgAoLAH[1-2039]G was constructed for expression of the AoLAH[1-2039] under the control of the *amyB* promoter. The MultiSite Gateway technique was used for construction of the plasmid.

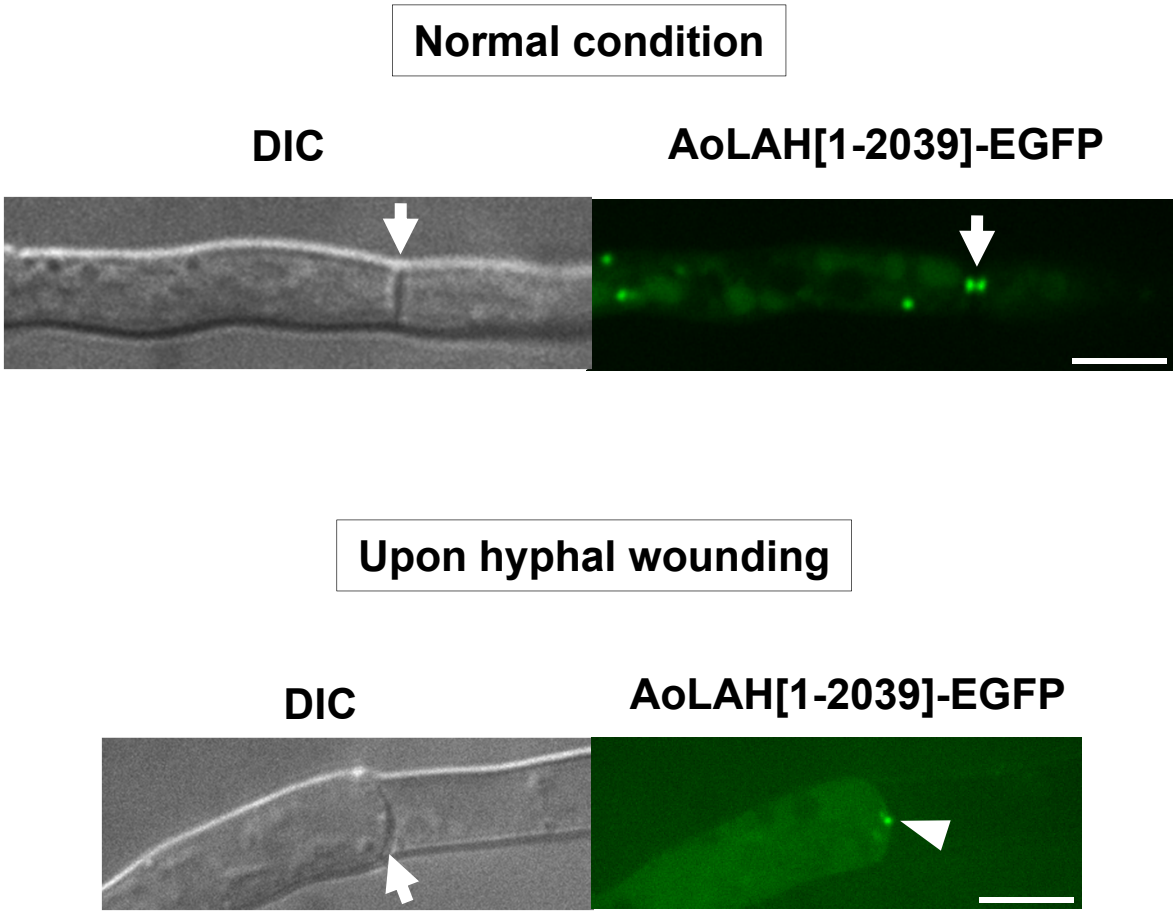


FIG. 2-4. Localization of AoLAH N-terminal regions fused with EGFP. (A) Localization of AoLAH[1-2039]-EGFP in the wild-type background around the septum (upper) and upon hyphal wounding (lower). Note that the fluorescent signal was located at a distance from the septum during normal growth (see upper). The lower image shows the localization of AoLAH[1-2039]-EGFP to the septal pore upon hyphal wounding. Hyphal tip bursting was induced by hypotonic shock, and the septal pore adjacent to the burst tip cell was observed by fluorescence microscopy. Arrows indicate the septum and arrowheads show the localization of AoLAH[1-2039]-EGFP to the septal pore. Bars: 5 μm.

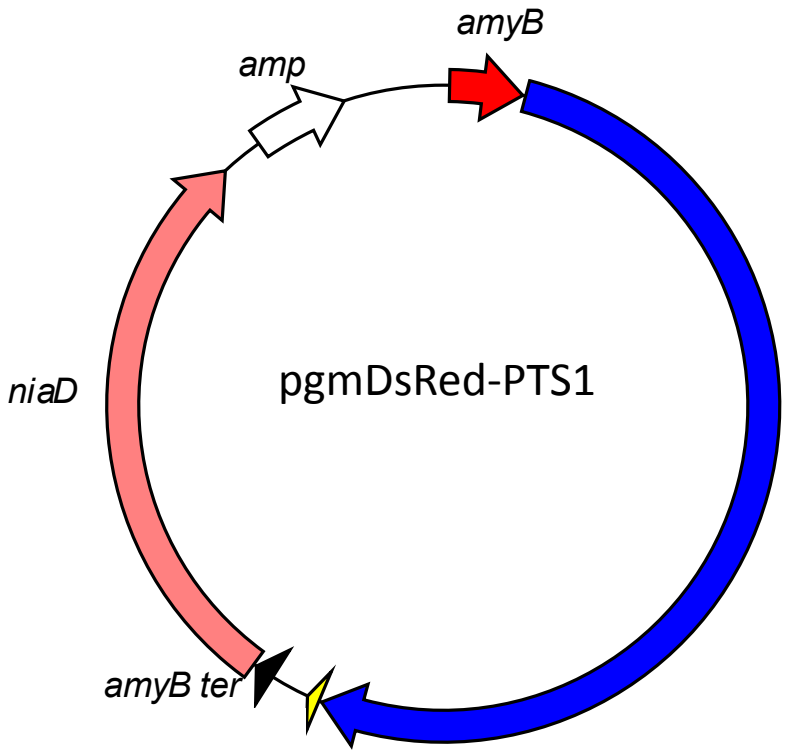


FIG. 2-5. Plasmid construct for expression of mDsRed-PTS1 protein. The MultiSite Gateway technique was used for construction of the plasmid.

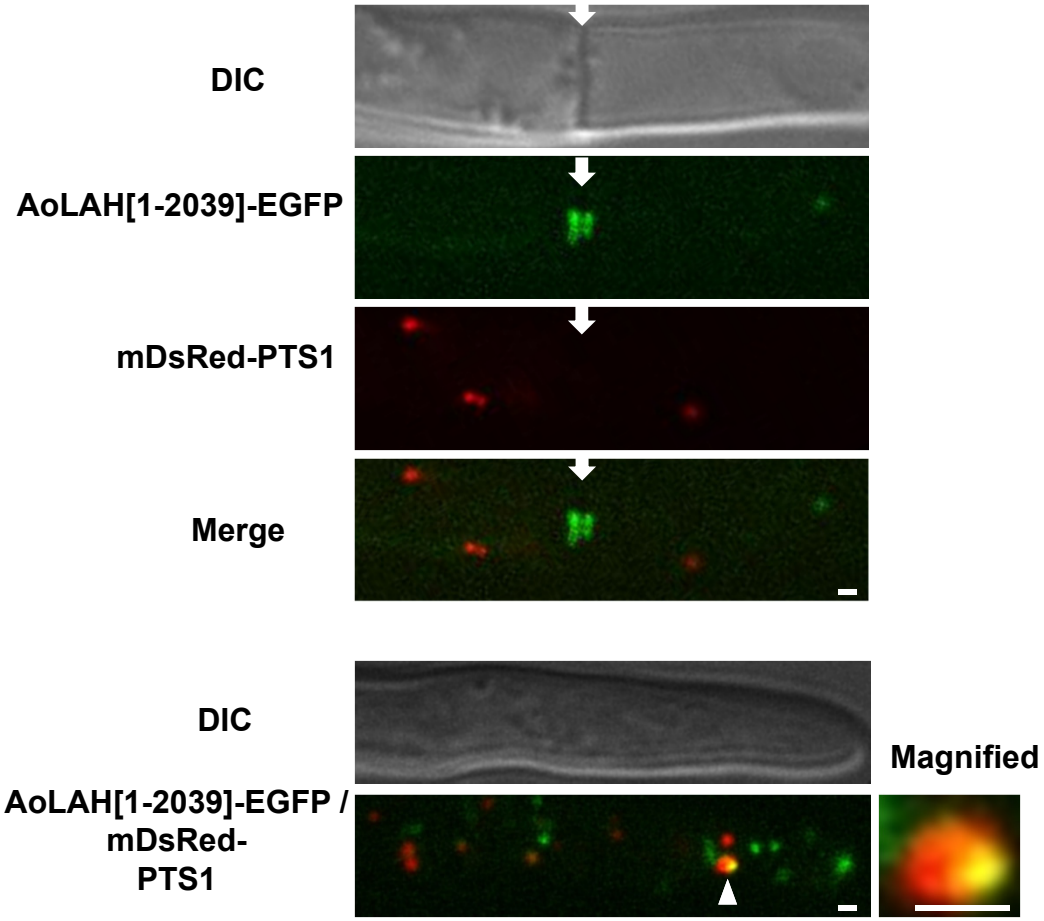


FIG. 2-6. Subcellular localization analysis of AoLAH[1-2039]-EGFP and peroxisomes. mDsRed fused with peroxisomal targeting signal 1 (PTS1) was expressed in an *A. oryzae* strain expressing AoLAH[1-2039]-EGFP. Fluorescence microscopic analysis revealed an independent distribution of the two fluorescent fusion proteins around the septum (upper) and in the apical cell (lower). The septum is shown by arrows. Peripheral association of AoLAH[1-2039]-EGFP with the peroxisome was also observed in the apical cell as shown by the arrowhead. Bars: 1 μm.

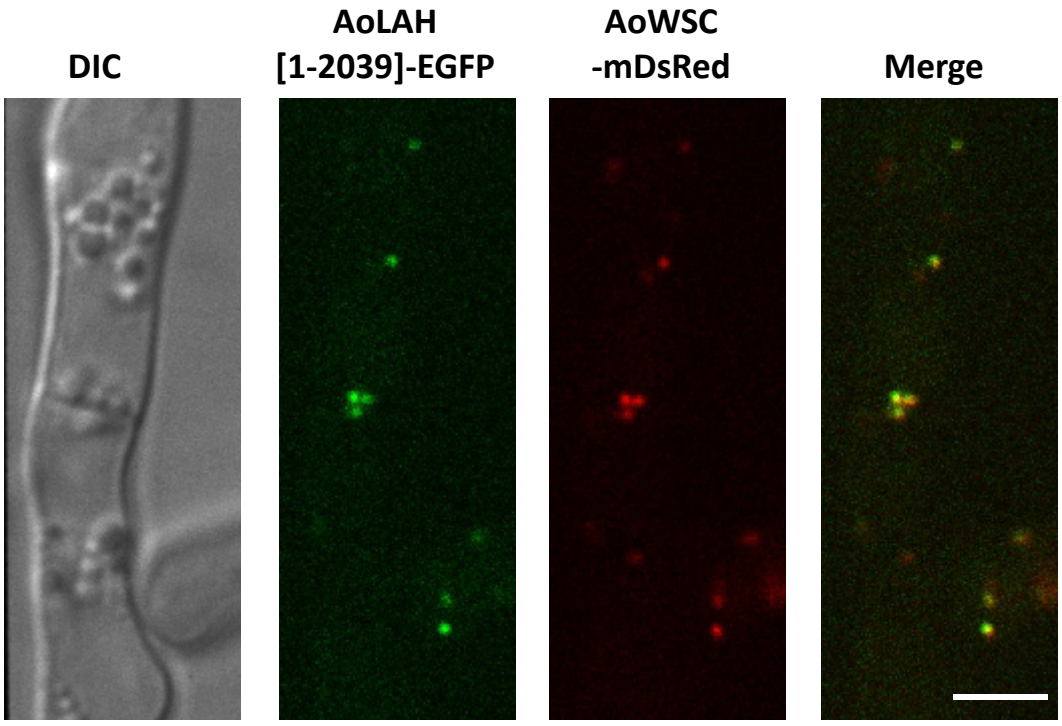


FIG. 2-7. Subcellular localization analysis of AoLAH[1-2039]-EGFP and AoWSC-mDsRed. AoWSC fused with mDsRed was expressed in an *A. oryzae* strain expressing AoLAH[1-2039]-EGFP. Fluorescence microscopic analysis revealed an colocalized distribution of the two fluorescent fusion proteins around the septum and in the cytoplasm. The septum is shown by arrows. Bars: 5 μm.

