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Comparison of Cell Wall Ultrastructures of *Aspergillus nidulans* in Presence and Absence of a MnpAp Mannoprotein

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Abstract: The ultrastructure of *Aspergillus nidulans* cell wall in relation to a mannoprotein was studied by scanning and transmission electron microscopy. An *mnpAp* null-mutant, DMPV1, was used as a negative control of a wild type VER7. To analyze whether the mannoprotein in the cell wall during the development of an *mnpAp* null-mutant is present or not, immunogold microscopy was also adopted. The surface sculpturing of various cell types - hyphae, conidium, Hülle cell, and ascospore - were not very different between the wild type and the *mnpAp*-null mutant (DMPV1) as examined by scanning electron microscopy. These results were comparable to those examined by transmission electron microscopy, in that the hyphal cell wall was not indentical between two strains, probably caused by the MnpA protein (MnpAp). MnpAp was absent in both the hyphal cell wall of the DMPV1 strain and the conidial cell wall of a wide type, but clearly recognized in the hyphal cell wall of a wild type.

Key words: *Aspergillus nidulans*, cell wall, *mnpAp*, ultrastructures

INTRODUCTION

The cell wall of a filamentous fungus, *Aspergillus nidulans*, has various functions such as protection from the internal and external environment, recognition of external stimuli, transportation and delivery of substances, and storage of carbohydrates (Bull, 1970; Aronson, 1981; Claveri-Martin et al., 1988). The fungus firstly develops tubular cells called hyphae, which then produce various forms of cells by an asymatric differentiation (Martinelli and Kinghorn, 1994; Adam et al., 1998). The polarised mode of growth could be possible in synthesizing cell wall components appropriately at an apical and/or subapical regions of hyphal cell. Analyses on the cell wall components of *Aspergillus* spp. have documented that cell walls are composed by β-1,3-glucan, β-1,6-glucan, linear β-1,3/1,4-glucan, chitin and mannoproteins (Bull, 1970; Zonneveld, 1971; Fontaine et al., 2000), but have simple structure compared to those of other organisms (Kaminskyj and Hamer, 1996). The fungus might be quite suitable organism for compatible studies on both genes relating to cell wall components and ultrastructures determined by their genetic informations.

Genes encoding for the synthesis of the cell walls in *A. nidulans* have been identified, such as *orlA*, *tsE*, *orlC*, *orlD* (Borgia, 1992; Borgia and Dodge, 1992), *chsA*, *chsB*, *chsC*, *chsD* (Bowen et al., 1992), *fksA* (Nam et al., 1998), *csmA* (Fujiwara et al., 1997) and *bimG11* (Borgia, 1992; Doonan, 1994). Four of these, *orlA* (Borgia and Dordge, 1992), *chsA* and *chsC* (Specht et al., 1996) and *fks*A (Nam et al., 1998), related to synthesize both carbohydrates and lipids, have cloned and characterized. However, little is known regarding genes encoding the structural protein in or on the cell walls of *A. nidulans* (Smith and Payton, 1994). We recently reported a gene, *mnpA*, encoding a mannoprotein *A. nidulans* (Jeong et al., 2003, 2004)

The *mnpA* gene has an open reading frame of 261 amino acids without intron, and the deduced amino acid sequence shows a 60% identity to that of *A. fumigatus* galactomannoprotein MP1. The *mnpA* gene has been proposed to be in vegetative mycelia, but much less in sexual structures, and not in conidia. Mannoproteins in fungal cell walls are important and abundant structural components that represent up to 10~40% of the total cell wall mass (Bull, 1970; Hearn, Cole and Susuki, 1993; Klis,

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1994; Hearn, 1997). Genes encoding mannoprotein have been also reported in *Candida albicans* (Lpez-Ribot et al., 1997), *Saccharomyces cerevisiae* (van der Vaart et al., 1995; Caro et al., 1997; Moukadiri et al., 1997) and *Penicillium marneffei* (Cao et al., 1998). To better understand the effect of MnpAp in cell wall in relation to ultrastructure, the comparative study using strains mutant for *mnpA* is required.

This study aims at the ultrastructure of the cell wall in *A. nidulans* in highlight to an *mnpA* null-mutant with immunoelectron microscopy.

