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Cellulase productivity of *Trichoderma reesei* mutants developed in Japan varies with varying pH conditions

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The ascomycete *Trichoderma reesei* is known to produce a variety of cellulases and hemicellulases and the hypercellulolytic mutants of this fungus are useful as industrial cellulase producers. In Japan, PC-3-7, derived from the early mutant QM9414, is well-known as a cellulase hyperproducing mutant. In addition to the productivity of enzymes, the composition of secreted enzymes greatly influences biomass saccharification. Therefore, we evaluated the cellulase productivity of *T. reesei* mutants in Japan at different pH as a factor influencing enzyme production. At higher pH values, QM9414 exhibited reduced cellulase productivity whereas PC-3-7 maintained high cellulase productivity and gene expression at the transcriptional level. The gene encoding the pH-responsive transcription factor PACI did not mutate in PC-3-7, and its expression pattern against different pH conditions was similar between QM9414 and PC-3-7. Furthermore, the deletion of *pac1* encoding PACI caused different expression patterns of cellulase genes between QM9414 and PC-3-7. Therefore, we suggest that *T. reesei* possesses a pH-responsive cellulase production mechanism that is different from the PACI-related mechanism. Finally, we identified that N-25, a strain developed at an early stage of mutant development acquired cellulase productivity at a higher pH. In this investigation, we also found and tested candidate genes possibly affecting pH response using comparative genome analysis.

[**Key words:** *Trichoderma reesei*; pH; Cellulase; Comparative analysis; Gene regulation]

Plant biomass is the most abundant resource on earth consisting predominantly of cellulose, followed by hemicellulose and lignin. In nature, cellulose is utilized as a carbon source by a variety of cellulose-degrading microorganisms. Monosaccharides are produced from cellulosic biomass by the cellulolytic enzymes in these microorganisms. Cellulosic biomass is a promising carbon source when converted to biofuel or other valuable materials through chemical- or bio-conversion. Moreover, through the effective production of cellulolytic enzymes with high saccharification activity from microorganisms, these processes could reduce global warming and environmental pollution by decreasing the demand for fossil fuels.

Among cellulolytic microorganisms, the filamentous fungi are used as cellulase producers. In particular, *Trichoderma reesei* (an anamorph *Hypocrea jecorina*), one of the most efficient producers of cellulolytic and xylanolytic enzymes, is widely used as a cellulase producer (1). For several decades, *T. reesei* enzymes have been used in a wide range of industrial applications, including in paper, pulp, food, and animal feed industries (2). Indeed, many studies have been carried out including those on cellulase enzymes, regulation of genes encoding cellulase and hemicellulase, and modification of strains with genetic recombination (3).

Previously, improvement of *T. reesei* strains to develop cellulase hyperproducing strains was carried out by mutagenesis using UV irradiation and chemical mutagens (4). From these efforts, mutant strains Rut C-30 and QM9414 (as well as their derivatives) obtained from the wild-type strain QM6a emerged and are being used as the basis for industrial enzyme-producing strains. In Japan, *T. reesei* mutants have also been developed with the support of a national project, Research Association for Petroleum Alternatives Development (RAPAD). PC-3-7, obtained through 5 generations of mutagenesis and screening from QM9414, shows enhanced cellulase production on a broad range of carbon sources (5), with twice as much cellulase activity as QM9414 when induced by sophorose, a potent inducer of cellulase expression (6). In comparison with QM9414, PC-3-7 has acquired high cellulase inductivity against not only microcrystalline cellulose and sophorose, but also bagasse, whey, L-sorbose, and cellobiose (5,7). Previously, we performed comparative genome analysis to elucidate genomic mutation(s) that brought about enhancement of cellulase production. Various aspects of cellulase production in PC-3-7 under specific carbon sources were investigated and mutations in *cre1*, *bglr*, and *bgl2* were identified as being involved in cellulase production (7–9).

