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

Cristopher Salazar Escaño,1‡ Praveen Rao Juvvadi,1‡§ Feng Jie Jin,2 Tadashi Takahashi,2 Yasuji Koyama,3 Shuichi Yamashita,3 Jun-ichi Maruyama,3 and Katsuhiro Kitamoto1*‡

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan1; Noda Institute of Scientific Research, 399 Noda, Noda-shi, Chiba 278-0037, Japan2; and Department of Agricultural and Environmental Biology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan3

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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 Aopex11-1 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 Aopex11-1 strain. Furthermore, distribution of AoHex1 was found to be peripheral in the enlarged peroxisome or junctional in dumbbell-shaped peroxisomes. Electron microscopy of the Aopex11-1 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 Aopex11-1 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’s 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 multimeric 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 ofMagnaporthe 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 role of NSRKb70-1-1 in the involvement of fungal peroxins for the biogenesis of 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 bodies. In addition to generating fluorescent protein (EGFP)-PTS1 fusion protein to concurrently visualize Woronin bodies and peroxisomes in the A. oryzae strain 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 (niaD- Δc' adeA ΔB2-Aopex11-2-3F ΔB3-Aopex11-1-3R ΔB4-Aopex11-1-5F Δku70 ΔargB ΔadeA) 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 (5′-TCAAGAGCTCAGGAGTAGTAAGGGTGGTTAGCGCC-3′) and argB-R (5′-TCAAGAGCTCAGGAGTAGTAAGGGTGGTTAGCGCC-3′), which was used as the control in phenotypic analyses. A. oryzae strains were cultured either in DPM 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% NH4Cl, 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 for the A. oryzae adeA transformants. Glucose as a sole carbon source in M-Met medium was replaced with oleic acid to induce peroxisome proliferation. DPM 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 micafungin (Astellas Pharma, Inc., Tokyo, Japan) was used as the control in growth experiments. A. oryzae was used as the control in growth experiments.

**Construction of the ΔAopex11 strains.** The plasmids pgDAPx11-1 and pgDAPx11-2 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 gene (2.0 kb) was amplified by PCR using the primers arB4-Aopex11-1-5F (5′-GGGAGCACTTGTTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAGAGTGCTGATTAGTAGTGAATGAGAg...
ATG-3) and attB-Aopex11-1-23R (5'-GGGACACATTTGTATAATAAGTG TACCTAGTCCATAGCATATAAACGTC-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 pgDPtrApX11-1, consisting of the Aopex11-1 gene deletion construct with the pyrimidine resistance gene (ptra) (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 p5 pDONR-P4-P1 and p3 pDONR-P2-P3 entry vectors (Invitrogen, Carlsbad, CA). Specifically, the obtained 5' and 3' entry clones together with the center entry clone plasmid, pgEAA (harboring the ade4 marker gene) (14) or pgEpIara (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-R43 (Invitrogen, Carlsbad, CA) to obtain the final plasmids pgDAPAx11-1, pgDAPAx11-2, and pgDPtrApX11-1, respectively. The deletion cassette for the Aopex11-1 and Aopex11-2 genes were amplified by PCR using the plasmids pgDAPAx11-1, pgDAPAx11-2, and pgDPtrApX11-1 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 NSRKu70-1 or NSRKDP11-2-12 strains.

Disruption 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.

Hypnotic 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 hyphal tip bursting, and the colony was observed using an IX71 inverted microscope (Olympus, Tokyo, Japan).

Complementation of the ΔAopex11-1 strain. To perform a complementation test for the Aopex11-1 disruption, the plasmid pEXN was constructed. The Aopex11-1 gene including the promoter, terminator, and coding region gene was disruption, the plasmid pPEXN was constructed. The gene including the promoter, terminator, and coding region gene was disrupted, the plasmid pPEXN was constructed. The following primer pairs, respectively, were constructed as follows. The 5'-GGGGACCACTTTGTACAAGAA and 3'-GGGGACCTTTGTACAAGAA TGACTCATGCCATAGCATATAAACGTC-3'). The underlined sequences in the primers above are the MultiSite Gateway attB recombination sites. Gateway LR reactions were accomplished by mixing pg5's Pab (PamyB), pgEFG-PTS1 (egfp-pts1), pg3's TnaAd (tnaD), and the destination vector pDEST-R43 (Invitrogen, Carlsbad, CA, USA), generating the plasmid pgDAEPN. The plasmid was then introduced into the control and ΔAopex11-1 strains having the mdsred-AoHex1 fusion to visualize the localization of Woronin body protein and peroxisome simultaneously. Conidia of the control (NSRKu70-1-1A-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 (1.4 numerical aperture); 485 nm (Furukawa Electric, Tokyo, Japan) and 561 nm (Melles Griot, CA) semiconductor lasers; GFP, DyRed, and DualView filters (Nippon Roper, Chiba, Japan); and a CSU22 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).