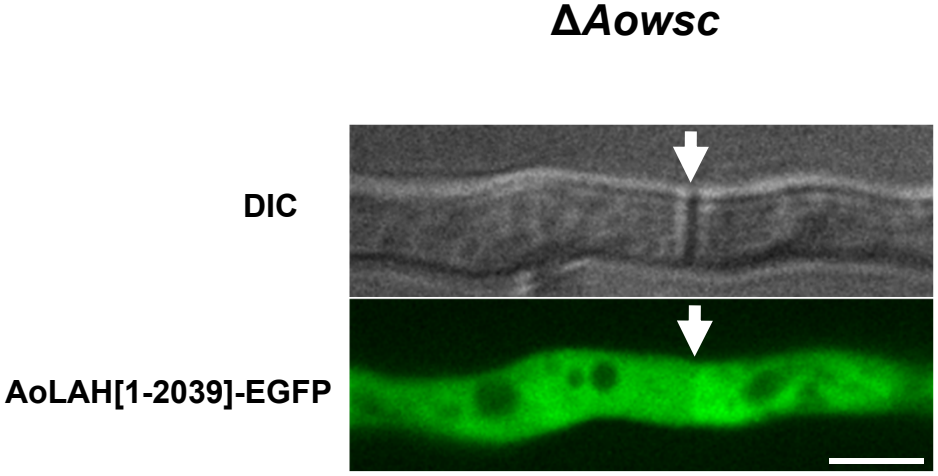


FIG. 2-8. Localization of AoLAH[1-2039]-EGFP in the *Aowsc* disruptants. Arrows indicate the septum. Bars: 5 μm.

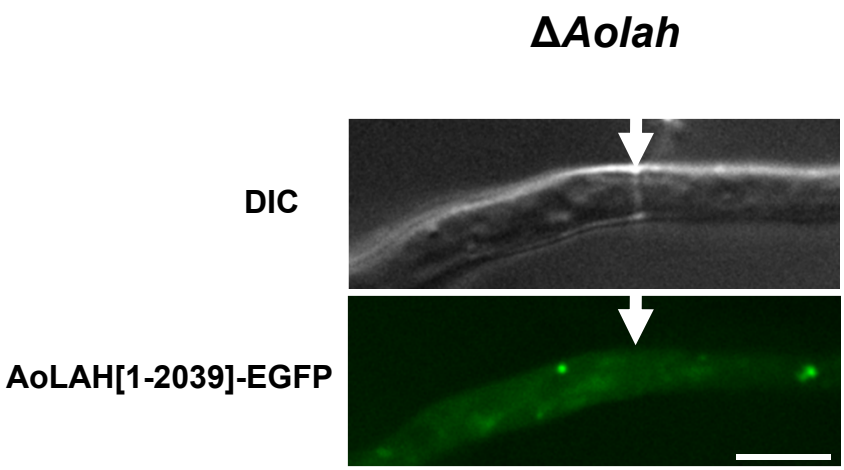


FIG. 2-9. Localization of AoLAH[1-2039]-EGFP in the *Aolah* disruptants. Arrows indicate the septum. Bars: 5 μm.

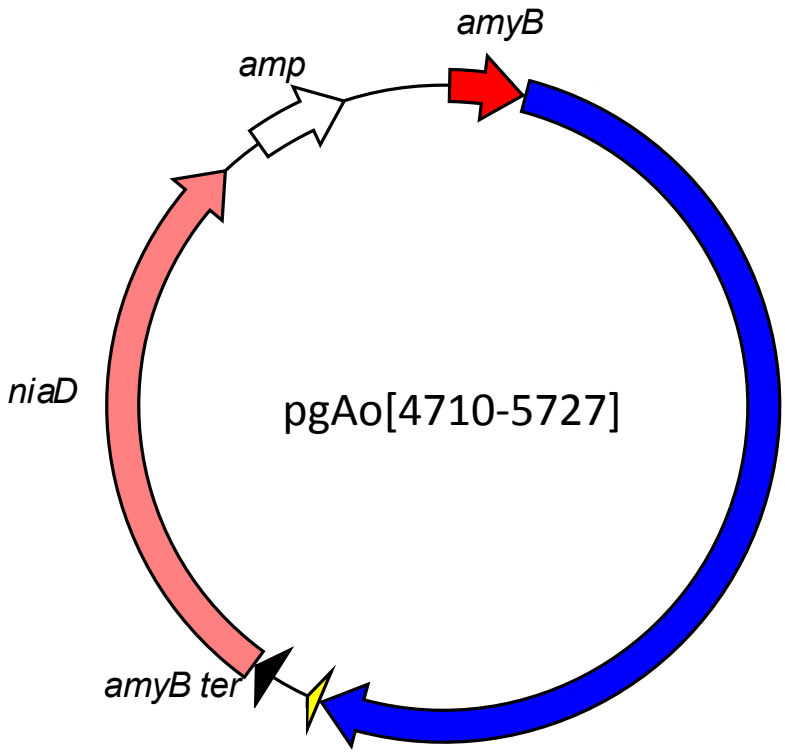


FIG. 2-10. Construction of AoLAH C-terminal-EGFP expression plasmid in *A.oryzae.*

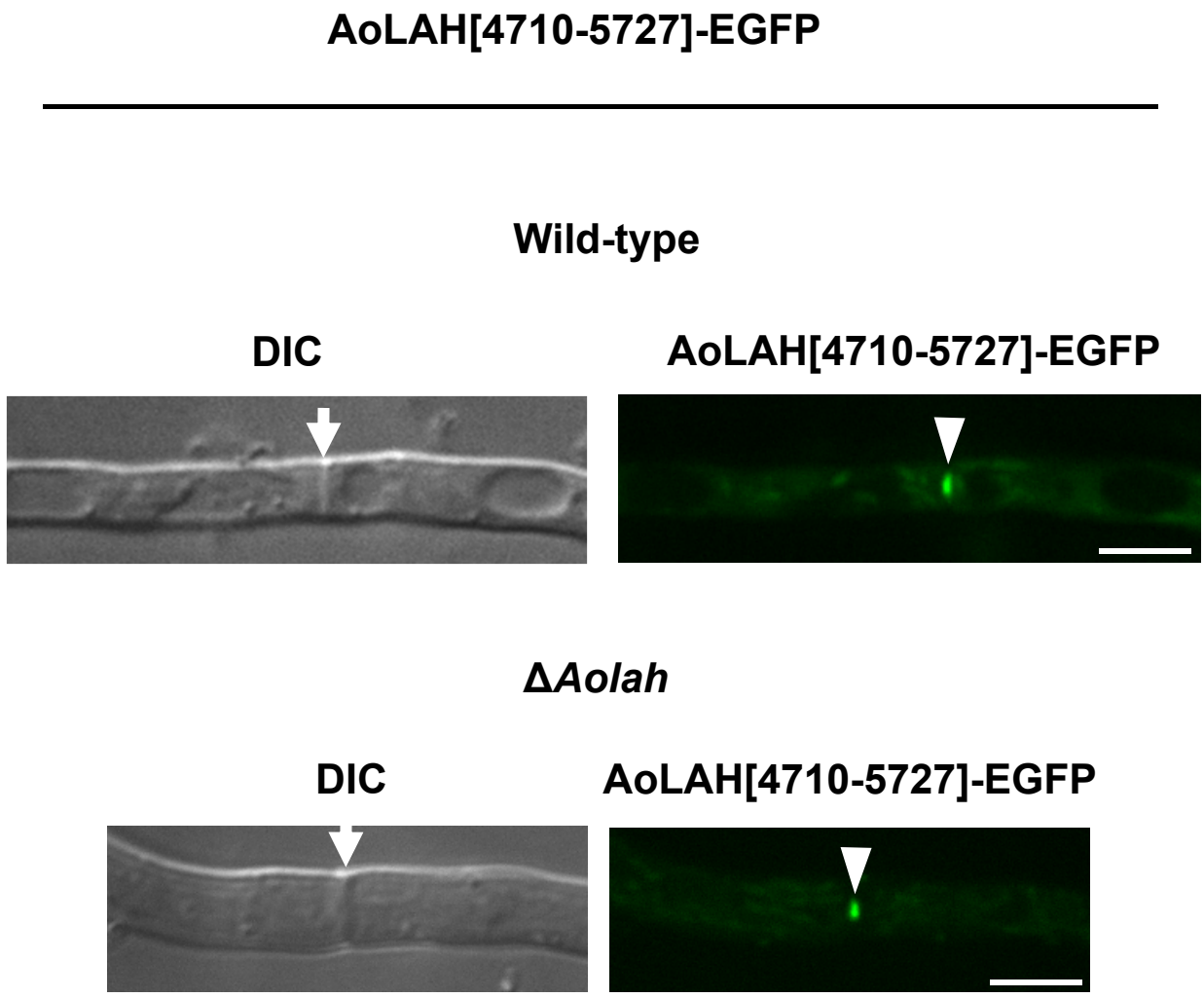


FIG. 2-11. Localization of AoLAH[4710-5727]-EGFP in the wild-type strain (upper) and *Aolah* disruptant (lower). Arrows indicate the septum and arrowheads show the close association of AoLAH[4710-5727]-EGFP with the septal pore. Bars: 5 μm.

Chapter 3

Functional analysis of AoLAH middle region

Introduction

Previously, in *Nectria haematococca* (Berns *et al*., 1992), Woronin bodies were pulled away from the septum with laser tweezers but went back to their original position. This suggests that there is an elastic filament tethering Woronin bodies to the septum. However, this elastic behavior of Woronin body tethering has not been observed naturally, and it is not characterized whether the LAH protein is required for the elasticity.

Based on homology comparison in Chapter 1 (Fig. 1-3), the N- (~2000 amino acids) and C- (~1000 amino acids) terminal regions of AoLAH share homologies with those of LAH proteins from *A. fumigatus* and *A. nidulans* (Fig. 1-3). However, the long-stretched middle region of AoLAH (~2500 amino acids) has low identities with the LAH proteins of *A. fumigatus* (~2300 amino acids) and *A. nidulans* (~2400 amino acids), and there has been no report of functional analysis on this large non-conserved middle region of LAH proteins.

According to results of the localization analysis in Chapter 2, N-terminal region of AoLAH associated with Woronin bodies dependently on AoWSC and endogenous AoLAH. C-terminal region of AoLAH targeted to the septal pore. Based on these results, here comes the hypothesis that the AoLAH N- and C-terminal fusion construct without the non-conserved middle region may be sufficient to tether Woronin bodies to the septum.

In this Chapter, to test this speculation, a middle-region deleted AoLAH construct (AoLAH[(1-2039)+(4710-5727)]) and full-length AoLAH were constructed and expressed in the *Aolah* disruptant. Western blot analyses of these two constructs

expressed strains were done to have more biochemical information about this large sized protein. Electron microscopy observation was performed to strains expressing middle-region deleted and full-length AoLAHs to examine the Woronin body localization pattern in detail. Moreover, Woronin body localization behavior was also observed by using AoLAH N-EGFP as a marker and time-lapse analysis of Woronin body in living cell was applied in middle-region deleted and full-length AoLAHs expressing strains. Finally, to investigate the functionality of the middle-region deleted AoLAHs, the strains were subjected to hypotonic shock experiment and the Woronin body plugging behavior was observed under fluorescence microscopy.

Results

1. Construction of strains expressing middle-region deleted and full-length AoLAH in the *Aolah*-disrupted background

MultiSite Gateway technique was used for construction of the plasmid for expressing middle-region deleted AoLAH (See Materials and methods under Chapter 3 for details). DNA fragments for AoLAH[1-2039] and AoLAH[4710-5727] were amplified and fused by fusion PCR, and then the resulting DNA fragment for AoLAH[(1-2039)+(4710-5727)] (middle-region deleted AoLAH) was tagged with 3×HA gene at the C-terminus under the control of *amyB* promoter. The expression plasmid pAoLAH[(1-2039)+(4710-5727)]N was obtained (Fig. 3-1).

To construct a cassette expressing full-length AoLAH, the 17.4-kb *Aolah* coding region (not including the stop codon) was PCR amplified using the PrimeSTAR GXL DNA Polymerase (TaKaRa; The DNA Polymerase allows amplification of products ≥ 30 kb in length while maintaining exceptionally high fidelity). The obtained DNA fragment was tagged with the 3×HA gene and use *amyB* as promoter to generate expression plasmid pAoLAHN (Fig. 3-2).

The obtained expression plasmids pAoLAH[(1-2039)+(4710-5727)]N and pgAoLAHN were introduced into the *Aolah* disruptants NSK-Δlah2 and NSK-Δlah-LAH[1-2039]EGFP. Positive transformants were selected on CD agar medium supplemented with 0.0015% methionine and unsupplemented CD agar medium, and the transformants were named as NSK-LAH[(1-2039)+(4710-5727)]-3HA, NSK-LAH[1-2039]G-LAH[(1-2039)+(4710-5727)]-3HA, NSK-Δlah-LAH-3HA, NSK-Δlah-LAH[1-2039]G-LAH-3HA, respectively. The plasmid pNR10 containing the

niaD marker was transformed into the wild-type strain NSRKu70-1-1A and *Aolah* disruptant NSK-ΔAolah as controls, resulting transformants named as NSRKu70-1-1AN and NSK-ΔlahN, respectively.

