Disruption of the Aopex11-1 Gene Involved in Peroxisome Proliferation Leads to Impaired Woronin Body Formation in Aspergillus oryzae

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The Woronin body, a unique organelle found in the Pezizomycotina, plugs the septal pore upon hyphal damage to prevent excessive cytoplasmic bleeding. Although it was previously shown that the Woronin body buds out from the peroxisome, the relationship between peroxisomal proliferation/division and Woronin body differentiation has not been extensively investigated. In this report, we examined whether Pex11 required for peroxisomal proliferation participates in Woronin body formation in Aspergillus oryzae. A. oryzae contained two orthologous PEX11 genes that were designated Aopex11-1 and Aopex11-2. Deletion of Aopex11 genes revealed that only the ΔAopex11-1 strain showed reduced growth and enlarged peroxisomes in the presence of oleic acid as a sole carbon source, indicating a defect in peroxisomal function and proliferation. Disruption of Aopex11-1 gene impaired the Woronin body function, leading to excessive loss of the cytosol upon hyphal injury. Dual localization analysis of the peroxisome and Woronin body protein AoHex1 demonstrated that Woronin bodies fail to fully differentiate from peroxisomes in the ΔAopex11-1 strain. Furthermore, distribution of AoHex1 was found to be peripheral in the enlarged peroxisome or junctional in dumbbell-shaped peroxisomes. Electron microscopy of the ΔAopex11-1 strain revealed the presence of Woronin bodies that remained associated with organelles resembling peroxisomes, which was supported from the sucrose gradient centrifugation confirming that the Woronin body protein AoHex1 overlapped with the density-shifted peroxisome in the ΔAopex11-1 strain. In conclusion, the present study describes the role of Pex11 in Woronin body differentiation for the first time.

Peroxisomes are single-membrane-bounded, ubiquitous intracellular organelles of eukaryotic cells ranging from the yeasts to humans, and their biogenesis is governed by a set of “peroxins,” the proteins encoded by PEX genes (7). The physiological relevance of these organelles is highlighted by their role in diverse metabolic activities including α- and β-oxidation of fatty acids, lipid biosynthesis, protein and amino acid metabolism (45), methanol degradation (46), and the glyoxylate cycle (23). Defects in the biogenesis of peroxisomes are the molecular cause for severe inherited diseases called peroxisome biogenesis disorders such as Zellweger syndrome, neonatal adrenoleukodystrophy, and Refsum disease (9). The yeast Saccharomyces cerevisiae, with at least 32 peroxins, has served as an excellent model to study peroxisome biogenesis (10). Of the 32 peroxins, 20 mammalian and 15 plant homologues have been identified to date (5, 10, 30, 31). While a majority of these peroxins are involved in matrix protein transport and peroxisomal formation (44), several others are required for peroxisome proliferation and division (48).

The Woronin body, a unique organelle found in the Pezizomycotina, plugs the septal pore in the event of hyphal damage (27). It is typically identified as a single-membrane-bounded structure in very close proximity to the septa (27). Jedd and Chua (12) first identified HEX-1 as the major protein of the Woronin body from Neurospora crassa. The genes encoding HEX-1 are well conserved in other members of the Pezizomycotina (1, 6, 12, 29, 38). Deletion of the hex-1 gene resulted in the disappearance of Woronin bodies and caused severe cytoplasmic bleeding upon hyphal damage (12, 29, 41), thus implicating HEX-1 in Woronin body formation and in plugging the septal pore. Self-assembly of HEX-1 into a hexagonal crystalline lattice provides the Woronin body with a stable and solid core (12, 49). Phosphorylation of HEX-1 is suggested to contribute to the formation of the multicellular core of the organelle (15, 41).

Woronin body formation occurs at the hyphal apex through a process involving apically biased hex-1 gene expression in N. crassa (42). The relationship between the peroxisome and Woronin body biogenesis is beginning to emerge from the fact that HEX-1 contains a peroxisomal targeting signal sequence 1 (PTS1) at its C terminus (12). While a study in N. crassa demonstrated the budding of the Woronin bodies from the peroxisome (42), later investigations on the Δpex6 strain of Magnaporthe grisea (32) and the Δpex14 strain of N. crassa (26) had revealed the absence of Woronin bodies. More recently, an in-depth report published by Liu et al. (24) on N. crassa...
emphasized the requirement of fungal peroxins for the biogenesis of the Woronin body apart from identifying the Woronin body sorting complex (WSC), which recruits the HEX-1 assembly to the peroxisomal membrane and facilitates the budding of the Woronin body. Another recent paper described a heterologous expression system of N. crassa HEX-1 in the yeast S. cerevisiae, suggesting that dynamin-related proteins required for the division of peroxisomes form independent Woronin bodies from peroxisomes (47). However, no studies had positively shown that the components involved in peroxisomal proliferation and division contribute to the differentiation of Woronin body.