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 microscopy (JEOL, Ltd., Tokyo, Japan).

The density gradient centrifugation 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 (Yasuiki 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 gradient dissolved in 50 mM Tricine, pH 7.4. After centrifugation, the organelles were collected and the pellets were collected, lyophilized, and dissolved in Tricine, pH 7.4, 100 μl of CD medium containing glucose as a carbon source.

RESULTS

Identification and disruption of the Aopex11-1 and Aopex11-2 genes. Kiel et al. (17) reported that filamentous fungi have three Pex11 isotypes (Pex11, Pex11B, and Pex11C). We searched the A. oryzae genome database (DOGAN; http://www.bio.nite.go.jp/dogan/Top) for genes encoding the protein showing similarity to Pex11. In this organism, two Pex11 orthologs were found to exist and were designated Aopex11-1 (DDBJ accession number AB440293) and Aopex11-2 (DDBJ accession number AB440294). In addition, one and two genes encoding isofoms Pex11B and Pex11C, respectively, were found in A. oryzae and named Aopex11B (DDBJ accession number AB440292), Aopex11C1 (AO0901020000109), and Aopex11C2 (AO0901020000101). Phylogenetic analysis (Fig. 1) shows that Pex11 can be classified into fungal, animal, and plant subclades, which are distant from other Pex11 subfamily
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 and other 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 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 ade4 marker gene, and their disruption was confirmed by Southern analysis (data not shown).

Earlier studies in yeast have demonstrated that a mutation in 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 ΛAopex11-1 and ΛAopex11-2 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 ΛAopex11-1 strain grown on an agar plate. (B) For complementation analysis, growth of NSRKu70-1-1A transformed with the ade4 marker (control), the Aopex11-1 disruptant (ΔAopex11-1), Aopex11-1 disruptant transformed with the Aopex11-1 gene in pPEN (ΔAopex11-1+[Aopex11-1]), and the Aopex11-1 disruptant transformed with the niaD marker (ΔAopex11-1+[Vector]) is shown. All agar plates were incubated for 5 days at 30°C.

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Since it was evident that the ΛAopex11-1 strain exhibited a phenotypic alteration in the presence of oleic acid, we next investigated whether the plasmid harboring Aopex11-1 transformed into the ΛAopex11-1 strain (NSRKDP11-1-10) would complement the observed defect in aerial growth and conidiation. As shown in Fig. 2B, the Aopex11-1 complemented strain grew normally in the presence of oleic acid, indicating that the Aopex11-1 gene is indeed functional and responsible for normal growth on oleic acid medium.

Susceptibility of the ΛAopex11-1 strain to cell wall-destabilizing agents. The absence of aerial hyphae and conidia in the
ΔAopex11-1 strain 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 Δpetx 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 AoHex1 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 constituents in the second compartment, respectively (Fig. 4B), the ΔAopex11-1 strain retained only a 50% ability to prevent 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 AoHex1 and expressed in the control and ΔAopex11-1 strains. We confirmed that expression of the

![FIG. 3. Growth sensitivity of the ΔAopex11 strains to cell wall perturbing agents. Conidia from the control, ΔAopex11-1, and ΔAopex11-2 strains were grown in the presence or absence of Congo red (100 μg/ml) or micafungin (20 ng/ml). Strains were grown for 2 days at 30°C on DPY medium. Cultures are identified according to the scheme in the top left panel.](http://ec.asm.org/)