The expression of middle-region deleted and full-length AoLAHs were confirmed by Western blot analysis (Fig. 3-3). The middle-region deleted AoLAH-3×HA was predicted to be ~330 kDa, but was detected around 460 kDa. The full-length AoLAH-3×HA was predicted to be a polypeptide of ~620 kDa, and a band were detected much higher than the maximum size (460 kDa) of the protein marker.

2. Electron microscopy of Woronin bodies in the strain expressing middle-region deleted AoLAH

Since the N-terminal region of AoLAH associate with Woronin body and the C-terminal region target to the septal pore (Chapter 2), I attempt to investigate whether AoLAH N- and C-terminal fusion construct without the non-conserved middle region is functionally sufficient to tether Woronin bodies to the septum, Woronin body localization near the septal pore was observed by transmission electron microscopy in *Aolah*-disrupted strain expressing middle-region deleted and full-length AoLAHs (Fig. 3-4).

Transmission electron microscopic analysis revealed that Woronin bodies were located near the septal pore of the *Aolah*-disruptant strain expressing the full-length AoLAH (Fig. 3-4 left, arrows). Similarly, in the strain expressing middle-region deleted AoLAH, Woronin bodies were also observed near the septal pore (Fig. 3-4 right, arrows), indicating that the N- and C-terminal fusion construct without the middle region is functional to tether Woronin bodies to the septum.

To more precisely investigate the effect of deleting the middle region of AoLAH on the tethering of Woronin bodies, the average distance of Woronin bodies from the septum was measured by transmission electron microscopy (Fig. 3-5). In the *Aolah*-disruptant strain expressing full-length AoLAH, Woronin bodies were located approximately 99 nm from the septum, whereas in the strain expressing the middle-region deleted AoLAH, the position of Woronin bodies had shifted approximately 50-nm closer to the septum (Fig. 3-5). These results suggest that the middle region of AoLAH has a role in regulating the distance of Woronin bodies from

the septum.

3. Time-lapse fluorescence microscopy of the tethered Woronin bodies

The tethering of Woronin bodies to the septum in *Nectria haematococca* was reported to exhibit elasticity, as demonstrated by the return of the Woronin bodies to the septum after their physical separation by laser trapping (Berns *et al*., 1992). As shown in Fig. 3-5, the distance of Woronin bodies from the septum was more variable in the *Aolah*-disruptant strain expressing full-length AoLAH compared to the strain expressing middle-region deleted AoLAH. Based on this finding, it was hypothesized that the middle region of AoLAH might confer the observed elastic characteristics of the Woronin body tether.

To answer this question, the movement of Woronin bodies in living cells expressing AoLAH[1-2039]-EGFP was examined by fluorescence microscopy (Fig. 3-6). In the strain expressing middle-region deleted AoLAH, Woronin bodies were located near the septum, and fluorescent dots were closer together across the septum than that in the strain expressing full-length AoLAH (Fig. 3-6). Time-lapse analysis revealed that Woronin bodies located near the septum exhibited positional changes in cells expressing full-length AoLAH (Fig. 3-7). In the subsequent experiments, I focused on the first septum adjacent to the apical cell as it was considered to be the most likely to allow cytoplasmic continuity between adjacent cells, as suggested by van Peer *et al.* (2012). Microscopic observation of the first septum of hyphae expressing full-length AoLAH showed that at least one of the tethered Woronin bodies moved rapidly back-and-forth between two positions in approximately 5 seconds. Two distinct movement patterns of Woronin bodies were observed: movement towards the septal pore and then back to the starting position (Fig. 3-7 upper), and movement away from

the septal pore and then back to the starting position (Fig. 3-7 middle). These observational analyses demonstrated that tethered Woronin bodies are capable of elastic movement. In the strain expressing the middle-region deleted AoLAH, however, such movement was not observed for Woronin bodies tethered to the septum (Fig. 3-7 lower). This finding indicates that the middle region of AoLAH is required for the elastic movement of Woronin bodies.

4. Ability to prevent the excessive loss of cytoplasm in strains expressing middle-region deleted AoLAH

Since the middle region of AoLAH influences the positional variation and elastic motility of tethered Woronin bodies (Figs. 3-5 and 3-7), I next investigated if the function of Woronin bodies was affected by deleting the middle region of AoLAH. Complementation analysis of the *Aolah* disruptant was performed with various AoLAH constructs, and marker-introduced strains in the wild-type (NSRKu70-1-1AN) and *Aolah*-disruptant (NSK-ΔlahN) backgrounds were used as positive and negative controls, respectively. The ability of cells to prevent the excessive loss of cytoplasm was analyzed by inducing hyphal tip bursting in each strain through hypotonic shock.

Although 82% of the wild-type cells prevented the excessive loss of cytoplasm in cells adjacent to the hyphal tip upon lysis, cells of the *Aolah* disruptant showed a lower ability (63%). Cells of the *Aolah* disruptant expressing full-length AoLAH displayed a restored ability (81%) (Fig. 3-8) that was comparable to the wild-type strain, indicating the functional complementation of the disruption. However, expression of middle-region deleted AoLAH in the *Aolah* disruptant did not restore the resistance of cells to cytoplasmic loss through the septum (62%) (Fig. 3-8). These results indicate that the middle region of AoLAH is needed for proper Woronin body position to prevent the excessive loss of cytoplasm upon hyphal wounding.

As the Woronin bodies visualized with the EGFP fusion of the AoLAH N-terminal region plugged the septal pore upon hyphal wounding (Fig. 2-4 lower), I also examined whether Woronin bodies plug the septal pore in the strains expressing full-length or middle-region deleted AoLAH. In the *Aolah* disruptant, although Woronin bodies did

not localize near the septum (Fig. 2-9), a fluorescent dot was observed at the septal pore upon hyphal wounding (Fig. 3-9 upper), suggesting that untethered Woronin bodies were pushed into the septal pore by cytoplasmic flow. In the strain expressing full-length AoLAH, a fluorescent dot was also observed at the septal pore (Fig. 3-9 middle), a finding that is consistent with the result presented in Fig. 2-4, lower In the *Aolah* disruptant and the strain complemented with full-length AoLAH, almost no fluorescence was detected near the septum, with the exception of the dot plugging the septal pore. In contrast, in the strain expressing the middle-region deleted AoLAH, Woronin bodies tethered to the septum were frequently observed after hyphal wounding, in addition to a fluorescent dot within the septal pore (Fig. 3-9 lower). Taken together, these results indicated that the non-conserved middle region of AoLAH is involved in proper spacing of tethered Woronin bodies around the septal pore, and this spacing is important for efficient plugging of the septal pore upon hyphal wounding.

Discussion

In addition to characterization of the conserved N- and C-terminal regions of AoLAH in Chapter 2, the functional roles of the non-conserved middle region of AoLAH were also examined in this chapter. When the AoLAH-EGFP fusion construct lacking the middle region (~2,700 amino acids) of AoLAH was expressed in the *Aolah* disruptant, Woronin bodies were re-tethered to the septum (Fig. 3-4), revealing that the middle-region deleted AoLAH is sufficient for the tethering of Woronin bodies to the septum. However, the Woronin bodies were located approximately 50-nm closer to the septum than those in the strain expressing full-length AoLAH (Fig. 3-5). Based on the fact that the giant protein titin, which is 4 mega Daltons, is 1 μm in length (Nave *et al*., 1989), the length of the deleted 2,670 amino acids of AoLAH is theoretically estimated to be 70 nm, which is consistent with the reduced distance of Woronin bodies from the septum that was observed in the *Aolah* disruptant expressing the middle-region deleted AoLAH.

In the *Aolah* disruptant, although no Woronin bodies were tethered to the septum, untethered Woronin bodies were found to plug the septal pore after hyphal wounding (Fig. 3-9 upper). This is likely pushed into the position by cytoplasmic flow upon hyphal wounding. However, untethered Woronin bodies were not able to plug the septal pore as quickly as the tethered Woronin bodies, which may explain why the loss of cytoplasm was greater in the *Aolah* disruptant as compared to the wild-type strain, but was lower than that of the *Aohex1* disruptant (Figs. 1-10 and 3-8). This is similar to the fact that an *A. fumigatus lah* mutant defective in the septal tethering of Woronin bodies exhibits no increased sensitivity to Calcofluor white, whereas the *hex1* disruptant is

sensitive (Beck *et al*., 2013). Unexpectedly, the ability to prevent the excessive loss of cytoplasm was not restored by expressing the middle-region deleted AoLAH construct in the *Aolah* disruptant (Fig. 3-8), even though Woronin bodies were tethered to the septum in this strain (Figs. 3-4 and 3-6). I specifically examined the septal plugging state by visualizing Woronin bodies bound to the AoLAH N-terminal region-EGFP fusion protein. Upon hyphal wounding, nearly all Woronin bodies tethered to the septum contributed to septal plugging in the wild-type and full-length AoLAH expressing strains (Figs. 2-4 down, and 3-9). However, the Woronin bodies remained tethered at a distance from the septal pore upon hyphal wounding in the strain expressing the middle-region deleted AoLAH (Fig. 3-9), indicating that they failed to plug the septal pore. It was likely that the untethered Woronin bodies plugged the septal pore, as was observed in the *Aolah* disruptant. This result may explain why the cells expressing the middle-region deleted AoLAH had a reduced ability to prevent the excessive loss of cytoplasm (Fig. 3-8), although it should be noted that the middle-region is not essential for the overall ability of Woronin bodies to plug the septal pore.

According to the transmission electron microscopic observations, the distance between Woronin bodies and the septum displayed greater variability in the presence of full-length AoLAH (Fig. 3-5), suggesting that Woronin bodies have positional flexibility. This speculation is consistent with the reported elasticity of the Woronin body tether in *N. haematococca*, as demonstrated by laser capturing experiments (Berns *et al*., 1992). It was recently reported that a limited lateral mobility of Woronin bodies tethered at the septum was found in *A. fumigatus* (Beck *et al*., 2013). In this study, by visualizing Woronin bodies with AoLAH N-terminal region-EGFP fusion protein, I observed that

some tethered Woronin bodies displayed back-and-forth movement that may reflect the presence of an elastic tether. The movement of tethered Woronin bodies was not seen in the strain expressing the middle-region deleted AoLAH (Fig. 3-7), suggesting that the middle region of AoLAH confers elastic movement activity to Woronin bodies. Moreover, the fixed tethering of Woronin bodies to the septum (Fig. 3-9) is attributable to the excessive loss of cytoplasm upon hyphal wounding and is due to a decreased movement activity by deletion of the middle region of AoLAH. Collectively, the results presented here suggest that efficient septal plugging requires not only that Woronin bodies are tethered to the septum, but also that they must have the movement activity conferred by the middle region of AoLAH.

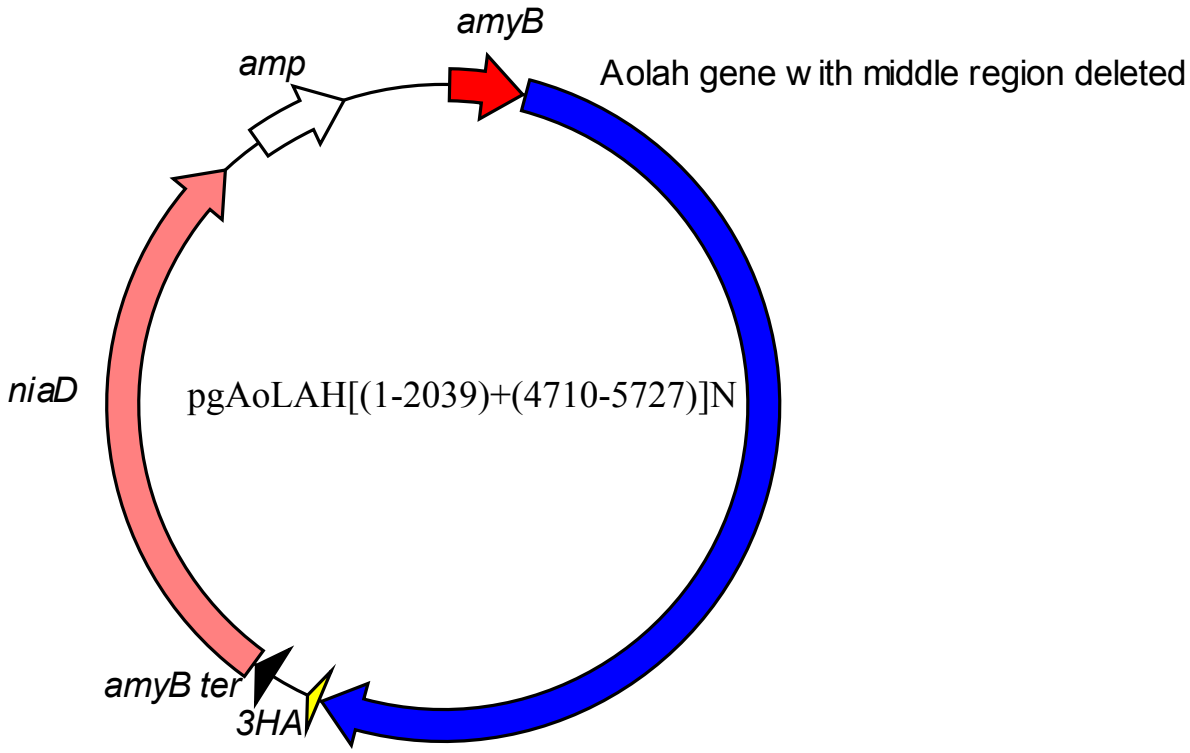


FIG. 3-1. Construction of middle-region deleted AoLAH expression plasmid in *A.oryzae.*