Pex11, a peripheral membrane protein of the peroxisome, was first identified as being implicated in peroxisome proliferation and division (8, 28). Lack of Pex11 in S. cerevisiae led to the formation of giant peroxisomes and resulted in a growth defect on oleate-containing medium (8). In contrast, overexpression of the PEX11 gene resulted in an increased number of enlarged peroxisomes (11). The knockdown of Pex11, Pex25, and Pex27 form the Pex11 family involved in peroxisome proliferation (43, 48). Filamentous fungus possess three isoforms of Pex11 (designated Pex11, Pex11B, and Pex11C) but no orthologs of Pex25 and Pex27 (17). In Penicillium chrysogenum overexpression of Pex11 induced peroxisome proliferation and increased penicillin production (16). In Aspergillus nidulans it was shown that loss of Pex11 (PexK) resulted in a reduced number of enlarged peroxisomes (11). The role of Pex11 in Woronin body differentiation has not been characterized yet.

In the present investigation, we disrupted the PEX11 orthologous genes in Aspergillus oryzae and studied their roles in the formation of Woronin bodies. In addition to generating strains expressing a monomeric Discosoma red fluorescent protein (mDsRed)-AoHex1 fusion protein and an enhanced green fluorescent protein (EGFP)-PTS1 fusion protein to concurrently visualize Woronin bodies and peroxisomes in the A. oryzae strains carrying deletions of pex11 (ΔAopex11), electron microscopy was also performed to gain a better insight into the involvement of AoPex11 in Woronin body differentiation.

**MATERIALS AND METHODS**

**Strains and growth media.** Strains used in this study are listed in Table 1. The wild-type A. oryzae RIB40 strain was used as a DNA donor. Escherichia coli DH5α was used for DNA manipulation. The A. oryzae Ku-deficient strain, NSRKu70-1-1 (niaDΔc adeAΔ Δku70::argB) was used as a host strain to disrupt Aopex11 genes. To construct this strain, the argB marker gene was amplified by PCR using primers argB-F and argB-R (5′-TCAAGATCGAGGATTAGAAAGGGTTGGAATTGCGTTTGGCC-3′ and 5′-TCAAGATCTGGGTTGTTGGCC-3′). The resulting fragment was digested with BglII, inserted into ligase BglII site of the plasmid pKu98pR (39), and transformed into the A. oryzae strain NSAR1 (niaDΔc adeAΔ Δku70::argB)-1 (13). The NSRKu70-1-1A strain, NSRKu70-1-1 transformed with adeAΔ was used as the control in phenotypic analyses. A. oryzae strains were cultured either in DPF medium (2% dextrin, 1% polypeptide, 0.5% yeast extract, 0.5% KH2PO4, and 0.05% MgSO4·7H2O; pH 5.5) or in M+ Met medium [0.2% NH4Cl, 0.1% (NH4)2SO4, 0.05% KCl, 0.05% NaCl, 0.1% KH2PO4, 0.05% MgSO4·7H2O, 0.002% FeSO4·7H2O, 0.15% methionine, and 2% glucose; pH 5.5], which was used as the selectable medium. A. oryzae adeAΔ transformants. Glucose as a sole carbon source in M+ Met medium was replaced with oleic acid to induce peroxisome proliferation. DPF medium containing 100 μg/ml calcofluor white (Sigma, St. Louis, MO) or 100 μg/ml Congo red (Nacalai Tesque, Inc., Kyoto, Japan) or 20 ng/ml micaflugin (Astellas Pharma, Inc., Tokyo, Japan) was used to inhibit the growth of fungal strains. Transformation of A. oryzae was performed according to the standard protocol (19).

**Construction of the ΔAopex11 strains.** The plasmids pgΔDAPx11-1 and pgΔDAPx11-2 were constructed to disrupt the Aopex11 genes using a Multisite Gateway cloning system (Invitrogen, Carlsbad, CA) (25). For the Aopex11-1 disruption, the upstream region of the Aopex11-1 gene (2.0 kb) was amplified by PCR using the primers attB4-Aopex11-1-5F (5′-GGGGGACAACTTTGTATAGAAAAGTTGCAGCTAGGACCAAGAGAAATTGTACG-3′) and attB4-Aopex11-1-3F (5′-GGGGACAACTTTGTATAGAAAAGTGGCACCCTCAAGTGTGGACTTGTTCTG-3′). For the Aopex11-2 disruption, the upstream region of the Aopex11-2 gene (2.0 kb) was amplified by PCR using the primers attB4-Aopex11-2-5F (5′-GGGGGACAACTTTGTATAGAAAAGTTGCAGCTAGGACCAAGAGAAATTGTACG-3′) and attB4-Aopex11-2-3F (5′-GGGGGACAACTTTGTATAGAAAAGTGGCACCCTCAAGTGTGGACTTGTTCTG-3′). For Aopex11-1 disruption, the upstream region of the Aopex11-1 gene (2.0 kb) was amplified by PCR using the primers attB4-Aopex11-1-5F (5′-GGGGGACAACTTTGTATAGAAAAGTTGCAGCTAGGACCAAGAGAAATTGTACG-3′) and attB4-Aopex11-1-3F (5′-GGGGGACAACTTTGTATAGAAAAGTGGCACCCTCAAGTGTGGACTTGTTCTG-3′). Disruption IMPAIRS WORONIN BODY FORMATION