![FIG. 4. Excessive loss of cytoplasmic constituents in the ΔAopex11-1 strain during hyphal lysis. (A) Differential interference contrast images of lysed hyphae showing blockage of extensive loss of the cytosol from the second compartment upon hyphal tip bursting. Note that approximately 50% of the ΔAopex11-1 hyphae observed were apparently devoid of the cytosol from the second compartment. Asterisks point to the first septa of the burst hyphae. Bar, 5 μm. (B) Percentage of hyphae preventing excessive loss of cytoplasmic constituents from the second compartment in the ΔAopex11 strains upon hypotonic shock. Fifty randomly selected hyphae showing hyphal tip lysis on a glass-based dish were observed and recorded. Standard deviations are indicated by the error bars. *** P < 0.001 (t test, n = 6).](http://ec.asm.org/)
mDsRed-AoHex1 fusion protein had only a moderate effect on Woronin body function in both the control and ΔAoHex1 strains (see Fig. S1A in the supplemental material). The mDsRed protein was fused to the N terminus of Woronin body protein AoHex1. The expression of the mDsRed-AoHex1 fusion protein was found at the septal pore adjacent to the lysed compartment upon hyphal lysis, suggesting the localization pattern typical of the Woronin body (see Fig. S1B in the supplemental material), indicating that the mDsRed-AoHex1 fusion protein was indeed functional. The expression of the mDsRed-AoHex1 fusion protein partially restored Woronin body function in the ΔAoHex1 strain, as evidenced by a 40% ability to prevent the loss of cytoplasmic constituents although the expression of the AoHex1 gene in the ΔAoHex1 strain exhibited an 80% ability at preventing excessive loss of the cytoplasm during hyphal lysis comparable with the wild type (76%), in contrast with the lower ability (20%) of the ΔAoHex1 strain having only the marker plasmid (unpublished data). The peroxisomes labeled with EGFP-PTS1 colocalized with the structures labeled with the mDsRed-AoHex1 fusion protein in both the control and ΔAoHex1 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), indicating that peroxisomes are not involved in the septal pore plugging process.

The control strain expressing EGFP-PTS1 and mDsRed-AoHex1 also displayed small red fluorescent dots of mDsRed-AoHex1 that were independent of the green-labeled peroxisomes, suggestive of Woronin bodies (Fig. S1B). However, the ΔAoHex1-1 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 ΔpexK (pex11) strain in 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 ΔAoHex1-1 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), indicating that peroxisomes are not involved in the septal pore plugging process.

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 ΔAoHex1-1 strain expressing EGFP-PTS1 and mDsRed-AoHex1 had only a few peroxisomes, and these were enlarged and contained both green and red fluorescence but no independent red fluorescent spots suggestive of Woronin bodies near the septum (Fig. 5B). Moreover, the distribution of AoHex1 was sometimes found to be peripheral inside the enlarged peroxisome or junction connecting two enlarged peroxi-
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 \( /H9004 AoPex11 \) 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 \( \Delta AoPex11-1 \) strain. In order to verify if Woronin bodies remain localized inside the peroxisome in the \( \Delta AoPex11-1 \) strain, sucrose density gradient centrifugation was performed. A PNS obtained from the control and \( \Delta AoPex11-1 \) strains was subjected to 30 to 60% sucrose gradient centrifugation to fractionate organelles. The cellular fractions from both the control and \( \Delta AoPex11-1 \) 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 \( \Delta AoPex11-1 \) strain, however, the EGFP-PTS1 was shifted to the denser fraction and overlapped with AoHex1 mostly in fraction 18 (density, 1.23 g/ml) (Fig. 7), suggesting the inclusion of undifferentiated Woronin bodies inside the

**FIG. 6.** TEM images of Woronin bodies in the \( \Delta AoPex11-1 \) strain. Hyphae of the control and \( \Delta AoPex11-1 \) strains were grown on DPY agar medium for 5 days at 30°C and processed for electron microscopy. M, mitochondria; N, nucleus; S, septum; V, vacuole; W, Woronin body; arrows, Woronin body associated with organelles resembling peroxisomes. Bar, 500 nm.
polyacrylamide gel electrophoresis gels are loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and analyzed by Western blot analysis. Note that AoHex1 and nonspliced AoHex1 (nsAoHex1) proteins are derived from the spliced and nonspliced transcripts, respectively, of the AoHex1 gene (29).

