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Ultrastructure of the Cell Wall of a Null Pigmentation Mutant, *npgA1*, in *Aspergillus nidulans*

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The null pigmentation mutant (*npgA1*) of *Aspergillus nidulans* was previously characterized by its production of no pigment at any stage of its life cycle, its reduction in hyphal branching, and its delay in the asexual spore development. The chemical composition of the cell wall was also altered in *npgA1* mutants that became more sensitive to Novozyme 234™, which is possibly due to a structural defect in the cell wall. To investigate the effects of the cell wall structure on these pleiomorphic phenomena, we examined the ultrastructure of the cell wall in the *npgA1* mutant (WX17). Scanning electron micrographs (SEM) showed that after being cultured for six days, the outermost layer of the conidial wall of WX17 peeled off. Although this phenotype suggested that the cell wall structure in WX17 may be modified, examination using TEM of the fine structure of cross-sectioned hyphal wall of WX17 did not show any differences from that of FGSC4. However, staining for carbohydrates of wall layers showed that the electron-translucent layer of the cell wall was missing in WX17. In addition, the outermost layer H1 of the hyphal wall was also absent in WX17. The ultrastructural observation and cytochemical analysis of cell walls suggested that the pigmentation defect in WX17 may be attributed to the lack of a layer in the cell wall.

***Key words*:** *Aspergillus nidulans*, *npgA*, ultrastructure

The cell wall of fungi confers not only a protective function on the cell, but is also involved in apical growth and hyphal branching. The wall of each cell type in the filamentous fungus *Aspergillus nidulans* contains a typical pigment that plays a role in protection from UV irradiation and some antifungal agents (Butler and Day, 1998). The conidia in wild type strains are colored dark green, while the conidiophores and ascospores are colored gray-brown and purple, respectively (Clutterbuck, 1990). The genes *wA* and *yA* that encode a polyketide synthase and laccase I, respectively, are involved in conidia coloration (Law and Timberlake, 1990; Mayorga and Timberlake, 1993). In electron microscopic observation, the conidial wall of the *wA9* mutant strain is observed to lack melanin and α-1,3-glucan in chemical composition, as well as the electron-dense outer layer that is normally present in the cell wall of wild type strains (Claverie-Martin, 1988). Pigmentation of conidiophores is controlled by the genes *ivoA* and *ivoB* (Clutterbuck, 1990).

Hyphal growth in filamentous fungi is accomplished by the synthesis of hyphal wall and cytoplasmic components in the apical region. The materials required for hyphal growth are synthesized in the subapical region and transferred to the apical area by exocytosis (Burnett, 1976). This indicates that hyphal growth is closely related to cell wall synthesis. Generally, the rate of cell wall synthesis is maximal at the tip of the hypha and gradually declines toward zero at the base of the extension zone (reviewed by Harold, 1990). Bartnicki-Garcia and Lippman (1969) reviewed the evidence that the apical extension depended on incorporation of precursor materials into the preexisting walls of uniform rigidity through the aid of apical vesicles. In the cell wall of the majority of fungi, chitin and β-1,3-glucan are believed to be the most important structural polysaccharides (Bartnicki-Garcia, 1968). The hyphal wall of *Schizophyllum commune* consists of a complex of chitin and certain β-glucans. Chitin and glucan are deposited separately at the apex, and become cross-linked into an insoluble complex by a time-dependent reaction, as they settle behind the extending tip. The tip of the hyphae remains plastic because of the absence of cross-links between polymers (Sietsma *et al.*, 1985). The conidial wall of *A. nidulans* showed four layers when stained for carbohydrates (Sewall *et al.*, 1990).

We have isolated and characterized a mutant from *A.*

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nidulans, designated as *npgA1*, which produces no pigment in any cell type throughout its entire life cycle (Han and Han, 1993). The phenotypic characteristics of the mutant included hypersensitivity to Novozyme 234™, negligible production of water-soluble viscous materials in liquid culture and the retardation of hyphal branching. These characteristics strongly suggested some defects in the cell wall structure of this fungus (Chung *et al.*, 1996). In this study, a combination of electron microscopy and cytochemical technique was used to compare the cell wall structures of conidia and hyphae in the *npgA1* mutant to those in the wild type strain. Our observations indicate that the *npgA* gene product might be involved in constructing the outer layer of the cell wall of conidia and hyphae.