MATERIALS AND METHODS

Strains, media, and culture conditions

Aspergillus nidulans strains used in the study were VER7 (*pabaA1*; *yA2*; *ΔargB::trpC*; *trpC801*) (Han et al., 2001), RMS011 (*pabaA1*; *yA2*; *ΔargB::trpC*; *trpC801*; *veA1*) (FGSC, Fungal Genetics Stock Center, Kansas, KS) and DMPV1 (*pabaA1*; *yA2*; *ΔargB::trpC*; *trpC801*; *ΔmnpA::argB*). The *A. nidulans* VER7 was a veA^{+} $argB^{-}$ segregant from the cross of RMS011 and Wx (Han et al., 2001). The *A. nidulans* DMPV1 was a null mutant of the *mnpA* gene constructed recently (Jeong et al., 2003). Complex medium (CM), minimal medium (MM) and CM containing 0.6 M potassium chloride (CMK) were prepared as previously described (Pontecorve et al., 1953; Jeong et al., 2000). Conidia were harvested by gentle rotating of plates with 0.08% (v/v) Tween 80. Sexual development was induced according to the method described previously (Jeong et al., 2000). Sexual structures were isolated by repeated sedimentation in the presence of conidia and mycelia in distilled water as described previously (Lee et al., 2001).

Scanning electron microscopy (SEM)

Mycelia grown in liguid CM and conidia grown on solid CMK were fixed in 2.5% (v/v) glutaraldehyde and 5% (v/v) acrolein in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 hr at 0°C. After dehydration through a graded series of ethanol, the specimens were transferred into *t*-butyl alcohol (1~2 ml) for three changes. The specimens in *t*-butyl alcohol were kept in a refrigerator (2~4°C) for butyl alcohol to be frozen within a few minutes. The specimens were transferred into the bell jar of a vacuum evaporator (HUS-4GB, Hitachi Ltd., Japan), and *t*-butyl alcohol was evacuated with a rotary pump alone. After frozen, *t*-butyl alcohol was completely sublimated, and the specimens were kept at room temperature for additional 20 min to be warmed up. The dried specimens were mounted on aluminum stubs with double-sided adhesive tape, and then sputter-coated with gold to a maximum thickness of 20 nm. The stubs were examined and photographed with an Akashi SR-50 SEM (Hidachi Ltd., Tokyo, Japan) operated at 10 kV.

Immnunogold staining and transmission electron microscopy (TEM)

The specimens for transmission electron microscopy were prepared as previously described (Jeong et al., 2004). The specimens, fixed and dehydrated in former steps, were embedded in LR White (Electron Microscopy Sciences, Washington, Pa.) following the manufacturer's instructions. After polymerization of the resin, ultra-thin sections were cut by a thickness of 60 nm with a diamond knife and mounted onto 300 mesh nikel grids for immunostaining. For immunostaining, sections were first blocked for 20 min in 0.5% (w/v) bovine serium albumin (BSA) (Sigma, St. Luis, Mo.) and 0.1% gelatin in PBS. Mouse anti-MnpAp antibodies (diluted by 1 : 500 in 20 mM Tris buffer (pH 8.2), prepared in a previous report (Jeong et al., 2004) with 0.1% BSA (TBSA)) was added and incubated with cell sections for 90 min. After rinsing with TBSA, the specimens were incubated with TBSA containing 1 : 25-diluted goat anti-mouse immunoglobulin G conjugated with gold particles of 10 nm in diameter (Amersham, Pharmacia Biotech. Ltd., Buckinghamshire, UK). After washing with TBSA, the sections were counterstained with uranyl acetate and lead citrate, followed by examination of electron microscope with a JEOL 1200EX TEM (Jeol Ltd., Tokyo, Japan) operating at 80 kV.

RESULTS AND DISCUSSION

We have recently isolated and characterized the *mnpA* gene encoding a mannoprotein of *A. nidulans*., and shown that the mannoprotein is located in the cell wall (Jeong et al., 2004). This result was consistent with the fact that its deduced amino acid sequence shares common sequence motifs of many cell wall proteins previously reported, such as a signal sequence, O-glycosylation sites and a glycosylated phophatidylinositol (GPI)-anchoring motif (Klis, 1994; Caro et al., 1997; Kapteyn et al., 2000). To understand the relationship between the MnpAp and cell walls during the growth and development, scanning and transmission electron microscopy were adopted. An *mnpA* null-mutant, DMPV1, was used as a negative control in comparing the cell wall ultrastructures with that of a wild type, VER7. And ultrastructural changes in the fungal cell wall along with aging of a cell were also observed with immunogold staining.