**TABLE 1. Strains used in this study**

| Strain | Genotype |
|--------|----------|
| RIB40  | Wild type |
| NSRKu70-1-1 | niaDΔc adeAΔ Δku70::argB |
| NSRKu70-1-1A | Δku70::argB adeAΔ |
| NSRKu70-1-1DRH | Δku70::argB adeAΔ |
| NSRKu70-1-1A-GFP-ARL | Δku70::argB adeAΔ | PanB::egfp-pts1(SRL)::niaD |
| NSRKu70-1-1AN | Δku70::argB adeAΔ |
| NSRKu70-1-1A-GFP-ARL | Δku70::argB adeAΔ | PanB::egfp-pts1(SRL)::niaD |
| NSRCP11-1 | Δku70::argB adeAΔ |
| NSRKP11-1 | Δku70::argB adeAΔ |
| RIB40  | Wild type |
| NSRKu70-1-1 | niaDΔc adeAΔ Δku70::argB |
| NSRKu70-1-1A | Δku70::argB adeAΔ |
| NSRKu70-1-1DRH | Δku70::argB adeAΔ |
| NSRKu70-1-1A-GFP-ARL | Δku70::argB adeAΔ | PanB::egfp-pts1(SRL)::niaD |
| NSRKu70-1-1AN | Δku70::argB adeAΔ |
| NSRKu70-1-1A-GFP-ARL | Δku70::argB adeAΔ | PanB::egfp-pts1(SRL)::niaD |
| NSRCP11-1 | Δku70::argB adeAΔ |
| NSRKP11-1 | Δku70::argB adeAΔ |
ATG-3) and aT3p-Aopex11-2-3R (5'-GGGGAACAATTTTTGATAAATAAGTTGACATGTTTACCATAAGC-3'). The underlined sequences in the primers are the Multisite Gateway attB recombination sites. For double disruption of the Aopex11-1 and Aopex11-2 genes, the plasmid pgDptAx1p1-1, consisting of the Aopex11-1 gene deletion construct with the pyrimidine resistance gene (AprA) (22) as a marker, was transformed into the ΔAopex11-2 strain, and transformants exhibiting resistance to pyrimidine (0.1 mg/l) were selected. All primers were designed based on the sequence data available in the A. oryzae genome database (National Institute of Technology and Evaluation DOGAN database; http://www.bio.nite.go.jp/dogan/Top). Using the genomic DNA of A. oryzae RIB40 as a template, the upstream and downstream sequences of both the Aopex11-1 and Aopex11-2 genes were individually cloned by BP recombination (recombination of attB and attP sites) into the 5' pDONR-P4-P1R and 3' pDONR-P2R-P3 entry vectors (Invitrogen, Carlsbad, CA, respectively). The obtained 5' and 3' entry clones together with the center entry clone plasmid, pgEaA (harboring the ade4 marker gene) (14) or pgEptA (harboring the ptr4 marker gene), were then treated with LR clonase (Invitrogen, Carlsbad, CA) for recombination of the attL and attR sites in the presence of the destination vector pDEST-R4-R3 (Invitrogen, Carlsbad, CA) to obtain the final plasmids pgDAEPN and pgDpApAx1p1-1, and pgDpApAx1p1-1, respectively. The deletion cassettes for the Aopex11-1 and Aopex11-2 genes were amplified by PCR using the plasmids pgDAEPN and pgDpApAx1p1-1, and pgDpApAx1p1-1 and the following primer pairs, respectively: attB4-Aopex11-1-5F and attB3-Aopex11-1-3R; attB4-Aopex11-2-5F and attB3-Aopex11-2-3R; and attB4-Aopex11-1-5F and attB3-Aopex11-3R. The amplified deletion fragments (6.0 kb) were transformed separately into the A. oryzae NRK70-1 or NSRKDP11-1. 2.2 Construction of the Aopex11-1 and Aopex11-2 genes was confirmed by Southern blotting. After electrophoresis, the digested genomic DNAs were transferred onto Hybond N+ membrane (GE Healthcare, Buckinghamshire, United Kingdom). An enhanced chemiluminescence direct nucleic acid labeling and detection system (GE Healthcare, Buckinghamshire, United Kingdom) and an LAS-1000 Plus luminescent image analyzer (Fuji Photo Film, Tokyo, Japan) were used for detection. 2.3 Hypoxic shock experiment. A. oryzae strains were point inoculated on DPY agar medium in a glass-based dish (Iwaki Glassware Co., Tokyo, Japan) and incubated at 30°C for 24 h. One milliliter of water was added to the fungal colony to induce hypophil tip bursting, and the colony was observed using an IX71 inverted microscope (Olympus, Tokyo, Japan). 2.4 Complementation of the ΔAopex11-1 strain. To perform a complementation test for the ΔAopex11-1 disruption, the plasmid pPFXN was constructed. The Aopex11-1 gene including the promoter, terminator, and coding region gene was amplified by PCR using the primers Aopex11-1-5F and Aopex11-1-3R and inserted by ligation with the plasmid pRSET-A as a cloning vector (Invitrogen). The resulting plasmid was used to transform the ΔAopex11-1 strain (Table 1), and the obtained strain was confirmed by PCR using the primers above are the Multisite Gateway attB recombination sites. Gateway LR reactions were accomplished by mixing pg5P′BaB (PunyB), pgEEG-PT5 (egg-gpt5), pg3TaNiAD (niiD), and the destination vector pDEST-R4-R3 (Invitrogen, Carlsbad, CA, USA), generating the plasmid pgDAEPN. The plasmid was then introduced into the control and ΔAopex11-1 strains having the mdred-AoHex1 fusion to visualize the localizations of Woronin body protein and peroxisome simultaneously. Conidia of the control (NRK70-1-1-1A-DREP-SRL) and ΔAopex11-1 (NSRKDP11-1-DREP-SRL) strains were inoculated in 100 μl of CD medium containing glucose as a carbon source in a glass-based dish (Iwaki Glassware Co., Tokyo, Japan) and cultivated for 20 h at 30°C. Confocal microscopy was performed with an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with 100× and 40× Neofluor objective lenses (1.40 numerical aperture); 488 nm (Furukawa Electric, Tokyo, Japan) and 561 nm (Melles Griot, CA) semiconductor lasers; GFP, DsRed, and DualView filters (Nippon Roper, Chiba, Japan); an 100×22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan); and an Andor iXon cooled digital charge-coupled-device camera (Andor Technology PLC, Belfast, United Kingdom). Images were analyzed with Andor iQ software (Andor Technology PLC, Belfast, United Kingdom). 2.5 Electron microscopy. A. oryzae conidial suspension was spot inoculated on DPY agar medium and incubated at 30°C for 5 days. Hyphae were prefixed in 0.1 M phosphate buffer (pH 7.0) containing 4% glutaraldehyde for 4 h and then postfixed in 1% OsO4 solution. After dehydration in a graded series of ethanol solutions, the samples were embedded in epoxy resin. Ultrathin sections were cut on an LKB ultratome using diamond knives and stained with uranyl acetate and lead citrate. The sections were observed using a JEM-1010 transmission electron microscope (ITEM JEOIL, Ltd., Tokyo, Japan). 2.6 Western blot analysis. The A. oryzae mycelia grown in DPY liquid culture medium at 30°C for 18 h were harvested by filtration, frozen in liquid nitrogen, and pulverized using a multibead shaker (Yasu kikai, Osaka, Japan). Proteins extracted in the homogenization buffer (150 mM Tricine, pH 7.4, 0.33 M sucrose, 10 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1:100 protease inhibitor cocktail (Sigma, St. Louis, MO)) were centrifuged three times at 500 × g for 5 min to remove cell debris. The resulting supernatant was considered as the postnuclear supernatant (PNS). To separate organelles by sucrose density gradient centrifugation, 100 μl of the PNS was layered on a 30 to 60% (wt/wt) 14-ml sucrose density gradient dissolved in the buffer containing 100 mM Tricine (pH 7.4)–1 mM EDTA. The gradient was subjected to centrifugation at 100,000 × g at 4°C for 5 h using a HIMAC CP-85 ultracentrifuge (Hitachi, Tokyo, Japan) equipped with a Hitachi P40ST swinging rotor (Hitachi, Tokyo, Japan). Fractions of 50 μl were collected from the top to the bottom. Sucrose density was determined refractometrically. Aliquots of each fraction were subjected to Western blot analysis. Sodium dodecy1 sulfate-polyacrylamide gel electrophoresis and immunoblot analysis were performed as previously described (9). Cellulose nitrate membranes (Immobilon-NC; Millipore, Bedford, MA) were either incubated with the anti-GFP (1:3,000 dilution; Funakoshi Co. Ltd, Tokyo, Japan) or the anti-AoHex1 (1:2,500 dilution) (29) antibody. Secondary antibodies were anti-mouse immunoglobulin G labeled with peroxidase (1:1,000 dilution; Funakoshi Co. Ltd, Tokyo, Japan) and anti-rabbit immunoglobulin G labeled with horseshadish peroxidase (1:1,000 dilution; Vector Laboratories, Burlington, CA). Detection was performed using the enhanced chemiluminescence procedure as described earlier.
members such as Pex11B, Pex11C, Pex25, and Pex27. AoPex11-2 belongs to the Pex11 subgroup of filamentous fungi and yeast including AoPex11-1 but is slightly diverged from the other members. It may be noted that only _S. cerevisiae_ contained just one isoform of Pex11. In contrast, filamentous fungi including _A. oryzae_ contained more than three Pex11 isoforms (Pex11, Pex11B, and Pex11C). This characteristic seemed to resemble the human and plant forms that contained several Pex11 (Pex11, Pex11B, and Pex11C). This characteristic seemed to resemble the human and plant forms that contained several Pex11, respectively. 