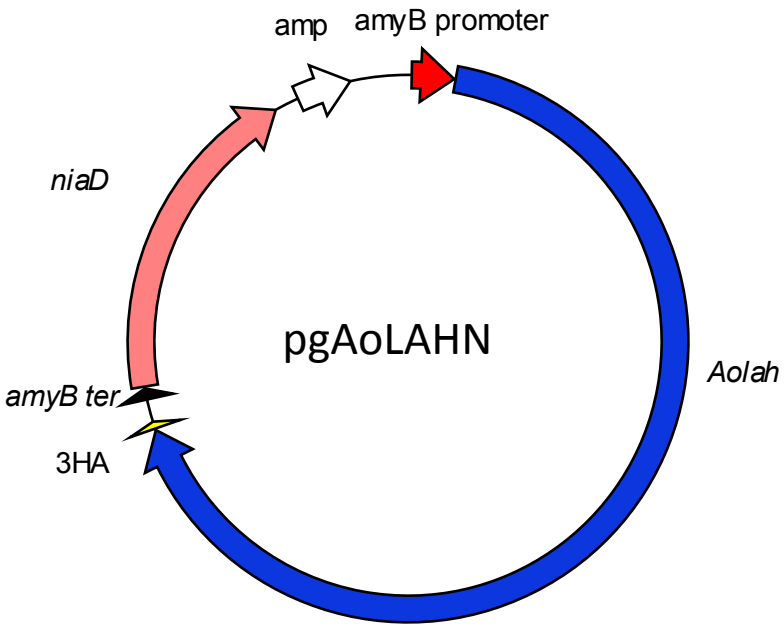


FIG. 3-2. Construction of full-length AoLAH expression plasmid.

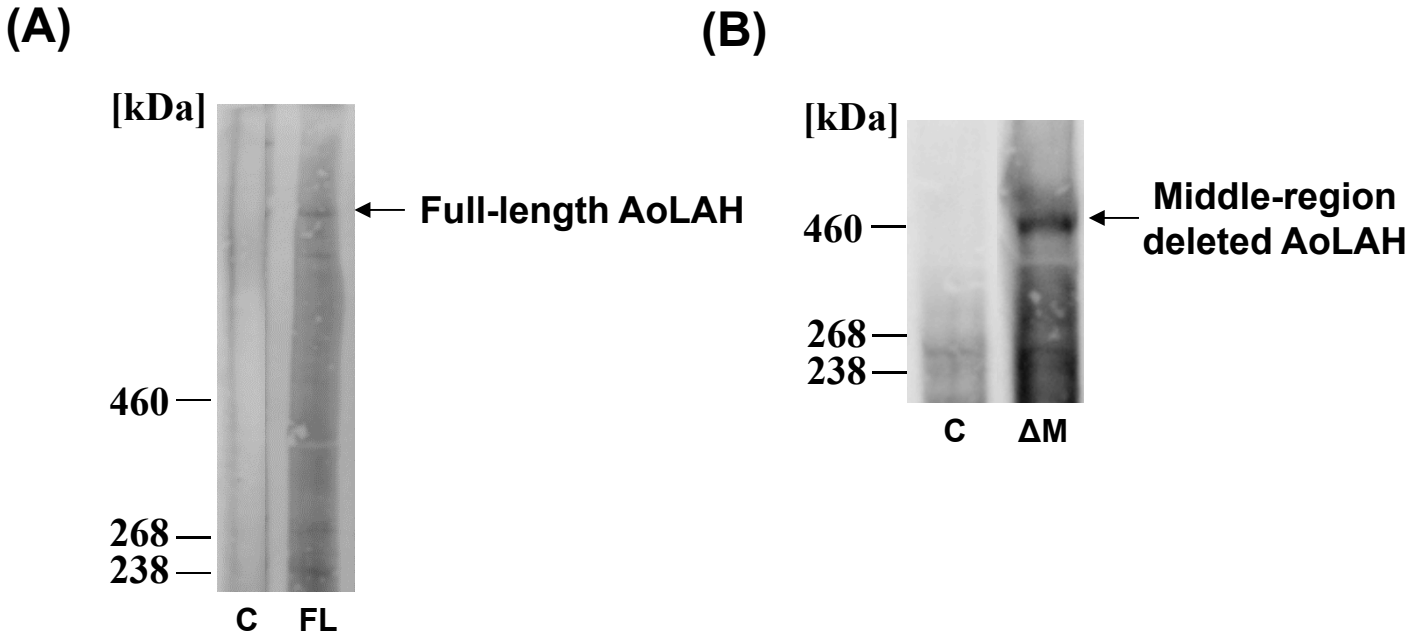


FIG. 3-3. Western blot analysis of full length (A) and middle-region deleted (B) AoLAHs. Arrowheads indicate the bands corresponding to each protein. Lanes: C, wild-type control strain (NSRKu70-1-1A); FL, AoLAH full-length expressing strain; ΔM: strain expressing middle-region deleted AoLAH.

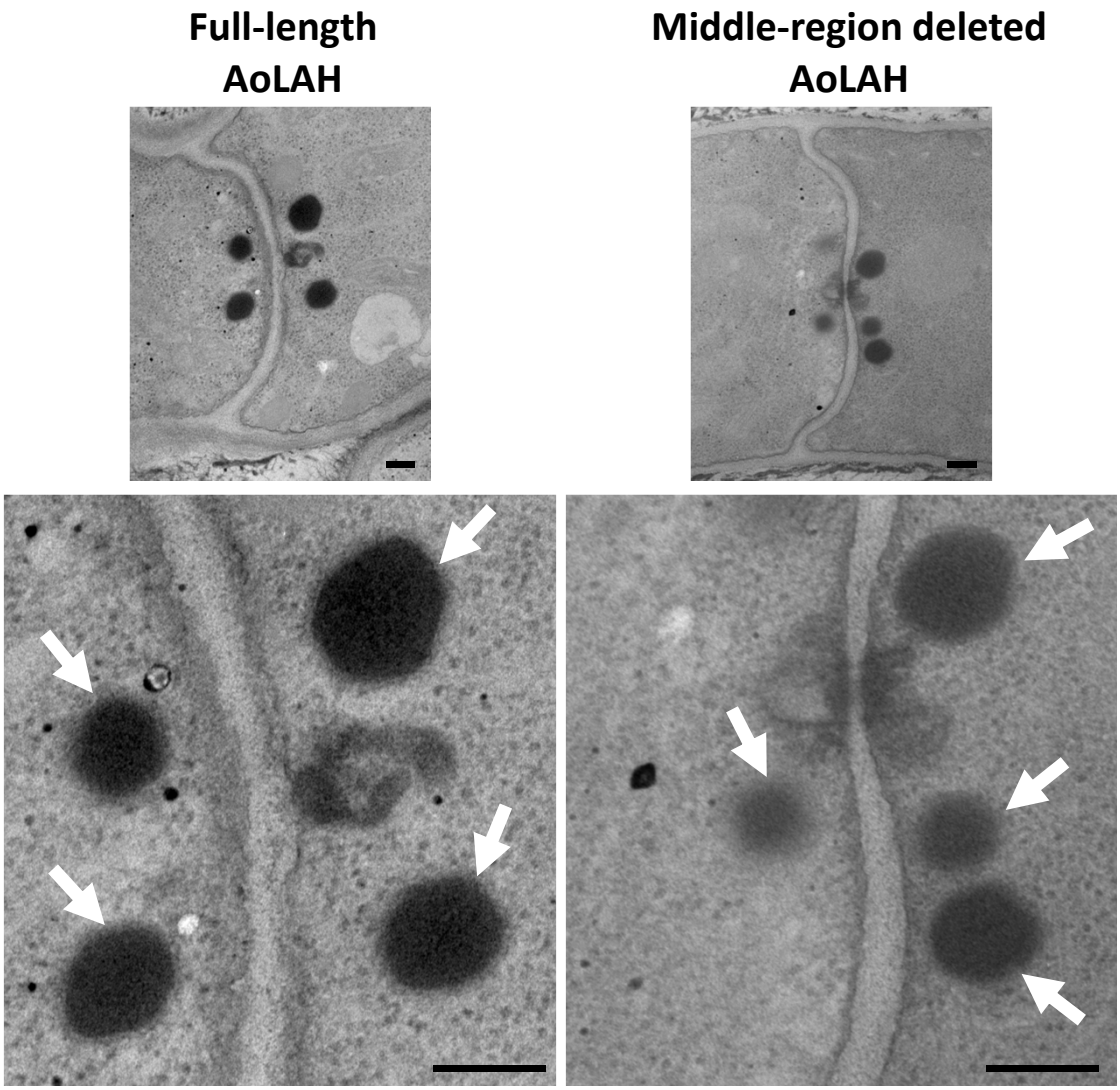


FIG. 3-4. Transmission electron microscopy of Woronin bodies in the *Aolah* disruptant strain expressing full-length or middle-region deleted AoLAHs. (A) Transmission electron microscopy of Woronin bodies located around the septum in the strain expressing full-length or middle-region deleted AoLAHs. Arrows indicate Woronin bodies. Bars: 200 nm.

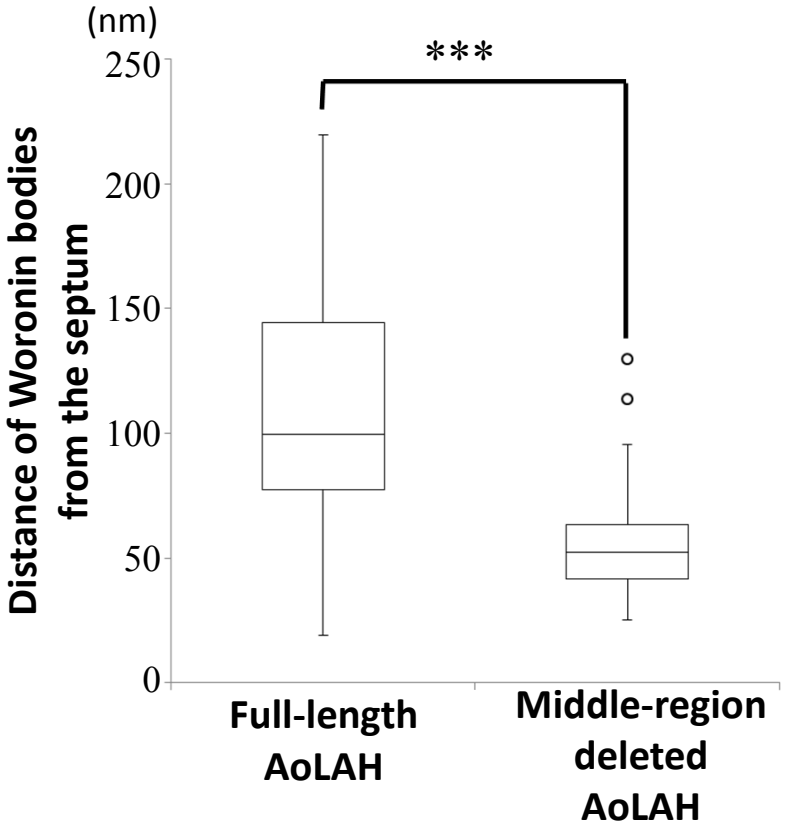


FIG. 3-5. Distance of Woronin bodies from the septum in the strain expressing full-length or middle-region deleted AoLAHs. The data are presented in the box plot chart. The top, bottom, and middle lines correspond to the 75th percentile, 25th percentile, and median, respectively; bars represent the limits of the upper (top) and lower (bottom) quartiles. The whiskers show the highest and lowest readings within a 1.5×interquartile range. The outliers are indicated as (○). n=44 and 48 for strains expressing full-length AoLAH or middle-region deleted AoLAH, respectively, ***: $p<0.001$.