SEM observation of various cell walls of VER7 and DMPV1

The surface sculpturing of the wild type was compared with that of the *mnpA* null-mutant to analyze whether there are differences in the surface sculptures of two strains (Fig. 1). The surfaces of hyphal cell were smooth and well branched in both of strains (Fig. 1A and 1B). After incubation on CM

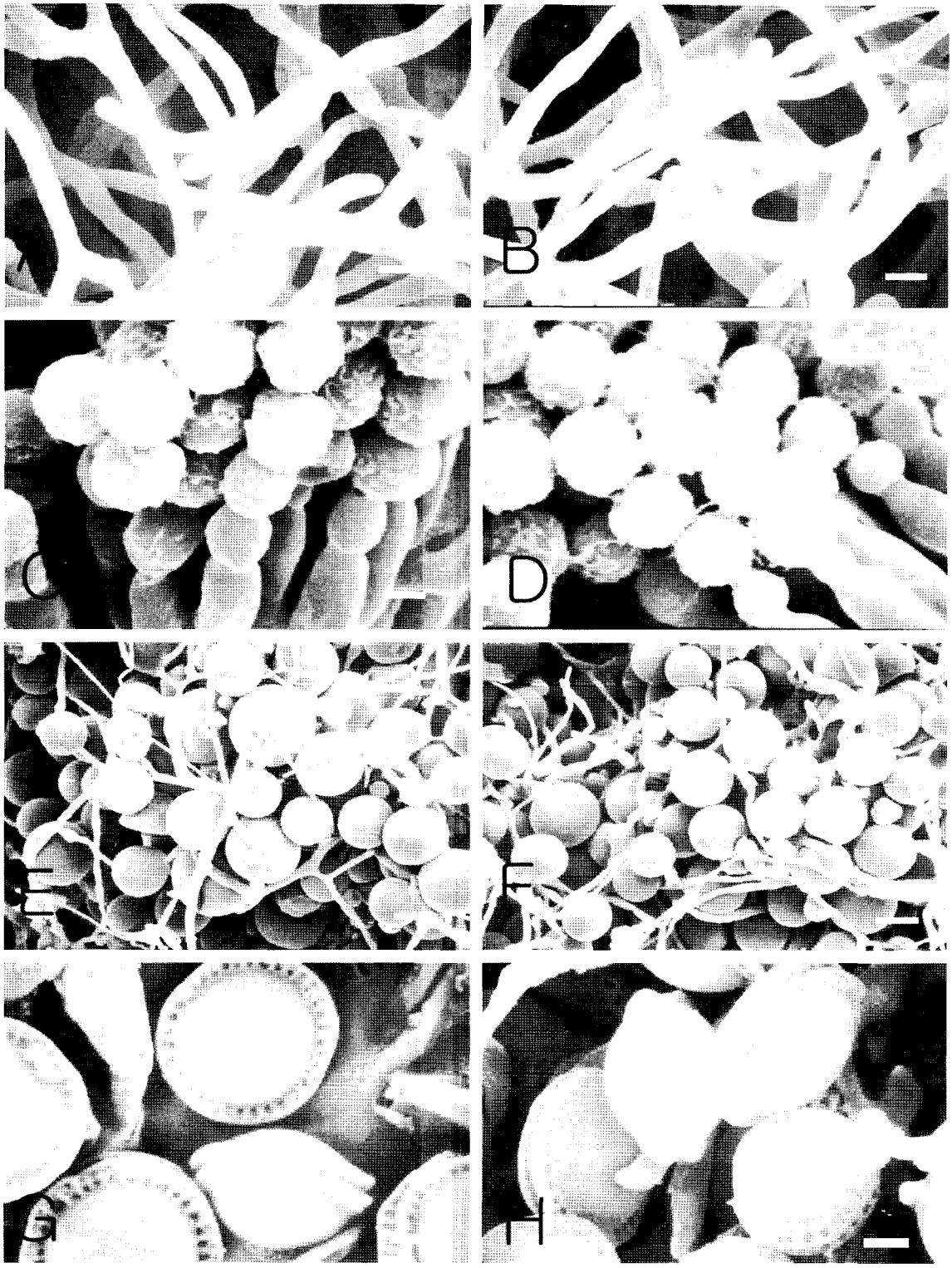

Fig. 1. Scanning electron micrographs of various cell types of *A. nidulans*. A, Mycelia of VER7 incubated on CM for 20 hr showing the network of hyphal cells. B, Mycelia of DMPV1 incubated on CM for 20 hr showing the network of hyphal cells. C, Conidia of VER7 incubated on CMK 50 hr showing the late stage of asexual development. D, Conidia of DMPV1 incubated on CMK for 35 hr showing the relatively early stage of conidiation. E, Hülle cells of VER7 incubated on CM for 50 hr showing the very early stage of sexual development. F, Hülle cells of DMPV1 incubated on CM for 50hr showing the very early stage of sexual development. G, Ascospore of VER7 incubated on CM for 60 hr showing the late stage of sexual development. H, Ascospore of DMPV1 incubated on CM for 60 hr showing the late stage of sexual development. Bars = 1 μm (C, D, G, H), 2 μm (A, B) and 10 μm (E, F).