S. cerevisiae and _A. nidulans_ contained more than three Pex11 isoforms (Pex11, Pex11B, and Pex11C). This characteristic seemed to resemble the human and plant forms that contained several Pex11 isoforms but have distinctly diverged from the fungal Pex11. While the AoPex11-1 and AoPex11-2 proteins showed a 54% homology between each other, they displayed a 26% and 21% similarity to _S. cerevisiae_ Pex11, respectively.

In order to understand the relevance of the existence of the two _PEX11_ genes in _A. oryzae_, we sought to perform a deletion analysis of the respective genes. The _Aopex11_ deletion strains were obtained by replacement of their respective coding sequences with the _adeA_ marker gene, and their disruption was confirmed by Southern analysis (data not shown).

Earlier studies in yeast have demonstrated that a mutation of _PEX11_ resulted in defective growth on oleic acid (8). To find any defect in peroxisomal function due to the deletion of _Aopex11_ genes, we first verified growth phenotypes of the _Aopex11_ strains by culturing them on minimal medium (M+Met) containing glucose or oleic acid as a sole carbon source. Growth of transformants on M+Met medium where glucose is replaced with 20 mM oleic acid is shown at right. The lower panel shows a side view of the control and _Aopex11_ strain grown on an agar plate. (B) For complementation analysis, growth of NSRKu70-1-1A transformed with the _adeA_ marker (control), the _Aopex11_ disruptant (_Aopex11_), _Aopex11_ disruptant transformed with the _Aopex11_ gene in pPENX (_Aopex11_+[_Aopex11]), and the _Aopex11_ disruptant transformed with the _niaD_ marker (_Aopex11_+Vector) is shown. All agar plates were incubated for 5 days at 30°C.

The absence of aerial hyphae and conidia in the _Aopex11_ strain exhibited a drastic reduction of aerial hyphal growth and blockage of conidiation in the presence of oleic acid (Fig. 2A), indicating the requirement of _Aopex11_ for the formation of aerial hyphae and conidia. This result is consistent with a reduction in the growth rate of the _peXK_ (peX11 homolog) strain in the presence of oleic acid as a sole carbon source in _A. nidulans_ (11). Disruption of the _Aopex11_ gene did not alter the phenotypes on minimal medium with oleic acid (Fig. 2A). Growth of the double deletion strain _Aopex11_ _Aopex11_ essentially resembled the _Aopex11_ strain when the strain was grown in the presence of oleic acid as a sole carbon source (data not shown). These results indicate that the _Aopex11_ gene is nonfunctional or redundant in peroxisomal function.

Since it was evident that the _Aopex11_ strain exhibited a phenotypic alteration in the presence of oleic acid, we next investigated whether the plasmid harboring _Aopex11_ transformed into the _Aopex11_ strain (NSRKDP11-1-10) would complement the observed defect in aerial growth and conidiation. As shown in Fig. 2B, the _Aopex11_ complemented strain grew normally in the presence of oleic acid, indicating that the _Aopex11_ gene is indeed functional and responsible for normal growth on oleic acid medium.

**Susceptibility of the _Aopex11_ strain to cell wall-destabilizing agents.** The absence of aerial hyphae and conidia in the

FIG. 1. Neighbor-joining phylogenetic tree of Pex11 protein sequences. The phylogenetic tree and molecular evolutionary analyses were determined using Mega software, version 4. Multiple alignments of the sequences were carried out in Clustal X V1. Ao, _A. oryzae_; Hs, _Homo sapiens_; Mg, _M. grisea_; At, _Arabidopsis thaliana_; Sc, _S. cerevisiae_; Yl, _Yarrowia lipolytica_.

FIG. 2. Growth phenotypes of the _Aopex11_ and _Aopex11_ strains. (A) Growth of control, _Aopex11_, and _Aopex11_ strains (as indicated in the schematic at left) on minimal medium (M+Met) containing glucose as a carbon source. Growth of transformants on M+Met medium where glucose is replaced with 20 mM oleic acid is shown at right. The lower panel shows a side view of the control and _Aopex11_ strain grown on an agar plate. (B) For complementation analysis, growth of NSRKu70-1-1A transformed with the _adeA_ marker (control), the _Aopex11_ disruptant (_Aopex11_), _Aopex11_ disruptant transformed with the _Aopex11_ gene in pPENX (_Aopex11_+[_Aopex11]), and the _Aopex11_ disruptant transformed with the _niaD_ marker (_Aopex11_+Vector) is shown. All agar plates were incubated for 5 days at 30°C.
ΔAopex11-1 strain during growth on oleic acid may be indirectly attributed to the inability of the fungus to carry out fatty acid metabolism, thus resulting in a deficiency in producing precursors needed for cell wall biosynthesis. Interestingly, the Δpex6 strain of M. grisea showed increased sensitivity to calcofluor white and Congo red (32) in addition to a complete absence of the Woronin bodies. In order to verify this notion, we treated the Aopex11 deletion strains with cell wall-perturbing agents such as calcofluor white, Congo red, and micafungin (4, 33). Interestingly, the ΔAopex11-1 strain showed greater susceptibility to Congo red and micafungin than the control and ΔAopex11-2 strains (Fig. 3). However, addition of calcofluor white did not significantly reduce the growth of all the strains (data not shown). The growth inhibition by the cell wall-perturbing agents suggested that the cell wall integrity might be affected by disruption of Aopex11-1 gene.