Materials and Methods

Strains and culture condition

A. nidulans wild type strain FGSC4 (Glasgow wild type) and a null pigmentation mutant strain WX17 (*npgA1 biA1*; *sB3*; *chaA1*; *veA1*) were used in this study. WX17 is a segregant from a cross between FGSC168 (*suA1adE20 adE20 biA1*; *ssbA3*; *sB3*; *choA1*; *chaA1*) and WX (*npgA1*). WX is an original null pigment mutant of FGSC4 (Han and Han, 1993). Strains were grown on complete medium (CM) plates (Harsani, 1976) at 37°C to obtain conidia. Conidia were inoculated (10^6/ml) into liquid CM and incubated at 37°C with vigorous shaking. Mycelium was harvested through Miracloth (Calbiochem, USA).

Electron microscopy

For scanning electron microscopy, mycelium was prefixed in a 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 4 h, washed 3 times in the same buffer and postfixed in 1% OsO_4 in the same buffer at 4°C for 4 h, and then washed 3 times in the same buffer, dehydrated in an ethanol series and an isoamylacetate series, dried with liquid CO_2 in a critical point dryer (Ladd, USA), and coated with gold in a sputter coater (Hummer, USA). Specimens were examined on a JEOL T330A scanning electron microscope (SEM) operating at 15-20 KV.

For transmission electron microscopy, samples were pre- and post-fixed, washed and ethanol dehydrated as described above. Samples were pelleted by centrifuging at 3,000 rpm and further dehydrated in propylene oxide, and embedded in Epon 812 resin. Specimens that were sectioned with a diamond knife on an LKB ultramicrotome, then stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined on a JEOL 1200EXII transmission electron microscope at 60 or 80 KV.

Enzyme digestion of mycelium

One hundred milligrams (in wet weight) of mycelial mass of *A. nidulans*, which was incubated at 37°C for 16 h with vigorously shaking, were obtained for enzyme treatment. The mycelial mass was separately treated at 37°C for 2 h with the following enzymes: 1) 2.0 U/ml laminarinase in 0.05 M Na-citrate buffer (pH 5.0), 2) 1.0 U/ml chitinase in 0.05 M Na-citrate buffer (pH 4.5), or 3) 3.5 mg/ml protease in 0.05 M PBS (pH 7.0) containing 0.01 M $MgCl_2$. Novozyme was prepared and treated as described in protoplast formation (Yelton *et al.*, 1984). To digest with combined enzymes, mycelium treated with laminarinase was washed 5 times in 0.05 M Na-citrate buffer (pH 4.5) and then digested in chitinase. Mycelium treated with protease was washed 5 times in 0.05 M Na-citrate buffer (pH 5.0) and then digested further in laminarinase.

Cytochemistry of cell wall

For the cytochemical analysis of the cell wall, thin sections of mycelia or conidia were collected on gold grids as described for TEM, and stained for carbohydrates using a modification of the method described by Sewall *et al.* (1990). Sections were oxidized for 1 h on droplets of 1% (w/v) periodic acid on strips of parafilm. Sections were washed briefly under a stream of distilled water. They were serially transferred to a droplet of 10% (v/v) acetic acid, to a droplet of 20% acetic acid, and to a droplet of 0.2% (w/v) thiocarbohydrazide in 20% acetic acid, 10% acetic acid, and finally distilled water for 15 min each. They were then washed under a stream of distilled water and placed on droplets of 1% (w/v, aqueous) mild silver protein solution. The mild silver protein solution was made immediately before use and centrifuged in 1.5 ml tubes in a microcentrifuge at 10,000 rpm for 2 min to remove particulate materials. Sections were stained with 1% mild silver protein solution for 2 h in the dark, then placed on three consecutive droplets of distilled water for 5 min each, washed under a stream of distilled water, and examined without post-staining as described in electron microscopy.

