Ultrastructure of the cellulolytic fungus *Trichoderma reesei*

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Summary: The cellulolytic fungus *Trichoderma reesei* is a potent cellulase producer and, therefore, cellulase hyper-producing mutants have been developed. However, morphological feature has still remained to be analyzed for understanding phenotypic change of *T. reesei* mutants. In this review, we show an electron microscopic observation of *T. reesei* to obtain new insights of morphological phenotypes of *T. reesei* mutants. We also successfully reconstructed the three dimensional structure of *T. reesei* hypha by using focused ion beam SEM technique.

Key words: filamentous fungus, *Trichoderma reesei*, cellulase, SEM, TEM, FIB-SEM

INTRODUCTION

The filamentous fungus *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) is highly potent in terms of cellulase production and the best studied among the cellulolytic fungi (Schuster and Schmoll 2010). In addition, its remarkable ability to produce large amounts of cellulolytic proteins has made this fungus an important commercial source of cellulases. Three types of cellulases are responsible for the conversion of cellulose to glucose. These include cellobiohydrolases (EC 3.2.1.91) and endoglucanases (EC 3.2.1.4), which act synergistically to degrade cellulose into cello-oligosaccharides (mainly cellobiose), and β -glucosidases (EC 3.2.1.21), which hydrolyze cellobiose into glucose. Glucose derived from cellulose can be converted into a variety of materials, such as alcohol, chemicals, or food via biological or chemical processes.

Generally, *T. reesei* produces cellulase when cellulose or its derivatives is available as the sole carbon source. Under cellulase producing conditions, the genes encoding cellulases are transcribed coordinately. This phenomenon suggests that a common regulatory machinery controls cellulase expression. To date, several *T. reesei* transcription regulators that control cellulase gene expression have been isolated and analyzed (Reviewed by Kubicek et al. 2009 and references there in, Shida et al. 2008, Furukawa et al. 2008, 2009, Nitta et al. 2012, Häkkinen et al. 2014). These investigations have made *T. reesei* a model fungus for studying the cellulase production mechanism.

Because of the industrial usefulness of *T. reesei*, cellulase hyper-secreting mutants of this fungus have been developed by systematic screening using strategies involving UV irradiation or chemical mutagens (Mäntylä et al. 1998). In Japan, a *T. reesei* mutant lineage has been developed with the support of a national project (Figure 1) (Kawamori et al. 1986a, b). In 2008, the whole genome information of *T. reesei* wild type,

QM6a, was obtained and made available to the public (<http://genome.jgi-psf.org/Trire2/Trire2.home.html>). In addition, next generation DNA sequence techniques have made rapid progress in recent years. Therefore, now it is possible to perform the comparative genomic analysis between *T. reesei* wild type and the mutants. Such comparative studies revealed that a few genes are responsible for the enhancement of cellulase production in *T. reesei* mutant PC-3-7 (Nitta et al. 2012, Porciuncula et al. 2013).

The molecular basis of *T. reesei* protein production has been widely analyzed, but a systematic morphological investigation of *T. reesei* mutants has not been carried out until now, despite

Figure 1 The lineage of mutant *T. reesei* strains developed in Japan. Conidia from each mutant were inoculated into an agar plate containing cellulose as a carbon source and incubated for 5 days. Right side panels are optical microscopic observations of hyphae derived from submerged culture of the standard strain QM9414 and the mutant strain PC-3-7. Scale bar indicates 10 μ m.

the awareness of a few conspicuous differences between the wild type and the mutant. For example, colony formation on the plate culture and the shape of the hyphae in the submerged culture are drastically different between the wild type strain and the mutants (Figure 1). In this paper, we attempted to examine the ultrastructure of the mutant and the wild type strains and investigated the possibility of some of these differences contributing to the enhanced cellulase production by the mutant strain.

MORPHOLOGICAL ANALYSIS OF *T. REESEI* HYPHAE BY SCANNING ELECTRON MICROSCOPY (SEM)