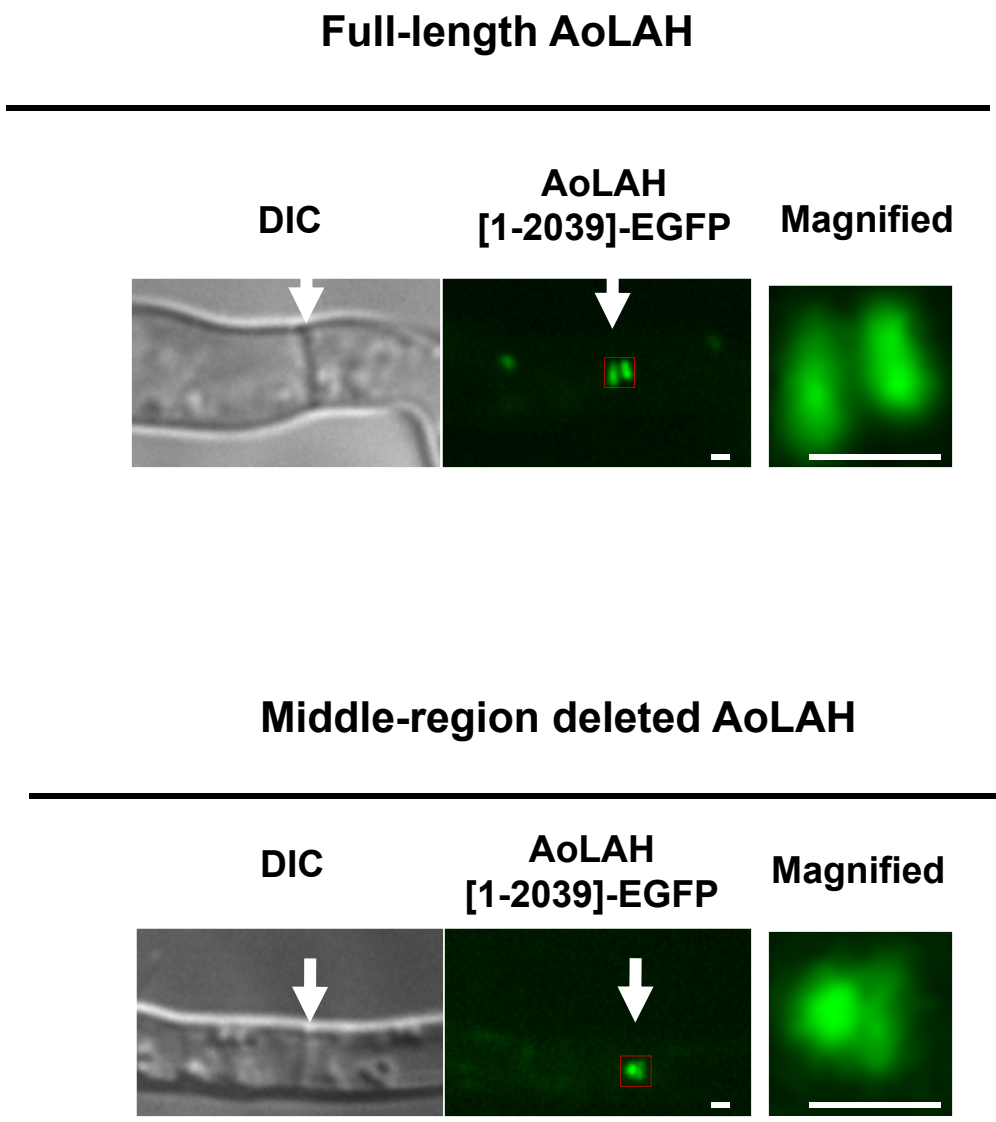


FIG. 3-6. Fluorescence microscopy of Woronin bodies visualized with the AoLAH N-terminal region-EGFP fusion protein in the *Aolah* disruptant strain expressing full-length or middle-region deleted AoLAH. Woronin bodies visualized by expression of AoLAH[1-2039]-EGFP near the septum in the strain expressing full-length or middle-region deleted AoLAHs. Arrows indicate the septum. Bars: 1 μm

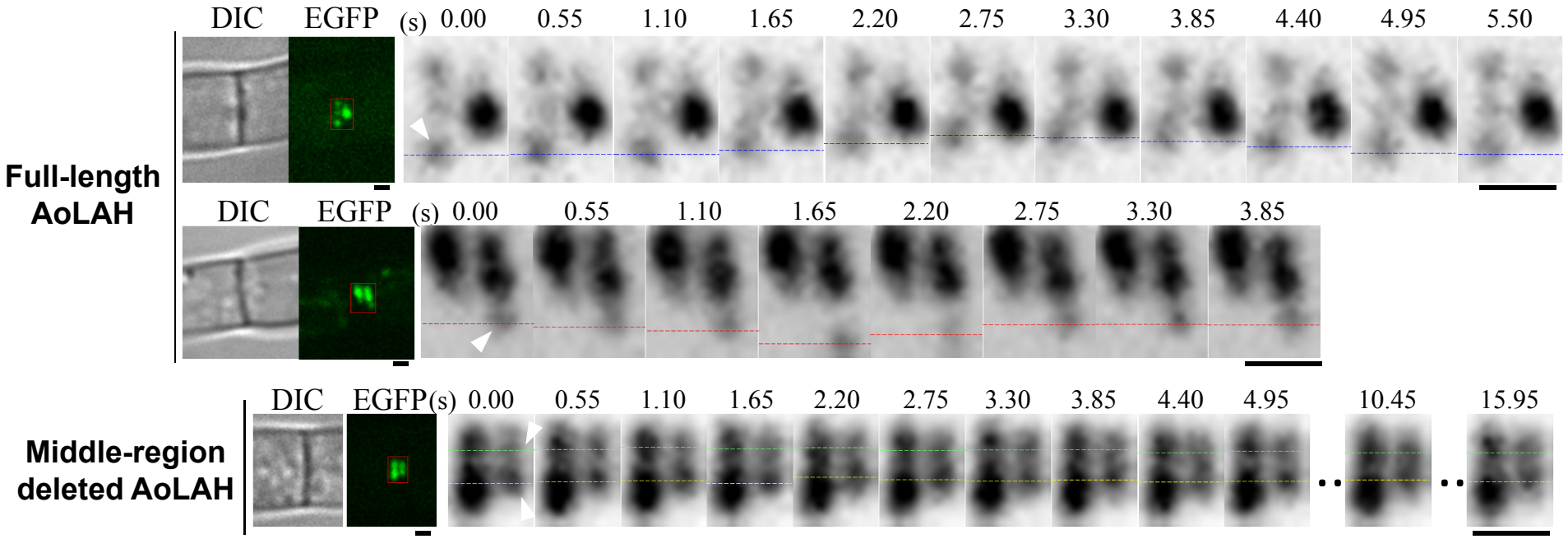


FIG. 3-7. Back-and-forth movement of the Woronin bodies visualized by AoLAH [1-2039]-EGFP. Woronin body movement at the first hyphal septum was analyzed by time-lapse analysis with a time interval of 550 ms. Tracked Woronin bodies are pointed by arrowheads in the first frame of time-lapse analysis. Blue, red, green and yellow dotted lines indicate the position of Woronin bodies in individual frames. Note that the back-and-forth movement was not clearly detected in the strain expressing the middle-region deleted AoLAH. Bars: 1 μm.

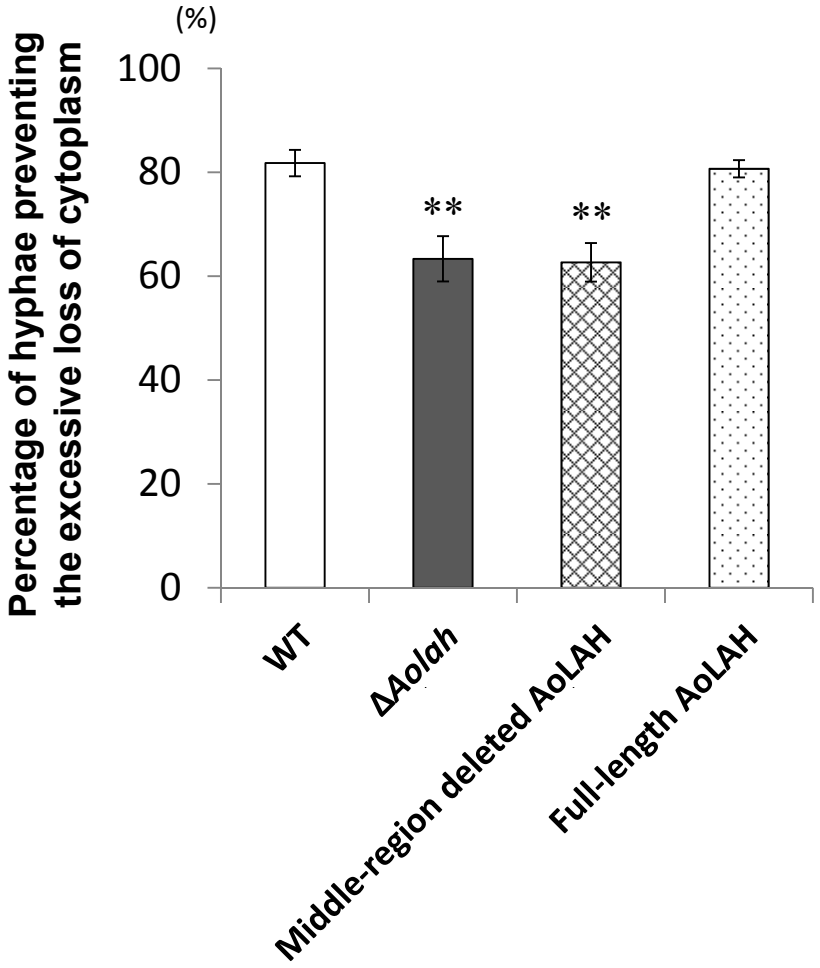


FIG. 3-8. Functional analysis of Woronin bodies in the Aolah disruptant strain expressing the middle-region deleted AoLAH. Colonies formed on agar medium were subjected to hypotonic shock and the ability of hyphae to prevent the excessive loss of cytoplasm was analyzed. The error bars indicate standard deviations. **, p <0.01 (One-way ANOVA, Dunnett's post hoc test. Comparison with the wild-type strain. n=9).

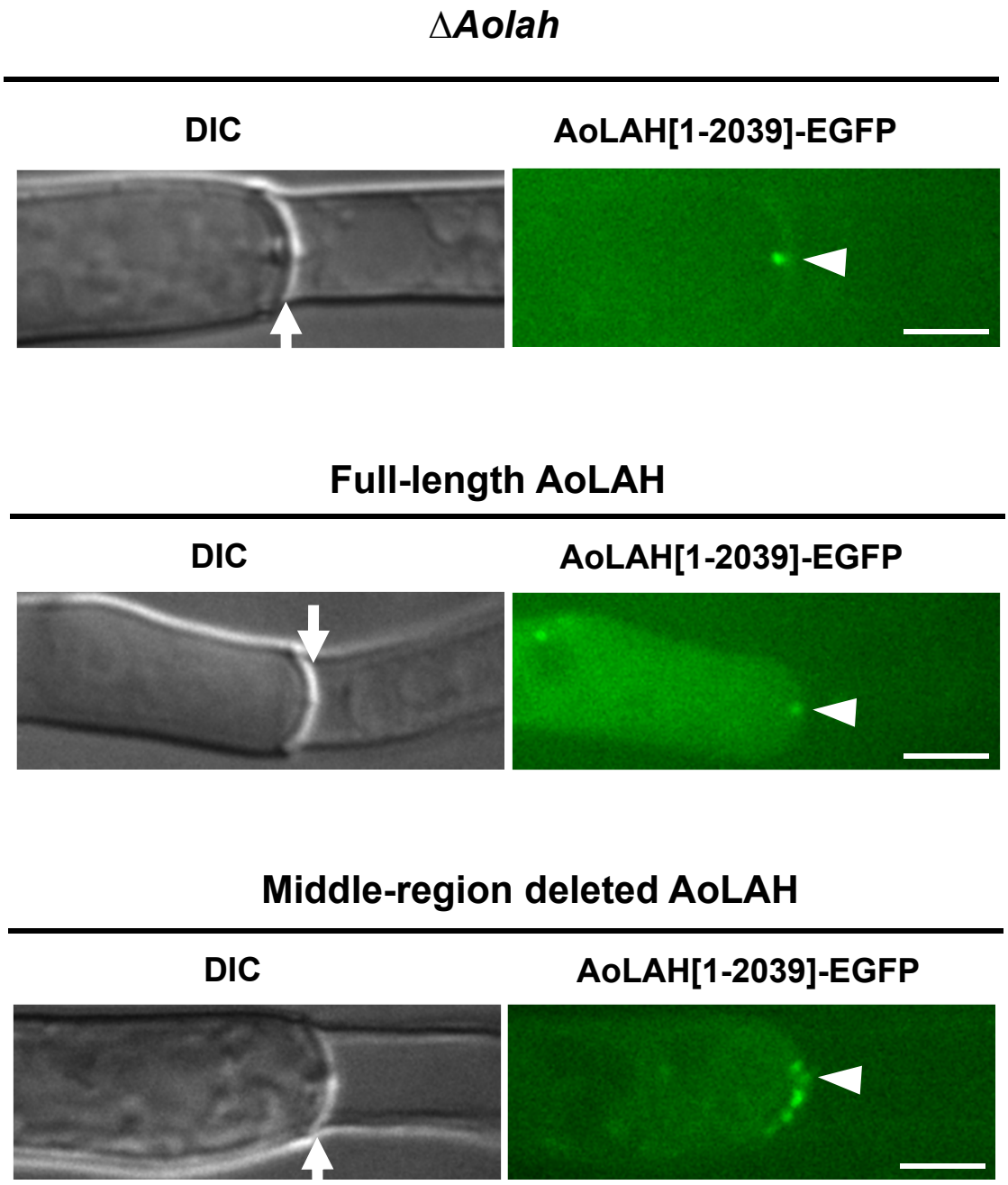


FIG. 3-9. Woronin body localization around the septum upon hyphal wounding in the *Aolah* disruptant, and *Aolah* disruptant strains expressing full-length AoLAH or middle-region deleted AoLAHs. Woronin bodies were visualized using AoLAH[1-2039]-EGFP. Arrows indicate the septum and arrowheads show the septal pore. Bars: 5 μm.