Results

Morphology of cell wall surface in WX17

Scanning electron micrographs (SEM) of the mycelium showed that the hyphal surface was smooth, and branching was well developed in FGSC4 (Fig. 1A); however, the hyphal surface of WX17 was relatively rough (Fig. 1B). Although it was not clearly shown in the picture, the hyphal branching was reduced in the mutant (data not shown). The reduction of hyphal branching likely caused the mycelial density to be lowered (Han and Han, 1994). The surface of the conidial wall also showed morphological changes in WX17 compared to that in the wild type. The many protuberances were evenly distributed on the conidial surface of the wild type strain after maturation (Fig. 1C). However, when the colonies of the *npgA1* mutant strain had aged for more than 6 days, the outer layer containing protuberances had peeled off leaving a smooth surface with some irregular protuberances on the

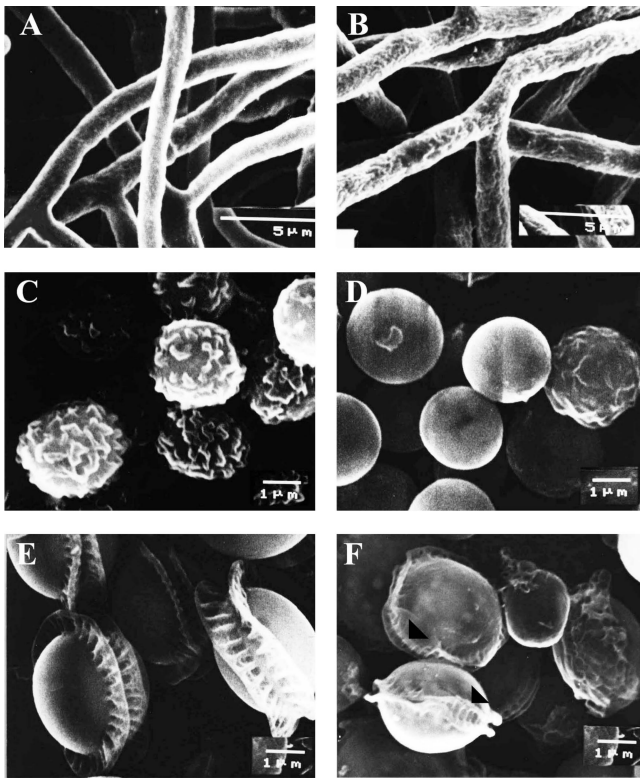

Fig. 1. Surface morphology of hyphae, condia and ascospores. The surfaces of hyphae (A and B), conidia (C and D) and ascospores (E and F) of the *npgA1* mutant strain WX17 (B, D, and F) were different from those of the wild type (A, C and E). Note that the outermost surface of mutant conidia had in many cases peeled off and there was a remnant of surface decoration left on the conidial surface (D). The girdles of the ascospores then developed to an aberrant shape (arrowheads on panel F). The hyphal surface of the mutant (B) was much rougher than in the wild type (A). Scale bar = 5 μm (A and B) and 1 μm (C, D, E and F).

great majority of conidia (Fig. 1D).

The ascospore of the *A. nidulans* wild type generally has two girdles around its surface (Fig. 1E). However the *npgA1* mutant strain differentiated the ascospores that had misshapen or broken girdles (Fig. 1F). Taking into account the data on such an abnormality in surface structure, we conclude that there may be structural defects in the cell wall of the *npgA1* mutant. The defects caused by *npgA1* mutation might appear in all cell types.

Ultrastructure of cross-sectioned conidia and hyphal wall

The cross-sections of conidia and hyphae stained with uranyl acetate and lead citrate were observed by the transmission electron microscope (TEM). The wall structures of cross-sections of wild type and the *npgA1* mutant strain were morphologically undistinguishable from each other except that the thickness of the hyphal wall of WX17 was slightly less than that of the wild type (Fig. 2). There were many electron-translucent structures in the conidial cytoplasm of WX17 (Fig. 2D).