One of the characteristics of filamentous fungi is the formation of distinct hyphae during growth and mycelial formation. Hyphae differentiate into conidiospores during asexual reproduction and their growth is highly polarized and restricted to the apical extensions in most ascomycetes. Similar growth patterns with apical extension are observed in other cells, such as neural cells, pollen tubes, and root hair cells, etc. Thus filamentous fungi can be used as model organisms among eukaryotes to study the apical growth of cells. Morphological analysis of conidiospores of *Trichoderma* species, such as *T. reesei*, *T. virens*, *T. viride* has been carried out previously for the purpose of taxonomic classification of different species (Meyer and Plaskowitz 1989, Gams and Bissett, 1998). Changes in the morphology of the cells and of organelles in fungi have been examined using optical microscopy and transmission electron microscopy (Peterbauer et al. 1992). In this report, we have performed extensive analysis of the morphological features of each of the mutant lineage that was developed in Japan. In order to determine the possible existence of distinct morphological phenotypes that can be associated with enzyme production capabilities, we made electron microscopic observations of the hyphal surface architecture. SEM observation of the hyphae of the standard strain QM9414 and the mutant strain PC-3-7, which has a 2-fold greater cellulase activity than QM9414 does, was carried out by low-voltage scanning electron microscopy (LV-SEM; S-5500, Hitachi) at 1.5 kV using a non-coating or slightly coating technique (Osumi et al. 1995). Hyphal samples of QM9414 and PC-3-7 were obtained from submerged culture with continuous agitation for one day in 1% (w/v) glucose or for three days in 1% (w/v) Avicel (micro-crystalline cellulose). Subsequently, hyphae were processed for SEM by fixation with glutaraldehyde–osmium tetroxide and staining with ruthenium tetroxide according to the method of Osumi et al. (Osumi et al. 1995). Figure 2 shows the hyphal surface architecture and branching feature of QM9414 and mutant PC-3-7. Both the strains exhibited a similar surface morphology when grown in glucose culture (Figure 2A, C). The hyphal surface of both the strains was partially and sparsely covered with a fibrous material. There are a few reports that recorded the appearance of such a fibrous material (Chapman 1983, Sprey 1986, 1988), but details of this material are not available in the morphological analysis of filamentous fungi, probably due to difficulties to reserve those fibrous structure using standard coating techniques for SEM.

Here, we adapted a different technique of uncoating or slightly coating the specimens, followed by low-voltage SEM (LV-SEM) observation that provided ultra-high-resolution images of the fibrous material. In contrast to the glucose cultivation, the hyphae of both strains obtained from Avicel cultivation were densely covered with plenty of fibrous material or covered with a layer that was knit out of fibrous material (Figure 2B, D). When the fibrous material in close proximity to the surface of the cell wall was magnified, it seemed as if the fibrous material was fused to the cell wall (Figure 3A) and a granular material was seen on the cell surface (Figure 3B). Although there have not been any experimental evidences, it is possible that the fibrous material synthesized on the cell wall or on the membrane was secreted to the surface of the hypha. Secreted fibrous materials may eventually form a layer by entangling with each other. Previously, studies using immuno-electron microscopic analysis reported that *T. reesei* produces a fibrous layer called as ‘exopolysaccharide layer’ on the cell wall and showed that it plays a role in trapping secreted β -glucosidases (Sprey 1986) and endoglucanases (Chapman 1983, Sprey 1988). It is probable that the fibrous material enables *T. reesei* to effectively harness the

Figure 2 LV-SEM images of *T. reesei*. QM9414 (A and B) and the mutant PC-3-7 (C and D) mycelia were grown in liquid culture containing 1% (w/v) glucose for one day (A and C) and in 1% (w/v) Avicel for three days (B and D). Note the appearance of the fibrous extracellular material, which sparsely covers the hyphae in glucose cultures, but is much more abundant in the Avicel cultures. Swollen cells were often observed particularly when grown in Avicel cultures. Scale bar indicates 1 μ m.

Figure 3 Surface structure of *T. reesei* QM9414 grown on glucose. (A) and (B) show another portion of the hyphal surface. (A) Fibrous material fused to the cell wall. (B) Elongating nascent fibers and emerging granules (arrows) being secreted from the cell wall surface. Scale bar indicates 50 nm.

available carbon and perform cellulose hydrolysis by trapping the essential enzymes in the vicinity of the cell wall. This might be one of the survival strategies of *T. reesei*. The composition of the fibrous material is not well understood yet and studies are needed in future to characterize this material.

ANALYSIS OF THE *T. REESEI* CELL BY TRANSMISSION ELECTRON MICROSCOPY (TEM)

Our observations of the massive fibrous material produced in the Avicel cultivation (Figure 2 B, D) suggest a correlation between cellulase production and the amount of fibrous material produced. From the viewpoint of hyphal morphology, PC-3-7 formed shorter, swollen, and highly branched hyphae compared to hyphae produced by QM9414. In addition, swollen beads-like cells with an oval shape were observed on PC-3-7 hyphae. It was obvious that the cellulase-producing hyphae underwent a drastic morphological change in the lineage of the mutant. Therefore, we predicted that the difference in hyphal morphology would have been accomplished due to associated cytological modifications and investigated the subcellular morphological changes by TEM. Figure 4 shows ultrathin sections of the samples fixed with glutaraldehyde - potassium permanganate (Osumi and Sand 1969) and observed by TEM (H-7000, Hitachi). We observed that the septal pore was closed by Woronin bodies (indicated by white arrows) and peroxisomes, which might differentiate into Woronin bodies (Escan et al. 2009) that were present nearby a septal pore in the QM9414 strain (Figure 4A, B). In general, hypha of filamentous fungus is composed of rows of cells separated by septa possessing small pores through which adjacent cells communicate with each other (Maruyama et al. 2005). The Woronin body, a unique organelle found in the *Pezizomycotina*, plugs the septal pore in the event of hyphal damage, such as osmotic damages. It is identified as a single membrane-bound structure situated in very close proximity to the septa (Markham and Collinge 1987).