ΔAopex11-1 strain exhibits increased loss of cytoplasmic constituents during hyphal lysis. The addition of water to A. oryzae cultures grown on agar medium induces hyphal tip bursting due to hypotonic shock (29). The absence of Woronin body in the ΔAopex11 strain increased cytoplasmic leakage from the hyphae. Since a Woronin body was found at the septal pore adjacent to the lysed hyphal compartment (29), such a strategy would reveal the role of the Aopex11 genes, if any, in the function of the Woronin body. In order to investigate this, we induced hyphal tip bursting by hypotonic shock in the ΔAopex11 strains by flooding the colony grown on agar medium with water. Within a few minutes after the addition of water, the cytoplasmic constituents leaked out from the lysed apical compartment in the control, ΔAopex11-1, and ΔAopex11-2 strains (Fig. 4A). A Woronin body visualized by the fluorescent AopHex1 fusion protein was previously observed to plug the septal pore adjacent to the lysed apical compartment upon hypotonic shock (29). Woronin body function was examined by the ability to retain cytoplasmic constituents in the second compartment upon differential interference contrast microscopic observation. In contrast to the control and ΔAopex11-2 strains that exhibited an 82% and 76% ability to retain cytoplasmic leakage from the second compartment. Considering the results obtained, it may be noted that deletion of only Aopex11-1 exclusively affected Woronin body function.

ΔAopex11-1 strain shows enlarged peroxisomes and undifferentiated Woronin bodies. Since we noted that the deletion of the Aopex11-1 gene affected the Woronin body function, we also sought to verify if it influences the formation of the Woronin body. While in the yeast the role of Pex11 in peroxisome proliferation is well established and its absence results in the formation of giant peroxisomes (8, 28), studies in N. crassa have demonstrated that the Woronin body buds off from the peroxisome (42). In order to understand if the disruption of Aopex11-1 gene would inhibit peroxisome proliferation and affect the Woronin body differentiation, we adopted a dual fluorescence strategy to simultaneously visualize the Woronin bodies and peroxisomes.

For visualization of Woronin bodies, mDsRed was fused at the N terminus of AopHex1 and expressed in the control and ΔAopex11-1 strains. We confirmed that expression of the
The mDsRed-AoHex1 fusion protein had only a moderate effect on Woronin body function in both the control and \( \Delta pex11-1 \) strains (see Fig. S1A in the supplemental material). The mDsRed protein was fused to the N terminus of Woronin body protein AoHex1. Control (A) and \( \Delta pex11-1 \) strains (B) expressing the EGFP-PTS1 and mDsRed-AoHex1 fusion proteins were cultured in 100 \( \mu l \) of CD medium containing glucose as carbon source for 20 h at 30°C. Asterisks denote the septa, and arrows indicate red fluorescent spots independent of the green-labeled peroxisomes, suggesting that they are Woronin bodies. Arrowheads represent peripheral distribution of the Woronin body protein AoHex1 in the peroxisomes. Bar, 5 \( \mu m \). Large spherical peroxisomes (C) and dumbbell-shaped peroxisomes (D) in the \( \Delta pex11-1 \) strain are shown. Arrowheads indicate peripheral or junctional distribution of the Woronin body protein AoHex1 in the peroxisomes. Bar, 2 \( \mu m \).

The control strain expressing EGFP-PTS1 and mDsRed-AoHex1 also displayed small red fluorescent dots of mDsRed-AoHex1 that were independent of the green fluorescence, suggestive of Woronin bodies (Fig. 5A). However, the \( \Delta pex11-1 \) strain expressing EGFP-PTS1 and mDsRed-AoHex1 had only a few peroxisomes, and these were enlarged (Fig. 5B). This result is consistent with an earlier study on the \( \Delta pexK (pex11) \) strain in \textit{A. nidulans} that showed only a few large peroxisomes (11).

The peroxisomes labeled with EGFP-PTS1 colocalized with the structures labeled with the mDsRed-AoHex1 fusion protein in both the control and \( \Delta pex11-1 \) strains (Fig. 5A and B). While the green fluorescence of EGFP-PTS1 was not observed at the septal pore during hyphal injury, only mDsRed-AoHex1 localized at the septal pore adjacent to the lysed compartment (data not shown), as reported earlier (29), indicating that peroxisomes are not involved in the septal pore plugging process.

The control strain expressing EGFP-PTS1 and mDsRed-AoHex1 revealed numerous green fluorescent spherical structures in various sizes, indicative of peroxisomes (Fig. 5A), the \( \Delta pex11-1 \) strain revealed only a few peroxisomes, and these were enlarged (Fig. 5B). This result is consistent with an earlier study on the \( \Delta pexK (pex11) \) strain in \textit{A. nidulans} that showed only a few large peroxisomes (11).