PC-3-7 strain has been used as the basis for industrial enzyme-producing strains as a cellulase hyperproducing mutant. Previously, we constructed a PC-3-7 strain expressing high performance BGLI under the control of *xyn3* (X3AB1 strain), *egl1* (E1AB1 strain) or

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modified *xyn3* (X3-2RB_AB1 strain) promoter, which has higher specific activity against cellobiose than PC-3-7 (10–12) (Fig. S1). JN11, the enzyme preparation produced by X3AB1 strain on cellulose, showed the highest saccharification ability of monosaccharides against hydrothermally pretreated biomass, such as the pretreated biomass containing only cellulose (10). In contrast, JN13H, the enzyme preparation produced by E1AB1 strain on cellulose and xylan, resulted in marked improvement in the saccharification ability against NaOH-pretreated biomass, such as the pretreated biomass containing cellulose and hemicellulose (11). It is suggested that this difference in saccharification ability was led by alteration of the enzyme ratio. Thus, it will be desirable to develop an industrial strain that can secrete cellulase preparation with a suitable enzyme ratio against each pretreated biomass. Using these facts, it is important to investigate mechanism(s) for controlling the ratio of secreted cellulases for industrial application of cellulases and hemicellulases. PC-3-7 strain has been studied for inducer responsibility, gene regulation, and genome analysis for a long time. However, previous investigations revolved around cellulase production using specific carbon sources without taking into consideration phenotypic changes of PC-3-7 under other environmental factors. Because each industrial strain was derived from PC-3-7, an understanding of the basic characteristics of this strain in detail could lead to more improvement in its industrial application.

The pH level on cultivation is an important factor in enzyme production by *T. reesei*. Several reports describe the effect of pH on enzyme production in *T. reesei* mutants. For instance, Bailey et al. (13) showed that xylanase production of Rut C-30 is favored by a high pH between 6.0 and 7.0 on cellulose- and xylan-based media whereas significant production of cellulase was obtained at a lower pH of 4.0. In addition, differences in cultivation pH have been reported for the production of xylanase I, II, and III, in which the pH of produced xylanase corresponded to the optimum pH of the particular enzyme on a lactose-based medium (14). Furthermore, different secretion responses of cellulases and glycoside hydrolases to pH were shown by the comprehensive analysis of secretome derived from QM6a, QM9414, Rut C-30, and QM9414MG5, the β-glucosidase high-producing mutant derived from QM9414 (15), on a cellulose-based medium (16). This investigation also demonstrated that the lower pH promotes higher protein expression, which did not affect cellulose degradation and enzyme activities. In addition, ambient pH affects not only cellulase production, but also the uptake of cellulase inducers such as sophorose and cellobiose in *T. reesei* QM6a (17). Therefore, understanding the pH response in *T. reesei* could lead to its better industrial use.

In the functional characterization of the *pac1* gene encoding PACI, an ortholog of *Aspergillus nidulans* PacC and a key transcription factor mediating a wide-range of gene regulation for adaptation to extracellular pH, He et al. (18) showed that cellulase genes and *xyr1* encoding their activator were repressed by PACI. In 2015, using genome-wide transcriptional profiling, Häkkinen et al. (19) found that few of the glycoside hydrolase-encoding genes showed direct PACI-mediated regulation and suggested that other regulatory mechanisms affected gene expression at different pH levels.

Evidence shows that pH-dependent cellulase production of PC-3-7 strain is different from its parent strain QM9414 (5,20). PC-3-7 strain showed enhanced BGL activity by shifting the pH from 4.0 to pH 5.0 or 6.0 in a jar fermentor (20). Interestingly, no mutations in *pac1* and genes encoding the *T. reesei* homolog of Pal proteins that are components of signal transduction of ambient pH in *A. nidulans* have been found in *T. reesei* PC-3-7 (21). Therefore, the pH-dependent cellulase production mechanism of PC-3-7 seems to function through another mechanism that is independent of the PACI regulatory system. Elucidation of this novel mechanism will provide important information for the improvement of this industrial strain.

In this study, we evaluated different *T. reesei* mutants to obtain new insights into the mechanism of pH-dependent cellulase productivity using flask cultivation, while taking into account the effect of PACI. We suggest that differences in the pH-dependent phenotype of each mutant and its cellulase production abilities at a high pH are acquired at an early stage of strain development.