We did not observe any significant cytological differences in hyphae of either strain when grown in glucose. Nuclei were typically delimited by a double-layered nuclear envelope with distinct nuclear pores, and characteristic Golgi structures were observed in both the mutants (Figure 4A, C). In contrast, when grown in Avicel, the hypha of PC-3-7 frequently exhibited long parallel-stacked layers of endoplasmic reticulum (ER), which were rarely seen in cells grown in glucose (Figure 4D). It has been reported that the proliferation of rough ER in *T. reesei* hypercellulotic mutants was accompanied by an increased secretion of cellulase (Ghosh et al. 1984). In addition to ER, vacuoles of differing sizes were observed to be randomly distributed in the cytoplasm of large numbers of cells in both strains. PC-3-7, in particular, had many vacuoles that were much larger than those of QM9414. A large number of electron dense spots, presumably proteins that should have been secreted, could be observed within those vacuoles (Figure 4D, black arrow). Several spots with a high electron density were also observed in addition to those confined to the vacuoles. A previous report using immuno-electron microscopic observation has shown

that xylanases were located in the vacuoles during cellulase production in high cellulase-producing mutant of *T. reesei*, the Rut-C30 strain (Kurzatkowski et al. 1993). Altogether, our results and previous reports suggest that the secreted proteins that were not completely folded or those that did not undergo posttranslational modifications might have been transferred to vacuoles.

Next, we measured the thickness of the extracellular fibrous material layer in *T. reesei* mutants grown on Avicel (shown as "F" in the Figure 4B inset) to investigate if there is any difference between QM9414 and the cellulase hyper-producing mutant PC-3-7 in regard with the fibrous layer production. We performed thickness measurements manually one hundred times on five scanned individual TEM images. The fibrous material layer of QM9414 and PC-3-7 were 301.2 nm (± 78.6) and 180.0 nm (± 74.0), respectively, indicating a 40% reduction in thickness in PC-3-7 compared to QM9414. It has been reported that the cell wall of cellulase hyper-producing mutant contains less chitin than that of the wild-type strains and tends to be thinner (Nevalainen et al. 1995). Our results are in agreement with this finding in that the cell envelope, including the fibrous material layer and the cell wall, of the hypercellulotic mutant PC-3-7 was thinner than that of QM9414 when cultured in Avicel-containing medium. The result is consistent with the SEM images (Figure 2) that show a massive amount of fibrous layer covering the hyphae grown on Avicel, particularly on QM9414, but the same was not true for cells grown on glucose. It is possible that cell expansion caused by the enlarged vacuole observed on Avicel culture contributes to the reduced thickness of the cell envelope in the PC-3-7 strain.

Figure 4 TEM images of the same samples shown in Figure 2. QM9414 (A and B) and the mutant PC-3-7 (C and D) mycelia were grown in liquid culture containing 1% (w/v) glucose for one day (A and C) and in 1% (w/v) Avicel for three days (B and D). Note the fibrous appearance at the outer surface of cell wall (shown as 'F' in the inset of image B) and enlarged vacuoles with electron dense spots (black arrows) particularly in Avicel cultures. Scale bar indicates 1 μ m. White arrows indicate Woronin bodies at the septal pores. CW, cell wall; ER, endoplasmic reticulum; F, fibrous layer; G, golgi body M, mitochondria; N, nucleus; P, peroxisome; V, vacuole.

3-DIMENSIONAL (3-D) IMAGE CONSTRUCTION OF *T. REESEI* HYPHA BY FOCUSED ION BEAM SEM (FIB-SEM)

In order to have a meaningful analysis of the morphology of a fungal cell, it is imperative to understand the topology of the membranous structures. Conventional 3-D analysis of cell structures uses serial sections of cell specimens. However, preparation of ultra-thin sections and reconstruction of the 3-D structure using those images requires expert skills and is extremely time consuming. Other alternative hybrid SEM techniques have been recently introduced for obtaining serial sections of biological samples embedded in resin. Notable among these are the serial block-face SEM (SBF-SEM) and the focused ion beam SEM (FIB-SEM) techniques. With SBF-SEM, serial section images can be obtained by repeating imaging and cutting the resin block with an ultramicrotome equipped inside the SEM chamber (Denk et al. 2004). FIB-SEM, on the other hand, allows a precise sectioning by ablation of material from the sample in between imaging scans. FIB-SEM uses an electron beam to capture the SEM image of the sample surface, followed by an ion beam to remove a layer of material (15–50 nm) that then exposes a new section for imaging (Bushby et al. 2011). The series of SEM images are then merged to form a 3-D reconstruction of the sample. With these techniques, a resolution of 20–40 nm is achieved for thick 3-D samples without a need for manual generation of serial sections. These technologies have also become useful for understanding a general picture of a microbial cell, and there have been several reports in which fungal cells were observed by FIB-SEM (Kamino et al. 2004, Ngamskulrungron et al. 2012, Kralj Kunčič et al. 2010).