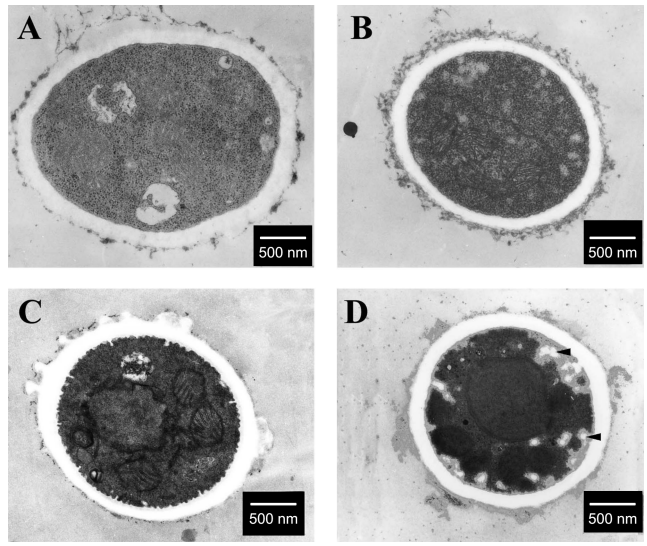

Fig. 2. Transmission electron micrographs of cross-sections of hyphae and conidia. Cross-sections of hyphae (A and B) and conidia (C and D) of the wild type (A and C) and the *npgA1* mutant strain (B and D) were stained with uranyl acetate and lead citrate. The thickness of the cell wall of the mutant hyphae (B) was slightly less than that of the wild type (A). Many electron-translucent structures were found in the cytoplasm of the mutant conidia (arrowheads on panel D). Scale bar = 500 nm.

Cytochemical analysis of cell wall

Since we could not distinguish differences in the wall structures between the wild type and WX17 by conventional electron stainings, we stained cross sections of the cell wall for carbohydrates before observing with the TEM. Hyphal walls stained for carbohydrates appeared differently layered in both WX17 and FGSC4 (Fig. 3). Cytochemical analysis of the hyphal wall in FGSC4 showed four layers as noted in Sewall's report (1990). We adopted Sewall's terminology to distinguish the following hyphal wall layers: based on the structure of the wild type strain, a strongly electron-opaque outermost layer of hyphal wall was designated H1; an electron-translucent outer layer was designated H3; an electron-opaque inner layer was designated H2; and an electron-opaque innermost layer that may be associated with the plasma membrane was designated H4 (Fig. 3A). However, the layers H1 and electron-translucent H3 were missing (Fig. 3B) in the hyphal wall of WX17. Similarly, the electron-translucent C2 layer of the conidial wall that was observed in the wild type was missing in WX17 (Fig. 3C and 3D). These results suggest that this translucent layer might be responsible for not only the pigment deposition in the walls of each cell type, but also the peeling off of the outer layer in the conidia. The electron-translucent layers in the hyphal wall (H3) and in the conidial wall (C2) might consist of the same constituents whose formation would be influenced by the *npgA* gene product.