MATERIALS AND METHODS

***T. reesei* mutant strains and culture conditions** *T. reesei* wild type QM6a (ATCC 13631) and its mutants QM9414 (ATCC 26921), N-25, K-14, KDG-12, and PC-3-7 (ATCC 66589) were obtained from Kao Co., Ltd. (Tokyo, Japan). QM9414 is an early cellulase-overproducing mutant. PC-3-7 is the cellulase hyperproducing mutant derived from QM9414. QM9414ΔP and PC-3-7ΔKP [*pyr4* gene deletants previously described (9)], and N-25 were used to construct the recombinants. Strains were maintained on Difco Potato Dextrose Agar (PDA; BD Diagnostics, Franklin Lakes, NJ, USA) plates, and conidia were stored at −80°C in 0.9% NaCl solution containing 10% glycerol until use. For enzyme production, 10^7 conidia of *T. reesei* strains were inoculated in 50 ml of the basal medium containing 1% (w/v) Avicel (Funakoshi Pharmaceutical Co., Tokyo, Japan) buffered at pH 3.5–5.5 using tartrate buffer and cultivated at 28°C with shaking at 220 rpm for 5 days in a shaking incubator Bioshaker G BR 300 (Taitec Corporation, Saitama, Japan) (22). Culture supernatant as enzyme preparations was obtained by centrifugation at 18,000 ×*g* and used for assaying cellulase activity. For transcriptional analysis, mycelia were harvested by filtration on Miracloth (Merck Millipore, Burlington, MA, USA) and immediately frozen in liquid nitrogen.

Plasmid construction In this study, three gene disruption cassettes were constructed for *pac1*, *tr22911*, and *tr75418* as follows: For the *pac1* disruption cassette, the 1.5 kbp upstream region and 1.6 kbp downstream region were amplified by polymerase chain reaction (PCR) using the genomic DNA derived from QM9414 as a template. The *Eco*RI site was introduced to the 5′ end of the upstream region and the 3′ end of the downstream region. The *pyr4* selective marker was released from pUpyr4F (9) by *Fsp*I excision. To yield pUΔpac1pyr4, these DNA fragments were cloned into the *Hinc*II site of pUC118 (Takara Bio, Shiga, Japan) using the In-Fusion cloning kit (Takara Bio) in the order: *pac1* upstream region, *pyr4* marker, and *pac1* downstream region. This fragment was used for transformation of strains QM9414ΔP, N-25ΔPA- (described later), and PC-3-7ΔKP.

For the *tr22911* disruption cassette, a DNA fragment containing the 1.5 kbp upstream region, *tr22911* ORF, and a 1.8 kbp downstream region was amplified by PCR using the genomic DNA derived from QM9414 as a template. *Sca*I sites were introduced into both termini of the PCR product. This product was cloned into the *Hinc*II site of pUC118 using the In-Fusion cloning kit. The resulting plasmid, pUtr22911, was opened by inverse PCR with a primer pair that was designed to anneal to the antisense and sense strand in close proximity to the start codon and stop codon, respectively. The *pyr4* selective marker, derived from pUpyr4 by *Eco*RI digestion, was cloned into this PCR product using the In-Fusion cloning kit, resulting in the plasmid pUΔtr22911pyr4. pUΔtr22911pyr4 was excised by *Sca*I to obtain the *tr22911* disruption cassette for *T. reesei* transformation. This fragment was used for transformation of strain QM9414ΔP.

For the *tr75418* disruption cassette, the DNA fragment containing the 1.9 kbp upstream region, *tr75418* ORF, and the 2.5 kbp downstream region was amplified by PCR using the PC-3-7 genome DNA as the template with tr75418_Fw and tr75418_Rv. The resulting PCR product was inserted into the *Hinc*II site of pUC118 to yield pUtr75418. To remove the part in *tr75418* containing the start codon, pUtr75418 was digested with *Bgl*II existing on the *tr75418* upstream region and ORF, and self-ligated. The resulting plasmid was named pUtr75418Bg. Then, the blunt-ended DNA fragment of *amdS* selective marker [derived from pBamdS (9) by digesting with *Spe*I and *Xba*I] was inserted into *Psh*AI sites of the remaining *tr75418* coding regions of pUtr75418Bg. From the resulting plasmid pUΔtr75418amdS, the *tr75418* disrupting cassette was released by digestion of *Nsi*I and *Fsp*I existing on the *tr75418* upstream region and downstream region, respectively. This fragment was used for transformation of strain QM9414.