Our results revealed that the hyphae partially expanded only in the cellulase hyper-producing mutant of *T. reesei*, PC-3-7 when cultured in Avicel and that the abundance of the hypertrophic vacuoles might have caused cell expansion and a reduction in the thickness of the cell membrane. These results do implicate a relationship between cellulase production and a morphological phenotype. However, obtaining intracellular information of *T. reesei* from a single section of an SEM image is quite difficult because of its multicellular nature and its apical growth. Therefore, we applied the FIB-SEM technique for serial sectioning of *T. reesei* QM9414 and PC-3-7 hyphae (data not shown). We used the same embedded hyphae used in our previous TEM analysis (Nitta et al. 2012). The target area (30 μm x 40 μm) of the block face was milled using FIB, and the SEM image acquisition (x 3,000 magnification) was carried out with 100 nm thick slices. About 500 serial cross section images were obtained for each specimen, which facilitated the observation of various organelles, such as the nucleus, vacuole, mitochondria, and the Woronin body (Figure 5). We reconstructed the 3-D structure of the hyphae using the TRI/3D-SRFB-FCS (Ratoc system engineering) from every fifth slice. Figure 6 shows the 3-D structure of QM9414 hypha from cellulose cultivation. Since extracellular fibrous materials were observed in serial cross section images, the 3-D structure of the hyphae and the fibrous material was reconstructed by utilizing the difference in contrast between the fibrous material and the

Figure 5 Serial cross section images of QM9414 grown on glucose. Keys to abbreviations are the same as those of Figure 4. Nucleus, vacuole, mitochondria, and fibrous material are clearly observed. The white arrow indicates the Woronin body. Scale bar indicates 5 μm .

Figure 6 Schematic presentation of the reconstructed QM9414 mycelia grown on Avicel. Front view, side view, and upper view are indicated. Mycelia with and without the fibrous material are drawn in green and blue, respectively. The fibrous material is drawn in red. Scale bar indicates 10 μm .

background. Consequently, it became clear that there are two types of QM9414 hyphae when grown in cellulose culture; those with and without the massive fibrous material. It could be that the production of fibrous material occurs only at a certain stage of hyphal growth. Otherwise, the site where the fibrous materials are produced may be restricted.

We selected targeted hyphae from each sample and painted the nuclei and vacuoles manually from every fifth slice using the Photoshop Elements version 11.0 (Adobe Systems), and the entire intracellular structure was also reconstructed. The vacuole was obviously enlarged in the PC-3-7 hypha derived from cellulose cultivation (Figure 7). Because we could not observe such a change in the vacuolar in the cellulose culture of QM9414, we propose that there is a relationship between the enhanced cellulase production and the hypertrophic vacuole in the *T. reesei* mutant (Figure 7B, D). Furthermore, in PC-3-7, the number of nuclei in the cells from cellulose culture was fewer than that from the glucose culture (Figure 7G, H). It is also possible that there is a correlation between the number of nuclei

Figure 7 3-D structure of targeted hyphae. QM 9414 hyphae (A, B, E, and F) and PC-3-7 hyphae (C, D, G, and H) were derived from the glucose medium (A, C, E, and G) and Avicel medium (B, D, F, and H). Scale bar indicates 10 μm . A, B, C, and D indicate the volumes of the vacuoles. Inset in D represents color range of cubic volume of the vacuole. E, F, G, and H show the distribution of nuclei. Inset in H shows the color range of the diameter of the nucleus.

and the production of cellulase. However, further studies are needed to confirm these hypotheses, as our observations have been restricted to the hyphae of these strains.

FUTURE PERSPECTIVES

Although *T. reesei* is extensively studied because of its industrial usefulness, there is a paucity of reports focusing on the morphological features of this strain. However, enough evidence derived from previous morphological analyses and our own investigations suggests that the hyphal morphology and intracellular architecture of these strains render selective advantages resulting in the enhanced enzyme production in *T. reesei*. As shown in this review, recently developed 3-D structure reconstruction techniques also allowed us to analyze the intracellular structure of these filamentous fungi to understand these possibilities further. The reconstruction of high-precision 3-D images of *T. reesei* mycelia is in progress. The systematic accumulation of morphological information or “morphome” of *T. reesei* will lead to further understanding of the morphological intricacies and how they could possibly aid in the high cellulase production of this fungus.

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