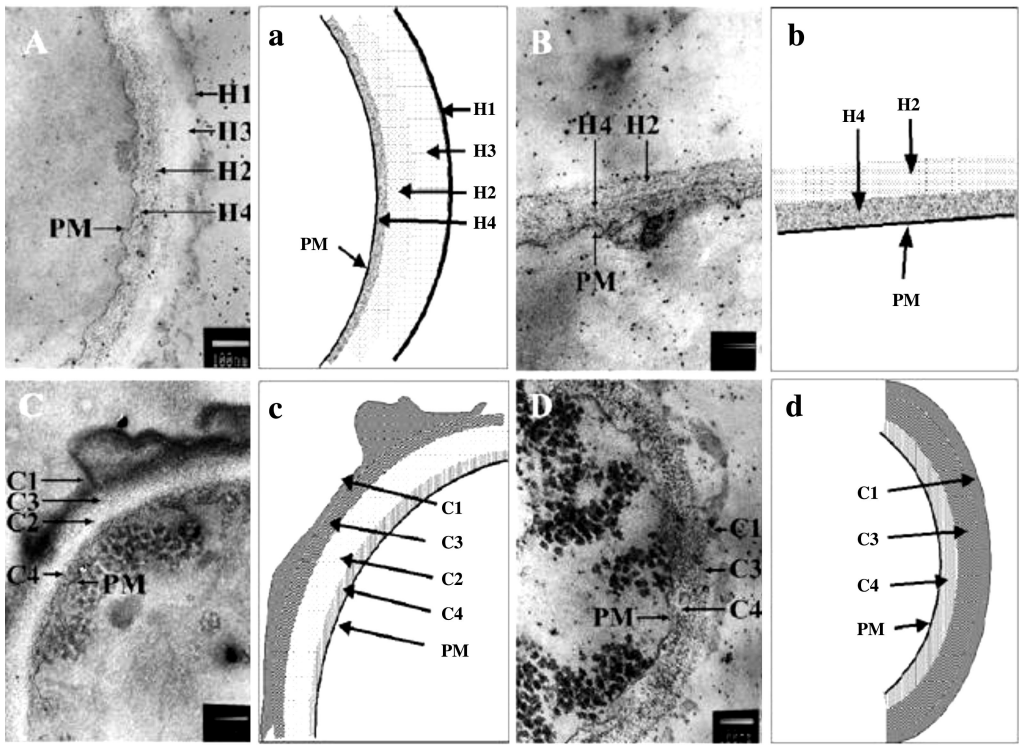

Fig. 3. The cell wall of hypha and conidium stained for carbohydrates. The cell wall layers of the wild type hypha (A) and conidium (C) are labeled as H1, H3, H2 and H4 and C1, C3, C2 and C4, respectively. Layers H1 and H3 in the hyphal wall (B) and C2 in the conidial wall (D) of the mutant strain WX17 are absent. The panels designated by lowercase letters (a, b, c and d) are the schematic diagrams of each photo, and are presented to make each layer be easily distinguishable. Note that H3 and C2 are electron-translucent layers. PM is plasma membrane. Scale bar = 100 nm.

Cytochemistry of hyphal wall digested with lytic enzymes

To determine which constituents make up the major components of each layer of the hyphal wall, Novozyme 234^{TM}, protease, laminarinase (β-glucanase) and chitinase were used either singly or in combination to digest the hyphal walls. Cross sections of hyphal wall were then stained for carbohydrates and observed by TEM.

Examination of the cytochemistry of the hyphal wall treated with protease revealed that layer H1 was lost from the wild type, in contrast to WX17 in which no changes were observed (Fig. 4A and 4B), implying that layer H1 may be composed of protein-containing constituents such as glycoprotein. However, consecutive treatment with protease and laminarinase (Fig. 4E and 4F) showed similar results as that of treatment with laminarinase alone (Fig. 4C and 4D). With these treatments, layers H1 and H3 appeared to be removed in the wild type but no changes were observed in WX17. This indicates that layer H1 may contain β-glucan in addition to protein and the major constituent of layer H3 is β-glucan and that there are no, or very little, proteineous materials or β-glucan in layers H2 and H4. Treatment with chitinase alone removed hyphal layers H2 and H3 in the wild type (Fig. 4G) and removed layer H2 in WX17 (Fig. 4H), meaning that layer H3 may contain chitin in addition to β-glucan. Consecutive treatment with laminarinase and chitinase showed that all layers except H4 were removed in the wild type and WX17 (Fig. 4I and 4J). The resistance of layer H4 to laminarinase and chitinase indicates that there is very little β-glucan or chitin in this layer. Novozyme 234^{TM} digested all hyphal wall layers in both the wild type and WX17 as expected (data not shown).

Treatment with chitinase removed 73.9% of the wall area, implying that chitin is a major component of hyphal wall in the wild type (Table 1). Chitinase treatment following after laminarinase digested H1, H3 and H2, and removed 91.3% of the cell wall area in FGSC4 (Table 1). However, treatment of the hyphal wall with protease, laminarinase, or both, removed none of the cell wall area of WX17 (Table 1 and Fig. 4B, 4D and 4F). Therefore, this data strongly implies that there is no, or very little, chitin in H1 and H4 layers, while layers H2 and H3 may contain chitin as structural components. From the results of the laminarinase digestion (Fig. 4C and 4D), it is proposed that layer H3 may be composed of a complex of β-glucan and chitin. Following this treatment, we observed only plasma membrane structures. Therefore, cytochemical analysis of the hyphal wall ultrastructure leads to the conclusion that layers H1, H2, and H3 are mainly composed of glucan-containing glycoprotein, chitin, and a β-glucan complex with chitin, respectively, whereas it is uncertain which components form the majority of layer H4.