To carry out transformation of N-25 as the host and to use the *pyr4* selective marker, it was necessary to prepare the uridine auxotroph of this strain. Several DNA fragments were prepared to construct the *pyr4* disruption cassette through intramolecular homologous recombination. An approximately 4.7 kbp DNA fragment containing 1.9 kbp of the *pyr4* upstream region, the *pyr4* coding region, and 0.5 kbp of the *pyr4* downstream region, as well as the *pyr4* downstream region of 0.5–1.5 kbp from stop codon was amplified by PCR and *Spe*I was introduced to the 5′ and 3′ termini. A 0.5 kbp DNA fragment (of −722 to −1221 bp from the start codon) was amplified by PCR and used as the direct repeat. The *amdS* selective marker was obtained by *Fsp*I digestion of pUamdSF (8). These four DNA fragments were cloned into *Hinc*II site of pUC118 using a Gibson Assembly Cloning Kit (New England Biolabs, Hitchin, UK) in the order of the 4.7 kbp fragment, *amdS* selection marker, direct repeat, and 1 kbp downstream region. The resulting plasmid, pUpyr4amdSRM, was digested by *Fsp*I to liberate the 8.1 kbp fragments for *pyr4* gene deletion by intramolecular homologous recombination (23). This fragment was used for transformation of strain N-25. One transformant was confirmed for introduction of this cassette by Southern analysis and was named N-25pyr4amdSRM. The primers used in this study are shown in Supplemental Table S1.

Fungal transformation and clone check of the transformants Transformation of *T. reesei* protoplasts was carried out by introducing the DNA fragment into *T. reesei* as previously described (24). Transformed protoplasts were plated on minimal medium containing acetoamide as the sole nitrogen source (for *amdS* selective marker) and without uridine (for *pyr4* selective marker) to screen for cells with uridine autotrophy. Transformants of each gene deletant candidate were streaked twice on minimal medium containing acetoamide as the sole nitrogen source and without uridine to obtain stable transformants. Homologous recombination and copy number of strains were confirmed by Southern hybridization analysis using an AlkPhos Direct kit (GE Healthcare Bio-Sciences Corp., Waukesha, WI, USA). Independent transformants were analyzed after digestion of genomic DNA. Restriction enzymes used for Southern analysis were *Sal*I and *Fsp*I for the *pac1* deletant, *Sca*I and *Spe*I for N-25pyr4amdSRM, *Nde*I and *Hind*III for the *tr22911* deletant of QM9414ΔP, and *Kpn*I and *Mfe*I for the *tr75418* disruptant of QM9414. After electrophoresis, the digested chromosomal DNA was transferred onto a Hybond N+ membrane (GE Healthcare Bio Sciences Corp.) and hybridized with an alkaline phosphatase-labeled *Pst*I fragment of the upstream region of *pac1* for each *pac1* deletant, *Nco*I fragment of *pyr4* for *tr22911* deletant and N-25pyr4amdSRM, and *Sal*I fragment of *tr75418* for *tr75418* deletant.

To remove the *pyr4* gene by intramolecular homologous recombination, conidia of N-25pyr4 and SRM were spread on PDA containing 5 mM 5-fluoroorotic acid (FOA; Funakoshi Pharmaceutical Co., Ltd., Tokyo, Japan), 20 mM uridine (Sigma−Aldrich, St. Louis, MO, USA), and 0.2% uracil (Wako Pure Chemical Industries, Osaka, Japan), and then incubated at 28°C. After cultivation, colonies were transferred onto another PDA medium containing 5 mM 5-FOA, 20 mM uridine, and 0.2% uracil. To confirm the removal of *pyr4*, genomic DNA from growing colonies was subjected to PCR analysis using IF_pyr4_up_Fw and IF_pyr4_ter_Rv. One transformant generated by intramolecular homologous recombination was named N-25ΔPA$^-$. The primers used in this study are shown in Supplemental Table S1.