The peroxisomes labeled with EGFP-PTS1 colocalized with the structures labeled with the mDsRed-AoHex1 fusion protein in both the control and \( \Delta pex11-1 \) strains (Fig. 5A and B). While the green fluorescence of EGFP-PTS1 was not observed at the septal pore during hyphal injury, only mDsRed-AoHex1 localized at the septal pore adjacent to the lysed compartment (data not shown), as reported earlier (29), indicating that peroxisomes are not involved in the septal pore plugging process.
somes, suggesting the possibility that assembly of AoHex1 in the peroxisome matrix is not affected by the deletion of the AoPex11-1 gene (Fig. 5C and D). Liu et al. (24) showed that HEX-1 self-assembles in the peroxisome and is recruited to the matrix face of the peroxisome membrane by the interaction with the WSC. Collectively, these data suggest that the assembled structure of AoHex1 remains as a fission or division precursor of a Woronin body inside the peroxisome in the absence of AoPex11-1.

Undifferentiated Woronin bodies remain associated with organelles resembling peroxisomes in the /H9004 AoPex11-1 strain. From the results obtained by simultaneous fluorescent imaging of the peroxisomes and the Woronin bodies, it became clear that, though assembled, the AoHex1 protein remained in an assembled structure within the peroxisome matrix in the AoPex11-1 strain. To further gain insight into these structures, we opted to examine the strains by TEM. The control and /H9004 AoPex11-1 strains grown on agar medium exhibited distinct spherical Woronin bodies near the septum (Fig. 6). Interestingly, some of the Woronin bodies in the /H9004 AoPex11-1 strain were found to be associated with organelles resembling peroxisomes (Fig. 6). While the Woronin body in A. oryzae is spherical and tends to be inherent near the septum, no Woronin bodies were seen in the vicinity of the septum in the /H9004 AoHex1 strain (29). The fluorescence microscopy data on the absence of independent Woronin bodies and the increased presence of Woronin bodies associated with peroxisomes observed by TEM support the view that AoPex11-1 contributes to formation of the Woronin body from the peroxisome and not the assembly of the Woronin body protein.

Sucrose gradient centrifugation analysis of the Woronin body protein AoHex1 and peroxisome in the ΔAopex11-1 strain. In order to verify if Woronin bodies remain localized inside the peroxisome in the ΔAopex11-1 strain, sucrose density gradient centrifugation was performed. A PNS obtained from the control and ΔAopex11-1 strains was subjected to 30 to 60% sucrose gradient centrifugation to fractionate organelles. The cellular fractions from both the control and ΔAopex11-1 strains expressing the EGFP-PTS1 fusion were examined by Western blot analysis using the AoHex1 and EGFP antibodies. In the control strain, the Woronin body protein AoHex1 was the most abundant in fraction 18, with a density of 1.23 g/ml (Fig. 7). The peak fraction of EGFP-PTS1 for the peroxisome was less dense (1.21 g/ml) than that of the Woronin body. These densities in the peak fractions of the Woronin body and peroxisome are in agreement with those reported in N. crassa (26). In the control strain, the Woronin body protein AoHex1 was the most abundant in fraction 18, with a density of 1.23 g/ml (Fig. 7), suggesting the inclusion of undifferentiated Woronin bodies inside the peroxisome matrix.
peroxisome. This result is congruent with the fluorescence and TEM observations on the point of Woronin body distribution inside the peroxisome in the ΔAopex11-1 strain.

DISCUSSION

The Woronin body is an organelle related to peroxisomes (12), and its formation governed by a budding process from the peroxisome has been demonstrated in N. crassa (42). In this study, we show that disruption of Aopex11-1 gene involved in peroxisome proliferation influences the differentiation and functioning of the Woronin body in A. oryzae.

In the presence of oleic acid as the sole carbon source, the ΔAopex11-1 strain exhibited aberrant growth defects, resulting in complete absence of aerial hyphae and a drastic reduction in conidiation (Fig. 2) and revealing that these abnormalities might be caused by a defect in fatty acid metabolism. Although the PEX mutants in Podospora anserina, A. nidulans, Colletotrichum lagenarium, N. crassa, and M. grisea displayed severe growth defects on oleic acid medium (3, 11, 18, 26, 32), the Pex11-deficient strains of A. nidulans and A. oryzae showed only a reduction but not complete inhibition of growth on oleic acid medium (11). While the deletion of Aopex11-2 exclusively did not show any phenotypic variation on oleic acid medium (Fig. 2), the double disruption of Aopex11-1 and Aopex11-2 resulted in a phenotype similar to that of the ΔAopex11-1 strain (data not shown). These results indicated that the Aopex11-2 gene may be nonfunctional or perform other unknown functions.