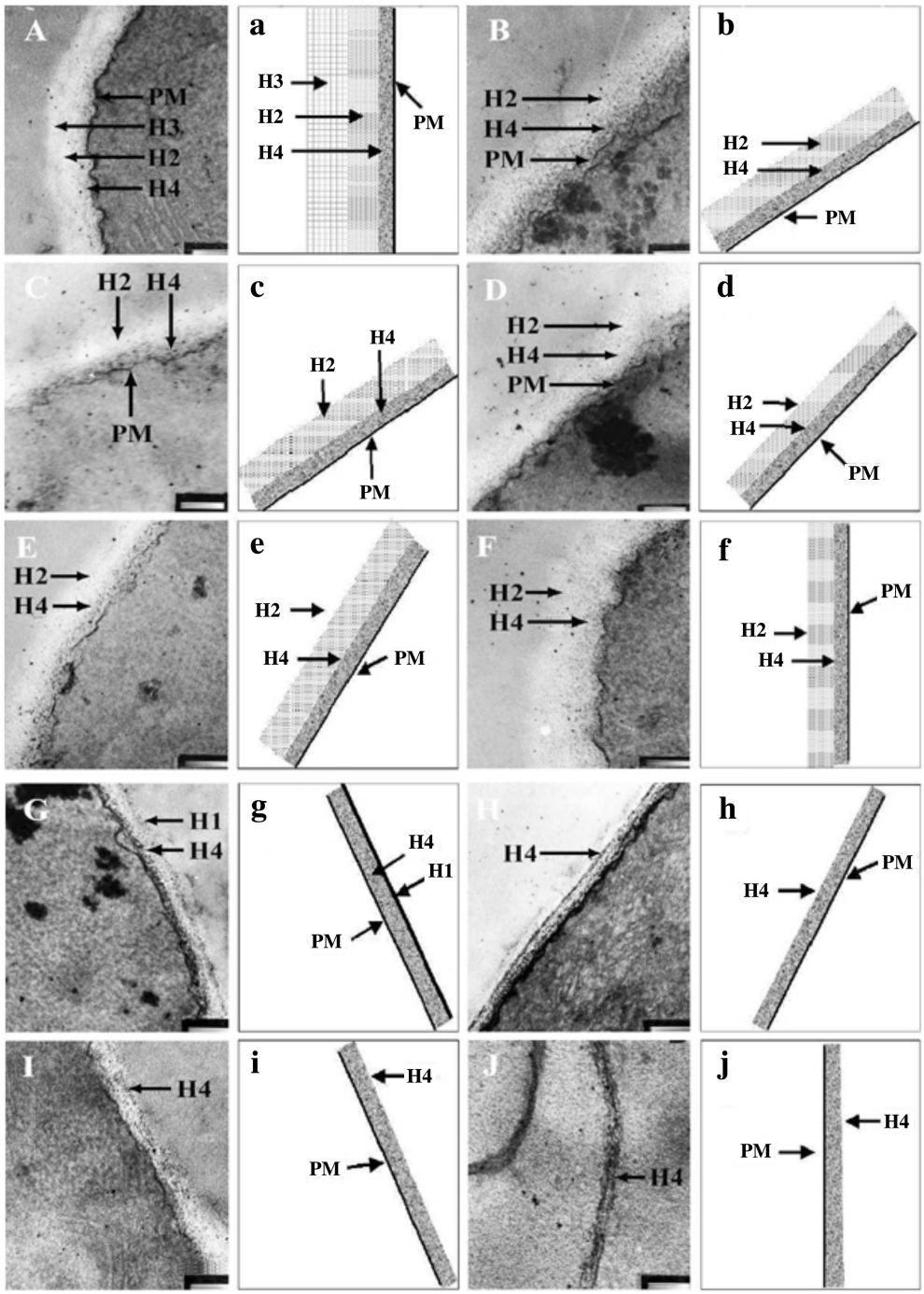

Fig. 4. The hyphal wall stained for carbohydrates after treatment with protease, laminarinase (β-glucanase), chitinase or a combination of treatments. When treated with protease the layer H1 that was normally observed in Fig. 3A disappeared in the wild type (A) but showed no changes in WX17 (B). Only layers H2 and H4 in the wild type (C) remained and there were also no changes observed in the mutant (D) after laminarinase treatment. Treatment with protease followed by laminarinase (E, wild type; F, mutant) showed similar results to laminarinase treatment alone. Treatment with chitinase removed layers H3 and H2 from the wild type (G) and H2 from the mutant (H). Treatment with laminarinase, followed by chitinase digestion, left only layer H4 in the wild type (I) and the mutant (J). The schematic diagrams of each photo identified by lowercase letters (a to j) are presented to provide clarification for each layer. Scale bar = 100 nm

Discussion

Although the mutant WX17 has the normal activity of several phenol oxidases, it can not develop pigment in any stage of its life cycle (Han and Han, 1993). The colorless phenotype was epistatic to other pigmentation mutants such as *wA9*, *yA2*, and *chaA1* and furthermore the hyphal branching of the mutant was remarkably delayed (Han

Table 1. Summary of cytochemical analysis of hyphal wall[a]

strain	enzyme treatment[b]				% of digested wall area[c]	digested layer
	P	L	C	N		
FGSC4	–	–	–	–	0.0±0.0	none
	+	–	–	–	2.78±0.8	H1
	–	+	–	–	52.0±1.3	H1, H3
	–	–	+	–	73.9±4.3	H2, H3
	+	+	–	–	63.4±2.5	H1, H3
	–	+	+	–	91.3±0.9	H1, H2, H3
	–	–	–	+	95.6±4.3	H1, H2, H3, H4
WX17	–	–	–	–	0.0±0.0	none
	+	–	–	–	0.0±0.0	none
	–	+	-	–	0.0±0.0	none
	–	–	+	–	84.4±4.4	H2
	+	+	–	–	0.0±0.0	none
	–	+	+	–	88.3±1.1	H2
	–	–	–	+	98.0±2.0	H2, H4

[a]Each mycelial mass was treated with enzyme(s) and then observed by TEM.