Biochemical analyses To obtain the pattern of secreted protein, the culture supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% polyacrylamide gel. The gel was then stained using Coomassie Brilliant Blue R250 dye (25). The Precision Plus Dual Color Standard Marker (Bio-Rad Laboratories, Hercules, CA, USA) was used for molecular weight determination. Protein concentration was determined by the Bradford method using bovine gamma globulin as the standard (26). Carboxymethyl-cellulase (CMCase) and xylanase activities of the culture supernatant were determined by measuring the reducing sugar using the 3,5-dinitrosalicylic acid (DNS) method (27). Avicelase activity was measured using a final concentration of 1% Avicel in 50 mM sodium acetate buffer (pH 5.0) at 50°C for 30 min. CMCase activity was measured at a final concentration of 1% CMC (low viscosity; Sigma−Aldrich, St. Louis, MO, USA) in 50 mM sodium acetate buffer (pH 5.0) at 50°C for 15 min. Xylanase activity was measured using birch wood xylan (Sigma−Aldrich) at a final concentration of 1% in 50 mM sodium acetate buffer (pH 5.0) at 50°C for 10 min. One unit of activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute in glucose or xylose equivalents. Cellobiase activity was determined using cellobiose (Sigma−Aldrich) at 50 mM sodium acetate buffer (pH 5.0) at 50°C for 30 min by measuring released glucose with a Glucose C2 kit (Wako Pure Chemical Industries). One unit of cellobiase activity was defined as the amount of enzyme that produces 2 μmol of glucose per minute. Each sample was examined in triplicate.

Quantitative reverse transcription PCR Total RNA was extracted from frozen mycelia by a modified hot-phenol method as previously described (9). Complementary DNA was synthesized from total RNA (1 μg) using a Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science, Bavaria, Germany). Quantitative reverse transcription PCR (qRT-PCR) was performed using a LightCycler 480 System (Roche Applied Science, Bavaria, Germany), as previously described (9). Expression levels were calculated relative to the value of the control, housekeeping gene (*act1*, the gene encoding actin). Each sample was examined in triplicate. PCR primers used for the expression analysis are listed in Supplemental Table S2.

Biomass saccharification The substrates used for saccharification was eucalyptus (a woody plant) subjected to a hydrothermal pretreatment. Hydrothermal-pretreated eucalyptus was kindly provided by Dr. Yoshinori Kobayashi (Japan Bioindustry Association, Ibaraki, Japan). Biomass pretreatment was carried out as described previously (28) with the modification in which the hydrothermal pretreatment was performed under operating conditions of 30 min at 210°C. The pretreated biomass was measured for components by using high-performance liquid chromatography (HPLC) described in the procedure published by the National Renewable Energy Laboratory (29). The saccharification by cellulase preparations of pretreated eucalyptus was carried out in 2-ml plastic bottles containing 5% (w/w) dry biomass in 100 mM sodium acetate buffer (pH 5.0) with enzyme loading at 10 mg protein/g dry biomass at 50°C for 72 h with constant shaking (150 rpm). After saccharification, the sample was boiled for 10 min to inactivate the enzymes, and the reducing sugar produced was measured using the DNS method. Samples were analyzed in triplicate.

Genomic DNA sequencing and bioinformatics analysis Chromosomal DNA was extracted from fine powdered mycelia in liquid nitrogen by combination of phenol/chloroform extraction and ethanol precipitation. Chromosomal DNA of QM9414 and N-25 was sequenced in the Illumina MiSeq platform. The genome sequence of wild-type strain QM6a was downloaded from the Department of Energy Joint Genome Institute (JGI) website (http://genome.jgi-psf.org/Trire2/) and used as a reference for comparative genomic analysis. Quality control of MiSeq reads was performed using trimmomatic ver. 0.36 (http://www.usadellab.org/cms/?page=trimmomatic) (30) and low quality reads were removed. After quality control, the numbers of reads for QM9414 and N-25 were 6.2 million and 5.5 million, respectively. Total nucleotide numbers were 6.5 Gbp with 45 folds depth for QM9414 and 1.3 Gbp with 40 folds depth for N-25. Read mapping onto the reference sequence was carried out using bwa ver. 0.7.12 by default parameters with mem algorithm. Overall mapping rates toward reference sequence were 95% and 94% for QM9414 and N-25. SNVs were extracted by samtools ver. 1.8 and bcftools ver. 1.2 (http://samtools.sourceforge.net/) (31). Thereafter, candidates of SNV were filtered with perl script, in which filtering criteria were that there were five or more leads supporting either reference or alteration, that there were one or more leads in both directions among them, and that the ratio of reads supporting reference or alteration was 80% or more. The effect of each SNV on mutated genes was judged using SnpEff ver. 4.3q (32) with reference to filtered gene models from *T. reesei* genome database of JGI.