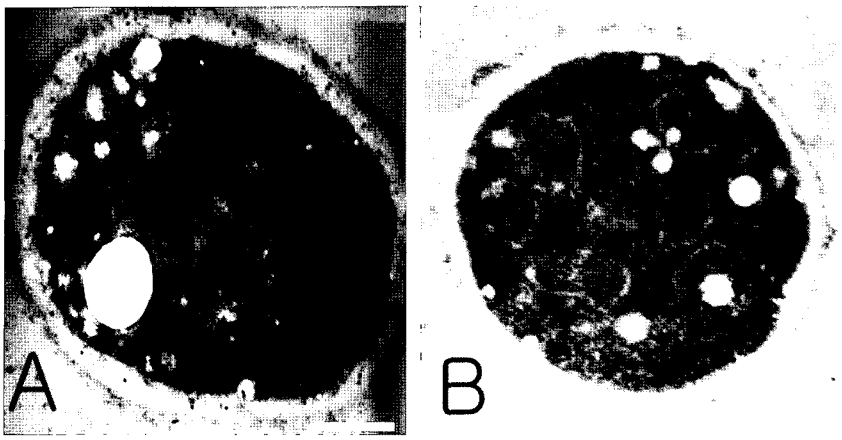

Fig. 2. Immunoelectron microscopy of two cell types of *A. nidulans* stained with mouse anti-MnpAp antibodies in view of cross section. A, Hyphal cell of VER7 showing immunogold particles specifically located at the cell wall. B, Conidium of VER7 showing the absence of immunogold particles in the cell wall. Bars = 200 nm.

for 35 hr, the surface sculpture of conidia in the *mnpA* null mutant showed the same textures as those of a wild type (Fig. 1C and 1D). The surfaces of Hülle cell, ascus and ascospore were also revealed to be identical in both of strains (Fig. 1E, 1F, 1G, and 1H). Smith and Payton (1994) have suggested that mannoproteins in *A. nidulans* may play a critical role in the maturation process of cell walls in which cross-links between the various cell wall components including mannoprotein needs to be formed, and that the absence or a reduced level of a structural mannoprotein in a newly formed wall may lead to the formation of balloons at growing hyphal tips by uncontrolled expansion of newly synthesized wall. Indeed, a *manA1* mutant forms balloons at hyphal tips and the *manA*-null mutant is lethal probably due to the loss of polarized growth (Smith and Payton, 1994). An *mnpAp*-null mutant in this study did not form balloons at the hyphal tips and was also not lethal, but had well branch hyphal cells and turned over from the asexual development into the sexual development after some degree of vegetative growth under various media, suggesting that *A. nidulans* has more than one gene for mannoprotein like other fungi (Caro et al., 1997). As a reference, 13 genes encoding cell wall proteins in *Saccharomyces cerevisiae* have been identified and several genes for mannoproteins have been described (Marguet et al., 1988; Lipke et al., 1989; Roy et al., 1991; Teunissen et al., 1993; Bidard et al., 1994; Kowalski et al., 1995; van der Vaart et al., 1995, 1996, 1997; Lo and Dranginis, 1996; Caro et al., 1997; Moukadiri et al., 1997; Kapteyn et al., 1999).

Immunoelectron microscopy to detect the distribution of MnpAp in cell wall of *A. nidulans*

To detect the distribution of MnpAp within the cell wall, fixed sections of various cell types were immunogold-stained with mouse anti-MnpAp antibodies (Fig. 2). MnpAp conjugated with immunogold particles was specifically found in the hyphal cell wall of a wild type (Fig. 2A), but absent in that of the mnpA null mutant (Jeong et al., 2004). And MnpAp in the conidial cell wall of VER7 was also absent (Fig. 2B). This distribution pattern of cell wall mannoprotein was similar to that of Pir-related protein in *C. albicans* (Kapteyn et al., 2000). Immunoelectron micrographs revealed further that MnpAp was evenly spread throughout the entire region of cell walls, but not localized in a specific layer or a region in cell walls (Fig. 2A, Jeong et al., 2004).

These results could be summarised that MnpAp was absent in the conidium of a wild type, but was specifically locate in the wall of hyphal cell.

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