The ΔAopex11-1 strain but not ΔAopex11-2 strain showed hypersensitivity to the cell wall-perturbing agents Congo red and micafungin (Fig. 3). Interestingly, the Δpex6 strain in M. grisea that lacked Woronin bodies also exhibited hypersensitivity to Congo red and calcofluor white (32). Disruption of the CRAT1/PTH1 gene encoding the peroxisome-associated carnitine acetyl-transferase in M. grisea increased sensitivity to calcofluor white but not Congo red, resulting in the formation of penetration hyphae deficient in chitin (2, 32). While these data suggest that peroxisomal metabolism involving carnitine acetyl transferase has a role in chitin synthesis, it is possible that peroxisomal biogenesis may also influence some other pathways in controlling cell wall integrity. Unlike the M. grisea Δpex6 strain, the ΔAopex11-1 strain did not show a growth defect when subjected to calcofluor white treatment (data not shown), implying that no severe defect in peroxisomal metabolism occurred due to the deletion of Aopex11-1. In accordance with these observations, a recent study in our laboratory has indicated that the ΔAhex1 strain lacking Woronin bodies is also sensitive to Congo red and micafungin but not to calcofluor white (unpublished data). The exact mechanism of action of the cell wall-perturbing agents on fungal strains defective of peroxisomes and Woronin bodies remains elusive or obscure.

By inducing hyphal tip bursting through hypotonic shock and visualizing the Woronin body plugging at the septal pore adjacent to the burst apical cell, we noted that, in comparison to the control strain which exhibited ~76% plugging efficiency, the ΔAhex1 strain lacking Woronin bodies showed a lower propensity (~20%) to prevent excessive loss of the cytoplasm (29; also our unpublished data). In the present study a similar induction of hyphal tip bursting revealed that, in contrast to the control and ΔAopex11-2 strains that displayed 82% and 76% plugging efficiency, the ΔAopex11-1 strain exhibited only a 50% ability to prevent the loss of the cytoplasmic constituents (Fig. 4), indicating that the Woronin body function was affected by the deletion of Aopex11-1. However, it may be noted that the plugging efficiency in the ΔAopex11-1 strain was not reduced to the extent observed with the ΔAhex1 strain lacking Woronin bodies.

Since the Woronin body originates from the peroxisome, we presumed that the deletion of a protein involved in peroxisome proliferation and division would lead to a reduction in peroxisome number and volume and, consequently, influence Woronin body differentiation. While the control strain displayed several peroxisomes with varied sizes along the hyphae, the ΔAopex11-1 strain contained only a few enlarged peroxisomes, which is in agreement with other studies on fungal Pex11 (8, 11). In contrast to the control strain wherein the Woronin body protein, AoHex1, could localize independently of the peroxisome (Fig. 5), indicative of normal differentiation of the Woronin body from the peroxisome, independent localization of AoHex1 was hardly seen in the ΔAopex11-1 strain (Fig. 5). Interestingly, AoHex1 was distributed on the inner periphery of the enlarged peroxisome or at junctions inside the dumbbell-shaped peroxisome, indicating that the undifferentiated Woronin bodies are retained inside the peroxisome. In N. crassa, the HEX-1 crystals associate themselves with the peroxisomal membrane via WSC, producing intermediate structures that undergo a maturation process involving membrane fission (24). In the present study we hypothesize that the deletion of Aopex11-1 impairs the division of peroxisomes, thereby retaining the undifferentiated Woronin bodies inside...
the peroxisomal membrane. However, it is likely that the self-assembly of HEX-1 occurs in the peroxisomal matrix of the \( \Delta \text{Aoxp}11-1 \) strain and that the self-assembled HEX-1 associates with the peroxisomal membrane probably through WSC, but the budding process of Woronin body from the peroxisome is repressed. A recent report employing a heterologous expression system in yeast suggested that dynamin-like proteins participate in the fission of HEX-1 crystals from the peroxisome (47) although loss of a single dynamin-like protein in N. crassa was not shown to alter Woronin body function (24). We speculate that Pex11 promotes the budding of the Woronin body from the peroxisome after attachment of the assembled HEX-1 to the peroxisomal membrane via WSC. How the assembled HEX-1 is recognized for its budding out from the peroxisome will be an interesting aspect to be investigated in the future.

In comparison to the electron micrographs showing Woronin bodies near the septum (Fig. 6), fluorescence microscopy revealed fewer Woronin bodies near the septum (Fig. 5). This result in addition to the colocalization of mDsRed-AoHex1 and EGFP-PTS1 leads to the assumption that the fusion of a fluorescent protein to AoHex1 may affect Woronin body differentiation and Woronin body targeting near the septum although we did not find a significant hindrance of Woronin body function upon expression of the mDsRed-AoHex1 fusion protein (see Fig. S1 in the supplemental material). The sucrose density gradient revealed that only a minor part of EGFP-protein (see Fig. S1 in the supplemental material). The sucrose though we did not find a significant hindrance of Woronin body function upon expression of the mDsRed-AoHex1 fusion protein (see Fig. S1 in the supplemental material). The sucrose density gradient revealed that only a minor part of EGFP-

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