RESULTS

The production pattern of cellulase and xylanase differed between QM9414 and PC-3-7 depending on culture pH at protein and transcripts level To evaluate the details of cellulase production against various ambient pH conditions, *T. reesei* QM9414 and PC-3-7 strains were cultivated in basal medium containing 1% Avicel in which the initial pH was adjusted from 3.5 to 5.5 using tartrate buffer. The obtained culture filtrate was subjected to SDS-PAGE and its protein concentration was measured (Fig. 1A and B). In QM9414, the electrophoresis pattern of secreted protein showed appreciably low band intensity at higher pH compared with that from lower pH conditions (Fig. 1A) and protein production at pH 5.0 and 5.5 reflected the pattern on SDS-PAGE (Fig. 1B). Higher protein productivity was observed in PC-3-7 than QM9414 under all pH conditions tested except at pH 3.5 (Fig. 1B). Additionally, a different trend in protein productivity was observed in PC-3-7 showing an increase in protein production corresponding with the increase in pH (Fig. 1B). From SDS-PAGE, a different secretome pattern was also observed between the strains with changes in culture pH (Fig. 1A). In the case of PC-3-7, almost all protein bands showed strong intensity at all of the cultivated pH conditions. XYNIII was not expressed at all pH conditions in QM9414, while it was expressed in PC-3-7 with a faint protein band at pH 3.5 and thick bands at pH over 3.5.

Enzyme activity of the supernatant was assayed by using Avicel, CMC, cellobiose, and xylan as the substrate for the evaluation of cellobiohydrolase, endoglucanase, β-glucosidase, and xylanase, respectively. For Avicelase and CMCase, QM9414 had lower activity at higher pH whereas PC-3-7 exhibited the highest activity at pH 4.5 and its activity was maintained at pH 5.0 and 5.5 (Fig. 1C and D). While high cellobiase activity was obtained from the cultivation at pH 4.0 to 4.5 in QM9414, its high activity range in PC-3-7 was from pH 4.5 to 5.5 (Fig. 1E). The xylanase activity of QM9414 was almost the same regardless of culture pH. In contrast, higher activity was obtained with the increase in cultivation pH in PC-3-7 (Fig. 1F).

To investigate the effect of different cultivation pH on the gene expression of biomass-degrading enzymes, the transcript levels of *cbh1*, *cbh2*, *egl1*, and *bgl1* encoding major cellulase genes, *xyn1* and *xyn2* encoding major xylanase genes, and *xyr1* encoding key cellulase activator were quantified by qRT-PCR. Total mRNA used in