General Overview

Woronin body is a Pezizomycotina-specific organelle typically tethered to the septum, and it plug the septal pore to prevent the excessive loss of cytoplasm upon hyphal wounding (Brenner and Carroll 1968; Trinci and Collinge, 1974; Collinge and Markham, 1985). In the majority of Pezizomycotina species, Woronin bodies are tethered to the septum, while in a small group including *Neurospora crassa* have evolved cell cortex association of Woronin bodies (Momany *et al*., 2002; Maruyama *et al*., 2005; Ng *et al*., 2009). *leashin* (*lah*) gene locus was originally identified in *N. crassa* to be required for the cell cortex association of Woronin bodies (Ng *et al.*, 2009). However, the role of the LAH protein in the process for septal plugging had not been characterized, and it was not known whether the tethering to the septum is sufficient to fulfill the Woronin body function. 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.

In Chapter 1, *Aolah* gene encoding a protein consisting of 5,727 amino acids with a similarity to *N. crassa* LAH proteins was functionally analyzed. By transmission electron microscopic analysis, it was concluded that AoLAH is required for the tethering of Woronin bodies to the septum. In the *Aolah* disruptant, the ability of preventing the excessive loss of cytoplasm was reduced. This indicates that AoLAH is involved in Woronin body function to prevent the excessive loss of cytoplasm upon hyphal wounding.

In Chapter 2, according to homology comparison among filamentous fungi, LAH protein consists of N- (~2,000 amino acids) and C- (~1,000 amino acids) terminal conserved regions and a long-stretched (~2,700 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. The AoLAH N-terminal region was normally localized at both sides of the septum and, upon hyphal wounding, plugged at the septal pore, which is a behavior similar to septal plugging of Woronin bodies. The AoLAH N-terminal region was recruited to Woronin bodies in AoWSC dependent manner (Fig. 2-7; Fig. 2-8). The presence of the endogenous AoLAH protein is required for the localization of the AoLAH N-terminal region near the septum, which is a behavior similar to the tethering of Woronin bodies as observed by electron microscopy. Collectively, the AoLAH N-terminal region exhibited localization patterns typical to those of Woronin bodies, and it was proposed that AoLAH[1-2039]-EGFP is a reliable marker of Woronin bodies. Localization analysis by expressing AoLAH[4710-5727]-EGFP revealed that the AoLAH C-terminal region itself is capable of being associated to the septal pore.

The long-stretched middle region is non-conserved among the LAH proteins (Fig. 1-3). As its involvement in the Woronin body function had not yet been investigated, I functionally characterized the middle region of AoLAH. In Chapter 3. When the N- and C-terminal fusion construct lacking the middle region (~2,700 amino acids) of AoLAH was expressed in the *Aolah* disruptant, Woronin bodies were re-tethered to the septum. This result indicates that the middle-region deleted AoLAH is sufficient for the tethering of Woronin bodies to the septum. However, the distance of Woronin bodies to the septum was shorter in the strain expressing the middle-region deleted AoLAH (Fig 3-5). This suggests that the middle region of AoLAH has a role in regulating the distance of Woronin bodies to the septum.

Although Woronin bodies were tethered to the septum by expressing the middle-region deleted AoLAH construct in the *Aolah* disruptant, the ability to prevent

the excessive loss of cytoplasm was not restored. This indicates that the middle region of AoLAH is involved in the Woronin body function. Moreover, in the strain expressing the middle-region deleted AoLAH, besides the septal plugging, Woronin bodies tethered to the septum were still frequently observed. 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 conferred by the AoLAH middle region.

Elasticity in the tethering of Woronin bodies to the septum was previously suggested in *N. haematococca*, in which Woronin bodies quickly moved back to the original position after pulled with laser (Berns *et al*., 1992). However, elastic movement of Woronin bodies had not been naturally observed. In this study, I sought to observe the Woronin bodies visualized with AoLAH[1-2039]-EGFP in living cells. When the first septum of *A. oryzae* hyphae was observed, at least one of the tethered Woronin bodies showed a quick back-and-forth movement in the full-length AoLAH expressing strain. This observation demonstrates 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.

Prospects

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. However, 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.

The mammalian muscle protein titin exhibits elasticity like a molecular spring via its intrinsically disordered region. AoLAH is also predicted to be highly disordered, particularly in the N-terminal and middle regions (Fig. 3-10). In Western blotting, the full-length and middle-region deleted AoLAHs were detected with the larger size than the predicted molecular weight (Fig. 3-3). This may be caused by the presence of intrinsically disordered characteristics in N-terminal and middle regions of AoLAH, which is in agreement with previous reports that there is slower migration of intrinsically disordered protein in SDS-PAGE (Uversky *et al.*, 2010; Iakoucheva *et al.*, 2012). Since the middle region of AoLAH is predicted to be intrinsically disordered (Fig. 3-10), it was hypothesized that the middle region might confer the elastic characteristics to the tethering linker of Woronin bodies.

The middle region of AoLAH does not share high similarity with other fungal LAH proteins (Fig. 1-3); however, several poly-lysine stretches are commonly present in these proteins, although their interval length is not conserved (Fig. 1-4). Glutamate was the most frequently found amino acid in the middle region of AoLAH, a feature that is also seen in other *Aspergillus* species (Fig. 3-11). The mammalian muscle protein titin exhibits molecular spring-like elasticity via its intrinsically disordered region, and contributes to the contraction-relaxation cycle of skeletal muscle (Li *et al.*, 2001). Calcium reduces the length of titin, and this calcium-dependent process

requires the presence of a glutamate-rich motif in the disordered region (Labeit *et al.*, 2003). The high content of glutamate in the middle region of AoLAH raises the possibility that this protein may also be calcium-sensitive, given the fact that the calcium concentration would be increased upon hyphal wounding, as has been reported in other organisms (McNeil *et al.*, 2005). I speculate that glutamates frequently found in the middle region of AoLAH may be involved in the extension and contraction of the Woronin body tether.

Intrinsically disordered proteins have recently been reported to have many biological roles (Rauscher *et al.*, 2012). A number of intrinsically disordered proteins are reported to localized to the septum in *N. crassa*, some of them associate with Woronin bodies at the occluded septal pore within minutes of cellular wounding, while some associate with Woronin bodies at the septal pore during cellular wound healing (Lai *et al.*, 2012). However, with the exception of a few studies (Lai *et al.*, 2012; Hurley *et al.*, 2013), little knowledge about the function of intrinsically disordered proteins has been accumulated in filamentous fungi. Here, I unexpectedly discovered the physiological importance of a non-conserved region in AoLAH that is predicted to be intrinsically disordered and potentially functions as a molecular spring, although further analyses of this protein are needed to confirm these findings. The present 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.

C-terminal region of AoLAH is conserved among the *Aspergillus* species, and itself functions to target to the septum. However, it is not known how it associates with the septum, and whether there is a receptor or it binds directly to the lipid on plasma

membrane. Investigation to this problem will provide a better understanding of the machinery for this Woronin body mediated septal plugging in the event of hyphal wounding.

Deletion of AoSO, AoSO-interacting proteins SoiA and SoiB, AoFus3 and AoFus3-interacting proteins FipA and FipB all displayed reduced ability of preventing the excessive loss of cytoplasm (Maruyama *et al*., 2010; Saeki, 2012; Sasaki, 2011; Yahagi, 2013)). It is unknown how these proteins function to limit the loss of cytoplasm, and possibilities could be that they affect tethering and septal plugging of Woronin bodies or that they have another machinery of septal plugging independently of Woronin body. Thus, it is very useful to employ the system that established in this study for observation of Woronin body in these protein disruptants. The appliance of this observation method of Woronin body will help us to unravel more features of this organelle in different conditions.

Through these plans I will direct to draw a whole picture of machinery for septal tethering and plugging of Woronin bodies.

A. oryzae
AoLAH

E (14.46%); A (11.05%); P (9.29%).

A. fumigatus
LAH

E (14.92%); A (10.82%); P (9.81%).

A. nidulans
LAH

E (15.79%); A (10.37%); P (8.98%).

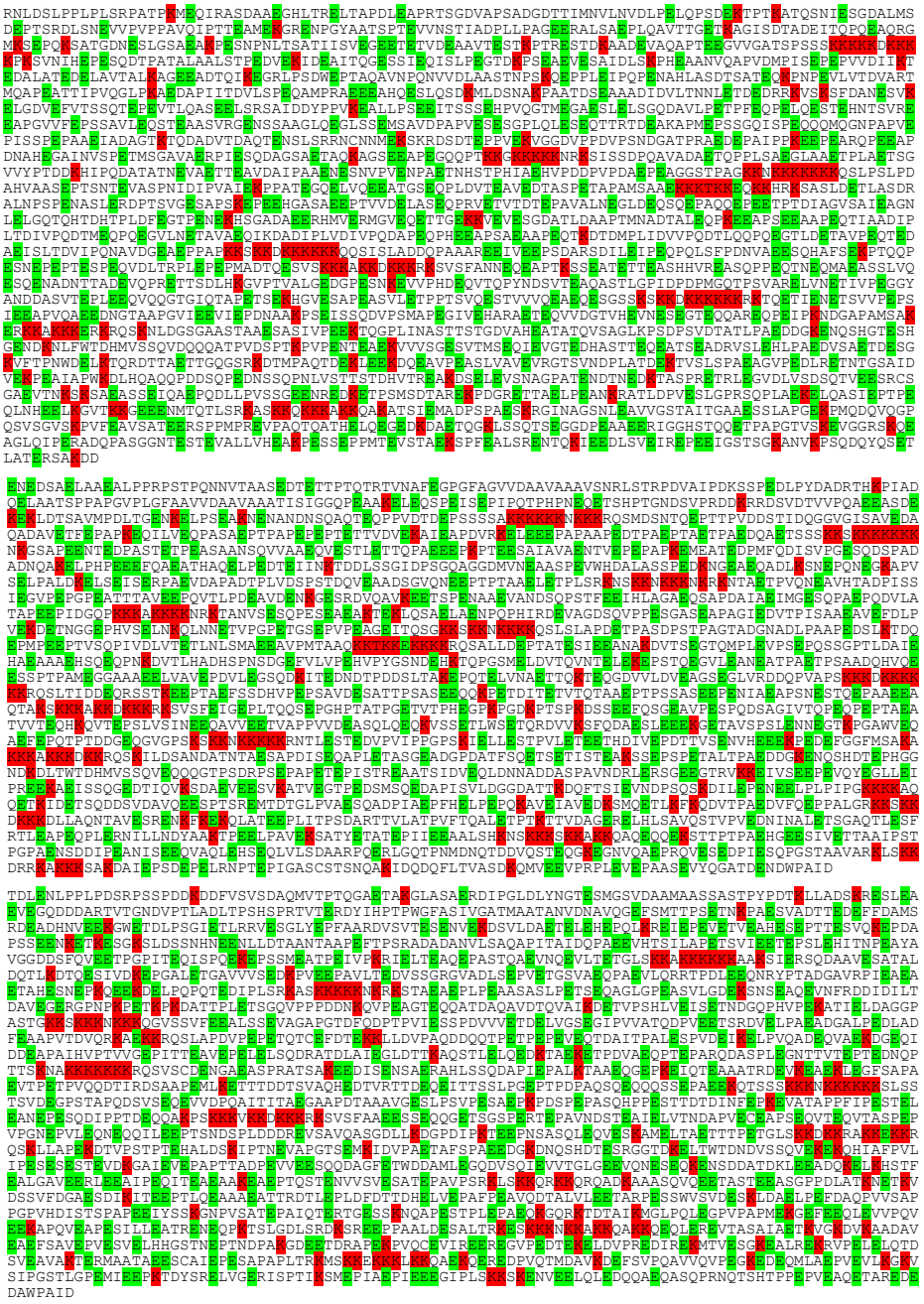


FIG. 5-2. Amino acid content of the middle region of LAH protein. The amino acid content of the LAH proteins was calculated and the three most abundant amino acids are listed. Important amino acids are highlighted by different colors; E and K are highlighted by green and red, respectively.