[b]L, laminarinase; C, chitinase; P, protease; N, Novozyme 234™. The symbol (+) and (-) indicate addition and no addition of each enzyme into reaction mixtures, respectively. Control mycelia were not treated with any enzyme.
[c]The area of cell wall after enzyme treatments shown in Figure 4 that was measured on the electronmicrographic photos. The percent of digested area was calculated by comparing the area of cell wall after enzyme treatments to that of untreated walls shown in Fig. 3A (wild type) and 3B (WX17).

and Han, 1993). The hyphal wall of WX17 was hypersensitive to Novozyme 234™, a cell wall degrading enzyme, and the viscosity of the culture broth of WX17 was reduced (Chung *et al.*, 1996). The chemical composition of the cell wall in the mutant was different from that of the wild type (Chung *et al*., 1996). The genetic analysis data that showed the epistatic activity to other pigmentation mutant genes and the chemical composition of the cell wall suggested that the cell wall structure might be altered in the mutant. Electron microscopic examination performed in this study supports this hypothesis. The surface morphology of the mutant's conidia showed that the outermost layer of the cell wall peeled off during aging. The hyphal surface of the WX17 was rougher than that of the wild type and its ascospores developed aberrant or deformed girdles (Fig. 1). Peeling off of the outer layer apparently did not cause the conidia to become osmotically unstable, since the old conidia did not rupture in hypotonic conditions (data not shown). These morphological and chemical phenotypes support the hypothesis that there must be a structural defect in the cell wall of the mutant strain.

Polacheck and Rosenberger (1977) reported that the mutant, *melB*$^{0}_{2}$, lacking both melanin and α-1,3-glucan but containing normal phenol oxidase activity, had lost the outermost layer of its cell wall as determined by TEM after staining with uranyl acetate and lead citrate. They suggested that the lack of α-1,3-glucan might cause the lack of melanin and cleistothecia.