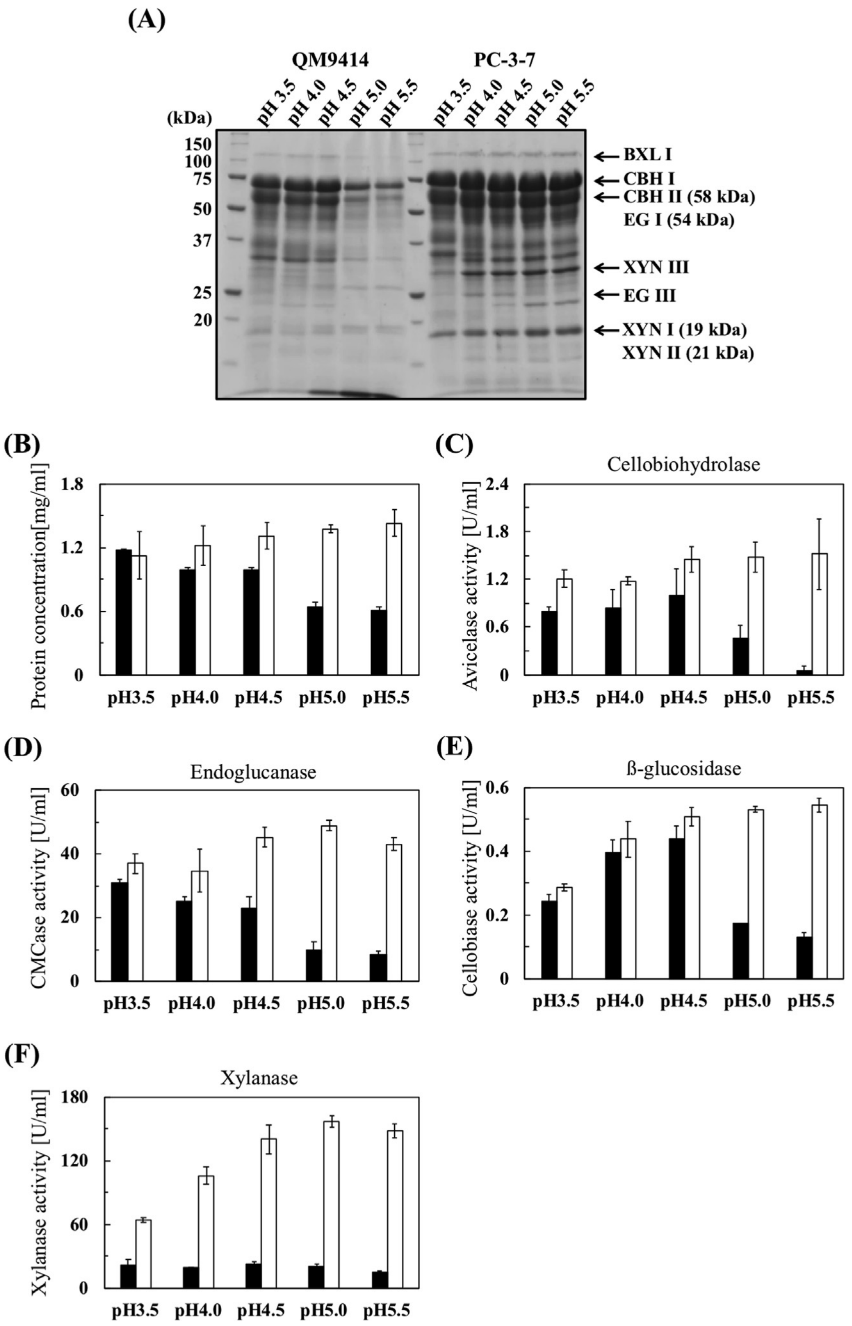

FIG. 1. Cellulase productivity of QM9414 and PC-3-7 at various pH conditions. *T. reesei* QM9414 (black bars) and PC-3-7 (white bars) strains were cultivated for 5 days in a medium containing 1% Avicel under pH 3.5, 4.0, 4.5, 5.0 and 5.5 using tartrate buffer. (A) Supernatants (16 μl) were analyzed by 12.5% SDS-PAGE. Gels were stained by Coomassie Brilliant Blue. Protein concentrations (B), and Avicelase (C), CMCase (D), cellobiase, and xylanase (E) activities were measured from supernatants. Values represent mean of triplicate experiments. Error bars indicate standard deviations.

this experiment was derived from mycelia cultivated on Avicel for 3 days at pH 3.5, 4.5, and 5.5. The transcript levels of cellulase genes in QM9414 decreased with increasing pH and were barely detected at pH 5.5. In contrast, PC-3-7 showed higher expression at pH 4.5 than at pH 3.5, and its transcript levels were generally much higher than QM9414 (Fig. 2A–C). The expression pattern of *bgl1* in QM9414 was different from other cellulase genes with the highest level observed at pH 4.5. This pattern was maintained in PC-3-7 with a dramatically increased expression at pH 4.5 and 5.5 (Fig. 2D). With respect to xylanase gene expression, the expression of *xyn1* and *xyn2* in QM9414 showed contrasting patterns in a subtle way, namely *xyn1* expression was lower at higher pH but that of *xyn2* showed the opposite pattern (Fig. 2E and F). An almost similar trend was observed in PC-3-7 as well (Fig. 2E and F). The *xyr1* expression pattern was roughly consistent with that of *cbh1*, *cbh2*, and *egl1* (Fig. 2G). These results suggest that the differences in pH-dependent cellulase production in both strains were due to regulation at the transcriptional level.