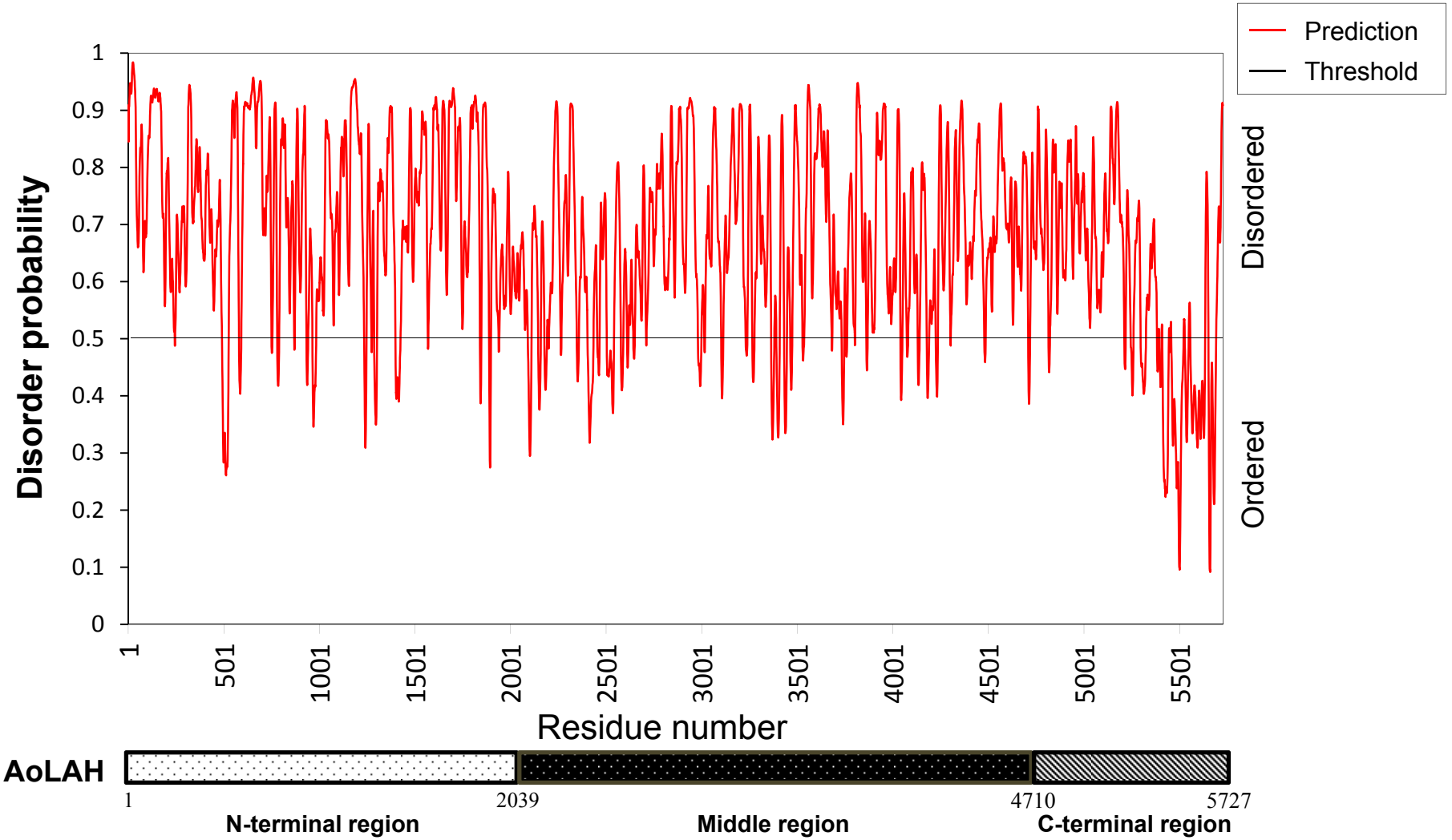


FIG. 5-1. Intrinsically disorder prediction analysis for AoLAH. The disorder probability of AoLAH was predicted by PrDOS (Protein DisOrder prediction System; http://prdos.hgc.jp/cgi-bin/top.cgi). The plot of disorder probability of each residue along the sequence is shown. Residues beyond the black threshold line in this plot are predicted to be disordered. Note that most of the AoLAH N-terminal and middle regions is predicted to be disordered, and that part of the AoLAH C-terminal region is predicted to be ordered.

Materials and Methods

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

Strains and growth media.

Strains used in this study are listed in Table M-1. The *A. oryzae* wild-type strain RIB40 (Machida *et al*., 2005) was used as a DNA donor. *Escherichia coli* DH5α was used for DNA manipulation. *A. oryzae* NSRKu70-1-1 (Escaño *et al*., 2009) was used as a host strain for gene disruption. DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.5) was used for liquid cultivation and growth analyses of the *A. oryzae* strains. Czapek Dox (CD) medium (2% glucose, 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, pH 5.5), M medium (2% glucose, 0.2% NH_4Cl, 0.1% $(NH4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, pH 5.5) and their methionine-supplemented media were used for transformation and growth analyses of *A. oryzae*. Transformation of *A. oryzae* was carried out as described previously (Maruyama *et al*., 2011). Potato Dextrose (PD) agar medium (Nissui, Tokyo, Japan) was used for the harvesting of conidia.

Construction of an *Aolah* disruptant.

For disruption of the *Aolah* gene, the plasmid pgdAoLAH was constructed using the MultiSite Gateway™ cloning system (Invitrogen, Carlsbad, CA, USA). The 1.5-kb upstream region of the *Aolah* gene was amplified by PCR using the primer pair (attB4)Aolah-up-F and (attB1)Aolah-up-R, and the 1.5-kb downstream region was amplified by PrimeSTAR HS DNA polymerase (Takara) using primers (attB2)Aolah-down-F_2 and (attB3)Aolah-down-R_2. The upstream and

downstream DNA fragments of the *Aolah* gene were cloned into the entry vectors pDONR-P4-P1R and pDONR-P2R-P3, respectively, using the BP clonase reaction of the MultiSite Gateway™ system. The obtained 5' and 3' entry clones and the center entry clone pgEaA, containing the *adeA* marker gene (21), were combined for the LR clonase reaction with the destination vector pDEST R4-R3 to obtain plasmid pgdAoLAH. A deletion DNA fragment of the *Aolah* gene was amplified by PCR using the plasmid pgdAoLAH as template and the primers (attB4)Aolah-up-F and (attB3)Aolah-down-R_2. The obtained PCR product was introduced into strain NSRKu70-1-1 (18) and M agar medium supplemented with 0.15% methionine was used for the selection of *adeA*$^{+}$ transformants. All six *Aolah* disruptants obtained in this study showed nearly identical phenotypes, and strain NSK-Δlah2 was used as the representative *Aolah* disruptant. Strain NSRKu70-1-1A, which was constructed by introducing the plasmid pAdeA containing the *adeA* marker gene into strain NSRKu70-1-1 (18), was used as a wild-type strain. An *Aohex1* disruptant strain NSK-Δhex1 was obtained using strain NSRKu70-1-1 as previously performed (13), and used for a comparative phenotype analysis under the same genetic background as the *Aolah* disruptant.

Construction of an *Aowsc* disruptant.

For disruption of the *Aowsc* gene, plasmid pgdAoWSC was constructed using the MultiSite Gateway™ cloning system. Briefly, the 1.5-kb upstream and downstream regions of the *Aowsc* gene were amplified by PCR using the primer pairs (attB4)Aowsc-up-F and (attB1)Aowsc-up-R, and (attB2)Aowsc-down-F and (attB3)Aowsc-down-R, respectively. The amplified upstream and downstream DNA fragments were cloned into the entry vectors pDONR-P4-P1R and pDONR-P2R-P3, respectively, by the BP clonase reaction. The obtained 5' and 3' entry clones were mixed with the center entry clone pgEaA, containing the *adeA* marker gene, for the LR

clonase reaction in the presence of the destination vector pDEST R4-R3 to obtain plasmid pgdAoWSC. The deletion fragment for the *Aowsc* gene was amplified by PCR using plasmid pgdAoWSC as template and the primers (attB4)Aowsc-up-F and (attB3)Aowsc-down-R. The PCR product was introduced into strain NSRKu70-1-1 (18), and an *adeA*$^{+}$ transformant, designated NSK-Δwsc1, was selected on M agar medium supplemented with 0.15% methionine.

Transmission electron microscopy.

Mycelia grown on DPY agar medium at 30°C for 4 days were fixed in 0.1 M phosphate buffer (pH 7.0) containing 4% glutaraldehyde at 4°C for 4 h, and were then washed three times in 0.1 M phosphate buffer (pH 7.0) for 10 min. The washed samples were treated with a solution of 1% osmium tetroxide, 1% potassium bichromate, and 0.85% sodium chloride (adjusted to pH 7.2 with potassium hydroxide) for 1 h. The fixed mycelia were washed with water at 4°C for 2 h and then incubated in 30%, 50%, 70%, 80%, 90%, and 100% ethanol for 10 min. After overnight incubation in a mixture of dehydrated acetone and epoxy resin (4:6), the samples were embedded in epoxy resin and heated at 40, 50, 60, and 70°C for 1 day. Ultra-thin sections were prepared using an ultramicrotome fitted with a glass knife and then stained with uranyl acetate and lead citrate. Samples were observed with a JEM-1010 transmission electron microscopy (TEM; JEOL, Tokyo, Japan) and images were analyzed with ImageJ v. 1.44 software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij). The nearest distance of the Woronin body margin from the septum was measured, and 44 and 48 independent Woronin bodies were analyzed for the strains expressing full-length AoLAH and truncated AoLAH, respectively.

Hypotonic shock experiment.

To induce hyphal tip bursting, 1 ml water was added to *A. oryzae* colonies cultured on a thin

layer of DPY agar medium at 30°C for 18 h. Randomly selected 50 hyphae showing hyphal tip bursting in each culture was observed by differential interference contrast (DIC) microscopy. In this assay, it was considered that the septal pore was effectively plugged by the Woronin bodies if the cytoplasm was retained by the cells, and conversely, the excessive loss of cytoplasm was attributed to Woronin body dysfunction. The ability to prevent the excessive loss of cytoplasm was analyzed by judging whether the cytoplasm in the cell adjacent to the burst tip cell was retained.

Southern blot analysis.

Southern blot analysis was performed using a probe amplified with the primers (attB4)Aolah-up-F and (attB1)Aolah-up-R for verification of *Aolah* gene disruption. For confirmation of *Aowsc* gene disruption, Southern blot analysis was performed using a probe amplified with the primers (attB4)Aowsc-up-F and (attB1)Aowsc-up-R. After electrophoresis, genomic DNAs digested with restriction enzymes *Eco*T22I or *Apa*I were transferred onto Hybond N+ membrane (GE Healthcare, Buckinghamshire, UK). The ECL (enhanced chemiluminescence) Direct Nucleic Acid Labeling and Detection system (GE Healthcare) and a LAS-1000plus luminescent image analyzer (Fuji Photo Film, Tokyo, Japan) were used for detection.

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

Construction of strains expressing AoLAH[1-2039]-EGFP.

A fusion protein consisting of the N-terminal 2039 amino acids of AoLAH and EGFP was constructed as follows. *Aolah[1-2039]* was amplified by PCR using the primers (attB1)Aolah_1-F and (attB2)Aolah_6243-R, and cloned into the entry vector pDONR221 using the BP clonase

reaction, generating the center entry clone pgAoLAH[1-2039]. The 5' entry clone pg5'PaB (P*amyB*) (22), center entry clone pgAoLAH[1-2039], 3' entry clone pg3'E (*egfp*) (22), and the destination vector pgDSO containing the *A. oryzae sC* gene as a selectable marker (18) were subjected to the LR clonase reaction. The generated plasmid, designated pgAoLAH[1-2039]G, was introduced into the wild-type strain (NSRKu70-1-1A) and *Aolah* disruptant (NSK-Δlah2), which were then plated on M agar medium for the selection of transformants. The resulting transformants were named NSK-LAH[1-2039]G and NSK-Δlah-LAH[1-2039]G, respectively.

Construction of strains for visualization of Woronin bodies and peroxisomes.

A DNA fragment for mDsRed fused with PTS1 (peroxisomal targeting signal 1) was amplified by PCR using the primers (attB1)-DsRed-M-F and (attB2)-PTS1-R, and cloned into pDONR221 by the BP clonase reaction, generating the center entry clone pgmDsRed-PTS1. The 5' entry clone pg5'PaB containing the *amyB* promoter, center entry clone pgmDsRed-PTS1, 3' entry clone pg3'TaN containing the *amyB* terminator and *niaD* marker, and the destination vector pDEST R4-R3 were mixed for the LR clonase reaction. The generated plasmid was named pgDPTS1N and introduced into strain NSK-LAH[1-2039]G. One of the resulting transformants was named NSK-LAH[1-2039]G-DPTS1 and was used in experiments to allow the visualization of Woronin bodies and peroxisomes.

Construction of strains expressing AoLAH[4710-5727]-EGFP.

A fusion protein consisting of the C-terminal region of AoLAH (amino acids 4710-5727) and EGFP was constructed as follows. *Aolah[4710-5727]* was PCR amplified using the primers Aolah-Cter-F(14254) and Aolah-Cter-R(17400), and cloned into pDONR221 using the BP clonase reaction, generating the center entry clone pgAoLAH[4710-5727]. The 5' entry clone pg5'PaB

(P*amyB*), center entry clone pgAoLAH[4710-5727], 3' entry clone pg3'E (*egfp*), and the destination vector pgDN (22) containing the *A. oryzae niaD* gene as a selectable marker were subjected to the LR clonase reaction. The generated plasmid, designated pgAoLAH[4710-5727]G, was introduced into the wild-type strain (NSRKu70-1-1A) and *Aolah* disruptant (NSK-Δlah2), which were then plated on CD agar medium supplemented with 0.0015% methionine for the selection of transformants. The resulting transformants were named NSK-LAH[4710-5727]G and NSK-Δlah-LAH[4710-5727]G, respectively.