**FIG. 7.** Sucrose density gradient centrifugation of peroxisome and Woronin body protein AoHex1. Mycelial extract of A. oryzae cells expressing the fusion EGFP-PTS1 were subjected to 30 to 60% (wt/wt) sucrose density gradient centrifugation at 100,000 × g for 5 h. Fractions (500 μl) were collected from the top to the bottom, and distribution of AoHex1 and GFP-PTS1 was examined by Western analysis using anti-AoHex1 and anti-GFP antibodies. Even-numbered fractions (numbers 4 to 26) were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and analyzed by Western blot analysis. Note that AoHex1 and nonspliced AoHex1 (nsAoHex1) proteins are derived from the spliced and nonspliced transcripts, respectively, of the AoHex1 gene (29).

**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 Aopex11-1 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, 24, 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 Aopex1-1-2 exclusively did not show any phenotypic variation on oleic acid medium (Fig. 2), the double disruption of Aopex1-1 and Aopex1-2 resulted in a phenotype similar to that of the Aopex11-1 strain (data not shown). These results indicated that the Aopex1-2 gene may be nonfunctional or perform other unknown functions.

The Aopex11-1 strain but not Aopex11-2 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 Aopex11-1 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 ΔAoHex1 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 ΔAoHex1 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 Aopex11-2 strains that displayed 82% and 76% plugging efficiency, the Aopex11-1 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 Aopex11-1 strain was not reduced to the extent observed with the ΔAoHex1 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 Aopex11-1 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, independent localization of AoHex1 was hardly seen in the Aopex11-1 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 ΔAopex11-1 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 succrose density gradient revealed that only a minor part of EGFP-PTS1 overlapped with AoHex1 in the control strain (Fig. 7), suggesting a complete differentiation of Woronin bodies from the peroxisome. Interestingly, in N. crassa the GFP-HEX-1 fusion protein overlaps with both the Woronin body (denser) and the glyoxisome (lighter) fractions on the density gradient, suggesting that the GFP moiety adversely affects the crystallization or condensation of GFP-HEX-1 (26).

In electron micrographs of the ΔAopex11-1 strain, some Woronin bodies were present near the septum that remained associated with organelles resembling peroxisomes (Fig. 6). Succrose density gradient centrifugation indicated that both AoHex1 and peroxisomal markers were recovered in the same fraction (Fig. 7), supporting the electron microscopy result that Woronin bodies were indeed confined to the peroxisome. In contrast to the ΔAopex11-1 strain in which undifferentiated Woronin bodies were found near the septum, the ΔAopex11-1 strain does not possess any Woronin bodies near the septum (29). These data indicate that in the absence of Pex11, the undifferentiated Woronin bodies localized inside the peroxisome could be targeted near the septum. In N. crassa, overexpression of WSC promotes its association with the plasma membrane (24), suggesting WSC-dependent guidance of Woronin body onto the plasma membrane. In A. oryzae some membrane components such as WSC might recruit the undifferentiated Woronin body near the septum in the ΔAopex11-1 strain. Considering this, the next question to be addressed would be why Woronin body function was partially impaired in the ΔAopex11-1 strain although the assembled AoHex1 was found near the septum in electron micrographs. At this juncture we presume that complete differentiation of the Woronin body from the peroxisome is required for its full ability to plug at the septal pore. Taken together, all our experimental evidence supports the view that in the ΔAopex11-1 strain, HEX-1 is assembled inside the peroxisomal matrix, but failure of the organelle to undergo division leads to abnormal differentiation and functioning of the Woronin body.

This study demonstrates for the first time that Pex11, involved in peroxisome proliferation and division, contributes to Woronin body differentiation and function. The other Pex11 isoform Pex11B is found to be exclusive in the Pezizomycotina (Fig. 1) (17), suggesting that an additional mechanism for peroxisomal proliferation processes such as Woronin body differentiation is present. Further characterization of peroxisomal proliferation in A. oryzae at a molecular level will provide new insights into the mechanisms involved in Woronin body formation and differentiation.

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