The ultrastructure of hyphal and conidial walls stained with conventional staining revealed that the walls were composed of several electron-opaque and electron-translucent layers, consistent with the reports elsewhere (Sewall *et al.*, 1990). When the wall of the wild type was stained for carbohydrates, it appeared as four separate layers both in the hyphae and the conidia. Using an analogy to the terms of Sewall *et al.* (1990) we designated the layers, from exterior side to interior, as H1, H3, H2 and H4 in the hyphal wall, and C1, C3, C2 and C4 in the conidial wall. Carbohydrate staining showed that the electron-translucent layers, H3 in the hyphae and C2 in the conidia, were missing in the mutant wall, indicating that the *npgA* gene may have a role in constructing this electron-translucent layer (Fig. 3). In the case of the hyphal wall, the outermost layer (H1) also disappeared in WX17, which might cause the reduction in the viscosity of the culture broth. Sewall *et al.* (1990) suggested that the conidial wall was differentiated into many layers, but the total thickness did not increase as the conidia matured. In this study, it was confirmed that the hyphal and conidial wall of the wild type and WX17 were also differentiated into multiple layers (Fig. 3) but WX17's thicknesses were slightly decreased when compared to the

wild type (Fig. 2).

The previous report showed that no melanin was detected in WX17 (Chung *et al*., 1996). Although we do not know whether the *npgA* gene is directly involved in the synthesis of melanin, the null pigmentation of the *npgA1* mutation might be due to the loss of the electron-translucent layers in the hyphal and conidial walls. One explanation for the null pigmentation is that these electron-translucent layers that may function as the place for melanin deposition in the wall of the wild type are not produced in the mutant. Treatment of the cell walls with cell wall-degrading enzymes and cytochemical analysis revealed that the H1 layer was digested by protease as well as laminarinase treatment in wild type hyphae (Fig. 4A and 4C). Chitinase treatment removed layers H2 and H3 of the wild type hyphal wall and layer H2 of WX17 (Fig. 4G and 4H). Laminarinase treatment digested layers H1 and H3 of wild type (Fig. 4C). All layers were completely digested by Novozyme 234™ treatment and protoplasts were formed in both the wild type and WX17 (data not shown). From these results, we can conclude that layer H1 is made up mainly of protein, β-glucan and some other carbohydrates; layer H3 was mainly composed of chitin and β-glucan; and the main components of layer H2 might be chitin.

During hyphal growth, many cell wall lytic enzymes that hydrolyze wall components such as α-glucan, β-glucan, chitin or protein are bound together in the cell wall structure (Pollacheck and Rosenberger, 1978). The activity of β-glucanase (laminarinase) on mutant hyphal wall fractions was not detected unless laminarin was added to the reaction mixture as its cognate substrate (Chung *et al*., 1996). This suggests that the presence of β-glucanase in the cell wall is normal, but there are no substrates for β-glucanase in the mutant cell wall fraction. Cytochemical analysis of mutant walls showing the absence of the electron-translucent layers H3 and C2 suggests that these layers may alter structures in which β-glucan is the main component of the hyphal wall. Although we do not know what the C2 layer of conidia consists, it may have a crucial role in the pigment deposition and the construction of the frame structure of the cell wall.

The *npgA1* mutation confers diverse phenotypes: including no pigmentation in all cell types, the retardation of hyphal branching, hypersensitivity to Novozyme 234™ and a reduction in the viscosity of the culture broth (Han and Han, 1993; 1994; Chung *et al*., 1996). This pleiomorphic phenotype implies that the *npgA* gene product functions as a central regulator that controls the diverse phenotypes that include cell wall construction. The gene *npgA* likely encodes a putative phosphopantetheinyl transferase (PPTase) that regulates the activity of various target enzymes involved in the synthesis of fatty acid, nonribosomal peptides, and secondary metabolites such as sterigmatocystin and siderophore (unpublished results; Mootz *et al*., 2002). Electron microscopic observation of the cell wall of the *npgA1* mutant showed the absence of the two layers H1 and H3 of the hyphal wall, and the layer C2 of the conidial wall in WX17, and this observation might be due to the defective actions of PPTase that resulted in the formation of the aberrant cell wall structure.

Acknowledgments

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