Confocal microscopy.

Conidia (1×10^3) of the strain expressing an EGFP or DsRed fusion protein were pre-inoculated into 100 μL CD liquid medium supplemented with 0.0015% methionine in a glass-bottom dish. After incubation at 30°C for 18-24 hr, the cultures were observed by confocal laser scanning microscopy using an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with a 100x Neofluor objective lens (1.40 numerical aperture), 488 nm semiconductor laser (Furukawa Electric, Tokyo, Japan), GFP and DualView filters (Nippon Roper, Chiba, Japan), CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan), and Andor iXon cooled digital CCD camera (Andor Technology PLC, Belfast, UK). Images were analyzed with Andor iQ software (Andor Technology PLC) and Meta-morph software (Molecular Devices, Sunnyvale, CA).

Chapter 3. Functional analysis of AoLAH middle region

Construction of a strain expressing *Aolah* with a truncated middle region.

DNA fragments for AoLAH[1-2039] and AoLAH[4710-5727] were amplified by PrimeSTAR HS DNA polymerase (Takara) using the primers (attB1)Aolah_1-F and Aolah_N-R-fusion, and

Aolah_C-F-fusion and Aolah-Cter-R(17400), respectively. The two DNA fragments were fused by PCR using the primers (attB1)Aolah_1-F and Aolah-Cter-R(17400), and the resulting DNA fragment for AoLAH[(1-2039)+(4710-5727)] was cloned by the BP clonase reaction into the entry vector pDONR-P221. The obtained center entry clone was mixed with the 5' entry clone pg5'PaB, containing the *amyB* promoter, and 3' entry clone pg3'HA3, containing the *3×HA* gene, for the LR clonase reaction in the presence of the destination vector pgDN to obtain plasmid pAoLAH[(1-2039)+(4710-5727)]N, containing *niaD* as a selectable marker. Plasmid pAoLAH[(1-2039)+(4710-5727)]N was introduced into the *Aolah* disruptants NSK-Δlah and NSK-Δlah-LAH[1-2039]EGFP, and positive transformants were selected on CD agar medium supplemented with 0.0015% methionine and unsupplemented CD agar medium, respectively. The expression of truncated AoLAH was confirmed by Western blot analysis.

Complementation of the *Aolah* disruptant.

For complementation of the *Aolah* disruptant (NSK-Δlah2), a plasmid expressing full-length AoLAH was constructed. The *Aolah* coding region (not including the stop codon) was PCR amplified using the primers (attB1)Aolah_1-F and Aolah-Cter-R(17400), and then cloned into the entry vector pDONR-P221 by the BP clonase reaction. PrimeSTAR GXL DNA Polymerase, a DNA Polymerase allows amplification of products ≧ 30 kb in length while maintaining exceptionally high fidelity was used for amplification of this long fragement. The obtained center entry clone, 5' entry clone pg5'PaB, containing the *amyB* promoter, and the 3' entry clone pg3'HA3, containing the *3×HA* gene, were subjected to the LR clonase reaction with the destination vector pgDN to obtain plasmid pAoLAHN, which contained *niaD* as a selectable marker. Plasmid pgAoLAHN was introduced into strains NSK-Δlah and NK-Δlah-LAH[1-2039]G, and positive transformants were selected on CD agar medium supplemented with 0.0015%

methionine and unsupplemented CD agar medium, respectively. The plasmid pNR10 containing the *niaD* marker was transformed into wild-type strain NSRKu70-1-1A and the *Aolah* disruptant NSK-ΔAolah as controls.

Protein extraction and Western blot analysis.

The A. oryzae strains expressing full-length AoLAH (AoLAH-3×HA) or middle-region deleted AoLAH (AoLAH[(1-2039)+(4710-5727)]-3×HA) were grown in DPY liquid nutrient medium as shaking cultures for 24 h at 30°C. Cell extracts were prepared by homogenizing the mycelia using liquid nitrogen in an elution buffer (50 mM Tris/HCl, pH 7.5, 1 mM PMSF, and 1:100 protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO]). Total cell lysates were centrifuged at 500×g for 3 min to remove cell debris, and the obtained supernatants were further centrifuged (10,000×g, 10 min, 4°C). The resulting pellet fraction was used for detection of middle-region deleted AoLAH. To prepare a fraction highly enriched with Woronin body proteins, the 10,000×g pellet fraction was re-suspended in the elution buffer supplemented with 0.5% Triton X-100, and then centrifuged at 20,000×g, 4°C for 10 min, of which the pellet fraction was used for detection of full-length AoLAH. The pellet fractions were dissolved in sampling buffer and analyzed by Western blotting. NuPAGE® Novex 3%-8% Tris-Acetate Gel, NuPAGE®LDS Sample Buffer (4X), and HiMark™ Pre-Stained Protein marker (Invitrogen Life Technologies, Carlsbad, CA) were used for Western blotting analysis. The primary antibodies used was mouse anti-HA monoclonal antibody (12CA5; Roche, Mannheim, Germany), and the secondary was peroxidase-conjugated anti-mouse antibody (Vector Laboratories, Burlingame, CA). Protein bands were detected and analyzed by the ECL detection reagents (Pierce, Rockford, IL) and a LAS-1000plus luminescent image analyzer.

Hypotonic shock experiment. The same procedure was followed as mentioned on Chapter 1 and Chapter 2.Material and methods section. 1 ml water was added to *A. oryzae* colonies cultured on a thin layer of DPY agar medium at 30°C for 18 h. Randomly selected 50 hyphae showing hyphal tip bursting in each culture was observed by differential interference contrast (DIC) microscopy.

Chapter4. Investigation of mechanisms for septal tethering of Woronin bodies

Construction of strains expressing AoLAH[1-2039]-EGFP.

The expression plasmid pgAoLAH[1-2039]G, was introduced into the Δ*soiA*, Δ*soiB,* Δ*fipA* strain and positive transformants have been selected and named as NSK-ΔsoiA-LAH[1-2039]G, NSK-ΔsoiB-LAH[1-2039]G, NSK-ΔfipA-LAH[1-2039]G.

Tables M-1. Strains used in this study for Chapter 1.

Name	Parental strain	Genotype
RIB40[a]		wild-type
NSRKu70-1-1[b]		*niaD⁻ sC⁻ adeA⁻* Δ*argB*::*adeA⁻* Δ*ku70*::*argB*
NSK-Δlah2	NSRKu70-1-1	*niaD⁻ sC⁻ adeA⁻* Δ*argB*::*adeA⁻* Δ*ku70*::*argB* Δ*Aolah*::*adeA*
NSK-Δwsc1	NSRKu70-1-1	*niaD⁻ sC⁻ adeA⁻* Δ*argB*::*adeA⁻* Δ*ku70*::*argB* Δ*Aowsc*::*adeA*
NSK-Δhex1	NSRKu70-1-1	*niaD⁻ sC⁻ adeA⁻* Δ*argB*::*adeA⁻* Δ*ku70*::*argB* Δ*Aohex1*::*adeA*
NSRKu70-1-1A[b]	NSRKu70-1-1	*niaD⁻ sC⁻ adeA⁻* Δ*argB*::*adeA⁻* Δ*ku70*::*argB* pAdeA (*adeA*)

a: Machida *et al.* 2005, b: Escaño *et al.* 2009

Tables M-2. Strains used in this study for Chapter 2.

Name	Parental strain	Genotype
NSK-LAH[1-2039]G	NSRKu70-1-1A	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* pAdeA (*adeA*)
		pgAoLAH[1-2039]G (P-*amyB*::*Aolah[1-2039]-egfp*::T-*amyB*::*AosC*)
NSK-Δlah-LAH[1-2039]G	NSK-Δlah	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAH[1-2039]G (P-*amyB*::*Aolah[1-2039]-egfp*::T-*amyB*::*AosC*)
NSK-Δwsc-LAH[1-2039]G	NSK-Δwsc	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aowsc*::*adeA*
		pgAoLAH[1-2039]G (P-*amyB*::*Aolah[1-2039]-egfp*::T-*amyB*::*AosC*)
NSK-LAH[4710-5727]G	NSRKu70-1-1A	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* pAdeA (*adeA*)
		pgAoLAH[4710-5727]G (P-*amyB*::*Aolah[4710-5727]-egfp*::T-*amyB*::*niaD*)
NSK-Δlah-LAH[4710-5727]G	NSK-Δlah	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAH[4710-5727]G (P-*amyB*::*Aolah[4710-5727]-egfp*::T-*amyB*::*niaD*)
NSK-Δlah-LAH-3HA	NSK-Δlah	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAHN (P-*amyB*::*Aolah-3HA*::T-*amyB*:: *niaD*)
NSK-Δlah-LAH[1-2039]G-LAH-3HA	NSK-Δlah-LAH[1-2039]G	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAH[1-2039]G (P-*amyB*::*Aolah[1-2039]-egfp*::T-*amyB*::*AosC*)
NSK-LAH[1-2039]G-DPTS1	NSK-Δlah-LAH[1-2039]G	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAH[1-2039]G (P-*amyB*::*Aolah[1-2039]-egfp*::T-*amyB*::*AosC*)
		pgDPTS1N (P-*amyB*::*mdsred-pts1*::T-*amyB*::*niaD*)

a: Machida *et al.* 2005, b: Escaño *et al.* 2009

Tables M-3. Strains used in this study for Chapter 3.

Name	Parental strain	Genotype
NSK-LAH[(1-2039)+(4710-5727)]-3HA	NSK-Δlah	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAH[(1-2039)+(4710-5727)]N (P-*amyB*::*Aolah[(1-2039)+(4710-5727)]-3×HA*::T-*amyB*::*niaD*)
NSK-LAH[1-2039]G-LAH[(1-2039)+(4710-5727)]-3HA	NSK-Δlah-LAH[1-2039]G	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA*
		pgAoLAH[1-2039]G (P-*amyB*::*Aolah[1-2039]-egfp*::T-*amyB*::*AosC*)
		pgAoLAH[(1-2039)+(4710-5727)]N (P-*amyB*::*Aolah[(1-2039)+(4710-5727)]-3×HA*::T-*amyB*::*niaD*)
NSRKu70-1-1AN	NSRKu70-1-1A	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* pAdeA (*adeA*) pNR10 (*niaD*)
NSK-ΔlahN	NSK-Δlah2	*niaD*⁻ *sC*⁻ *adeA*⁻ Δ*argB*::*adeA*⁻ Δ*ku70*::*argB* Δ*Aolah*::*adeA* pNR10 (*niaD*)

a: Machida *et al.*2005, b: Escaño *et al.* 2009

Table M-4. Primers used in this study

Name	Sequence (5'-3')
(attB4)Aolah-up-F	GGGGACAACTTTGTATAGAAAAGTTGGGAGGATTGCCTCCGCATACAATAC
(attB1)Aolah-up-R	GGGGACTGCTTTTTTGTACAAACTTGGCCTTGATCGCTCTCTGCCCACT
(attB2)Aolah-down-F_2	GGGGACAGCTTTCTTGTACAAAGTGGGATGACTATGATGACTGCCAACATCTCC
(attB3)Aolah-down-R_2	GGGGACAACTTTGTATAATAAAGTTGCGATATGGAACCGATTCAAAGTCAACTCC
(attB1) Aolah_1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTTTAAGGCCTTATTGGCCGGGGGCCGT
(attB2) Aolah_6243-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCCAGCCCGCCATCGTCTTTTACACCGA
Aolah-Cter-F (14254)	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGAATGGCCTTTGATAGATTGGGAGAAGGAA
Aolah-Cter-R (17400)	GGGGACCACTTTGTACAAGAAAGCTGGGTCAATCACATTGCTCATGTCCATGGTCGACGA
Aolah_N-R-fusion	TATCAAAGGCCATTCCTCCAGCCCGCCATCGTCTTTTACACCGAC
Aolah_C-F-fusion	GATGGCGGGCTGGAGGAATGGCCTTTGATAGATTGGGAGAAGGAA
(attB4) Aowsc-up-F	GGGGACAACTTTGTATAGAAAAGTTGAGATGAGAGCATAGCGCGGTACC
(attB1) Aowsc-up-R	GGGGACTGCTTTTTTGTACAAACTTGGATGGCGGTTGATGCGGTTGCGT
(attB2) Aowsc-down-F	GGGGACAGCTTTCTTGTACAAAGTGGATAGCGTTACGACCAACGTCGCG
(attB3) Aowsc-down-R	GGGGACAACTTTGTATAATAAAGTTGTGCCTCCAAGAGGCGAAAGTCAGT
(attB1)-DsRed-M-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGGACAACACCGAGGACGTCATC
(attB2)-PTS1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCATAATTTGGACTGGGAGCCGGAGTGGCG

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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

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