Cellulase hyperproducing mutants derived from the fungus Trichoderma reesei QM9414 were analyzed. They exhibited higher filter-paper degrading activity and a lower growth rate than the wild-type QM9414 strain. Transcription of the celllobiohydrolase I and endoglucanase I genes in the mutants was also greater than that of QM9414, suggesting that cellulase hyperproduction by these mutants was regulated at the transcriptional level. Furthermore, it remains unclear why these mutants exhibit higher cellulase activity than wild-type strains. Understanding the mechanisms underlying cellulase hyperproduction by these mutants might lead to the generation of a fungal strain with higher cellulase activity. In the present study, we characterized cellulase production at enzymatic and transcriptional levels using the mutants PC-1-4 and X31 and cellulose materials to induce cellulose production.

We obtained the T. reesei strain QM9414 (the wild-type) and cellulase hyperproducing mutant strains X31 and PC-1-4 from the American Type Culture Collection (Manassas, VA). We then cultured these strains in basal medium containing 10 g/l of polypeptone, 0.5 g/l of yeast extract, 1.4 g/l of (NH₄)₂SO₄, 24 g/l of KH₂PO₄, 0.3 g/l of CaCl₂, 0.3 g/l of MgSO₄, and 0.1% trace element solution (pH 4.0), and 40 g/l of sorbitol, a non-inducing and non-repressing carbon source for cellulase genes (3) at 30°C for 2 d at 220 rpm. The resulting mycelium were transferred into basal medium containing 40 g/l of Avicel PH-101 (Fluka, Buchs, Switzerland) or ball-milled eucalyptus, and cultured at 30°C for a further 7 d at 220 rpm. Ball-milled eucalyptus was prepared as described by Inoue et al. Next we investigated extracellular filter paper degrading enzyme (FPase) activity by a previously described method. When the X31 and PC-1-4 mutant strains were cultured in Avicel PH-101 or eucalyptus for 7 d, the mutants exhibited 1.5- and 3.5-fold higher FPase activity respectively levels than the wild-type QM9414 strain (Fig. 1). Avicel PH-101 induced a higher maximum FPase activity in the mutants than was induced by eucalyptus, although QM9414 demonstrated similar maximum FPase activity in the Avicel PH-101 (1.8 U/ml, 6 d) and eucalyptus (1.5 U/ml, 7 d, Fig. 1). These data suggest that cellulase production in the mutants cultured with pure cellulose was more effective than that with the impure cellulose. These findings are consistent with previous results that demonstrated higher cellulase induction by alkali-treated bagasse than by untreated bagasse. Next we measured the intracellular ATP concentration, as described previously, as it is well established that intracellular ATP concentration reflects fungal growth. The maximum intracellular ATP concentration was measured using a fluorometric method. ATP concentrations were measured using a Microplate reader (Model 3550, PerkinElmer, MA) according to the manufacturer's instructions. The ATP concentration was expressed as nanomoles of ATP per milligram of mycelium dry weight. The results are shown in Fig. 2. The ATP concentration was significantly higher in the mutants than in the wild-type strain. The ATP concentration was also higher in the mutants cultured with eucalyptus than in those cultured with Avicel PH-101. These results suggest that cellulase production in the mutants is associated with increased ATP production, which may be due to increased fungal growth. In conclusion, the cellulase hyperproducing mutants derived from T. reesei QM9414 showed higher cellulase activity than the wild-type strain. These mutants may be useful for the production of cellulase for applications such as fuel ethanol production.
concentration of the mutants was found to be lower than that of the QM9414 strain, except when X31 was cultured in Avicel PH-101, indicating that the growth rate of the mutants was not as great as that of QM9414. These results suggest that the cellulase activities of the mutants on a per-cell basis are higher than that of QM9414. The use of Avicel PH-101 in the culture medium also resulted in higher intracellular ATP concentrations in the mutants than when eucalyptus was added to the medium, while the maximum ATP concentrations of QM9414 cultured in Avicel PH-101 and in eucalyptus remained similar. This indicates that the growth of the mutants in pure cellulose was enhanced as compared to growth in impure cellulose, and confirms the results obtained in the present study that demonstrated increased FPase activity in mutants cultured with Avicel PH-101 as compared to eucalyptus.

Next we investigated the transcription of the cellobiohydrolase I and endoglucanase I genes, the major cellulase genes of T. reesei, in each of the strains by real-time PCR analysis. First, total RNA from frozen cells was extracted using the FastRNA Pro Red kit (MP Biomedicals, Irvine, CA). After DNase I (Takara Bio, Otsu, Japan) treatment, the extracted RNA solution was purified with an RNeasy Mini kit (Qiagen, Hilden, Germany) and cDNA synthesized from 10 µg of total RNA using reverse transcriptase XL (Takara Bio) with oligo (dT) 20 (Toyobo, Osaka, Japan) as the primer. Real-time PCR analysis was undertaken using a 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The specific primers used to amplify the actin gene were 5’-caactgggacgacatggaga-3’ and 5’-gggattgacttggtgggtg-3’. Those used for the additional genes analyzed have been described previously.14) The conditions for thermal cycling have also been described previously.14) The relative expression ratio was normalized to the actin housekeeping gene. We found that transcription of the cellobiohydrolase I and endoglucanase I genes was highest in the PC-1-4 strain, followed by the X31 and QM9414 strains, with the exception of the strains cultured with Avicel PH-101 for 72 h (Fig. 2). These findings indicate that cellulase hyperproduction by the mutants was regulated at transcriptional level. Earlier reports have suggested that transcription of the cellulase genes in the PC-3-7 strain was higher than QM9414 when the strains were incubated with sophorose or L-sorbose.10) Our results demonstrating that transcription of the cellulase genes in X31 and PC-1-4 were up-regulated by Avicel PH-101 and eucalyptus indicate that the transcription of cellulase genes in the mutants obtained from Kyowa Hakko were also up-regulated by various inducers to a higher level than the wild-type strain. Transcription of these genes was also higher in PC-1-4 than X31, although the FPase activity of these mutants was largely unchanged. This suggests that cellulase production per cell in the PC-1-4 strain increased as compared to X31. However, the cell growth of PC-1-4 was reduced in X31 (Fig. 1C and D), a result that likely contributed to the finding that the FPase activity in these mutants was similar value based on the culture supernatant (Fig. 1A and B).

We also sequenced the promoter region of the cellobiohydrolase I gene in the X31 (AB524354), PC-

![Fig. 1. Extracellular FPase Activity and Intracellular ATP Concentrations in the T. reesei Strains.](image-url)
production observed in the mutants (Fig. 3). However, the level of transferase activity of beta-glucosidase in the strains remains unknown. The reasons for the differences in the transcription of the genes in the observed strains are an important question to be addressed in the future.

In the present study, we found that X31 and PC-1-4 produced large amounts of cellulase at transcriptional level. This finding might prove useful in investigating the mechanisms underlying cellulase hyperproduction in the mutants obtained from Kyowa Hakko.

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