Evaluation of the pH transduction pathway for cellulase production in *T. reesei* To investigate the relationship between the pH transduction pathway and the differences in pH responses between QM9414 and PC-3-7, the expression of genes encoding factors correlating to the pH transduction pathway was analyzed on cellulase producing conditions. The putative homologs of *palA*, *palB*, and *palH* were searched from the genome

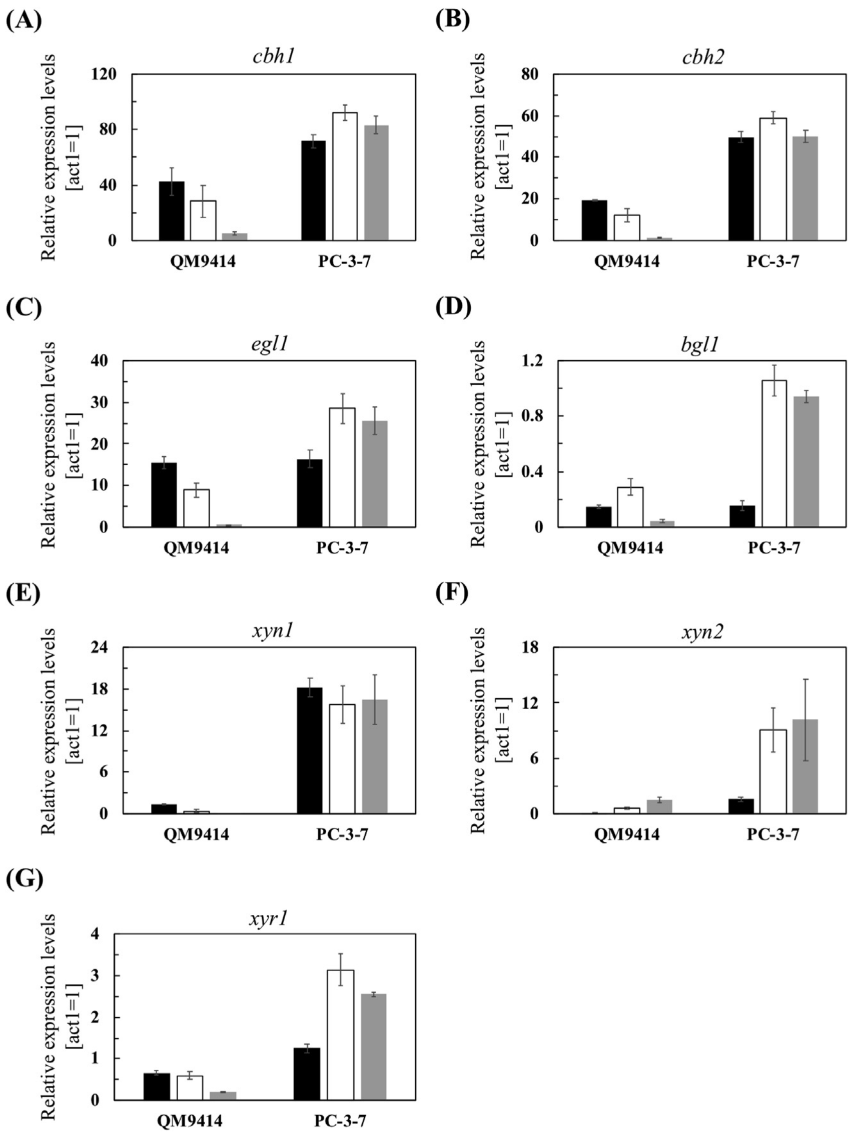

FIG. 2. Expression profiles of *T. reesei* glycosyl hydrolase genes and *xyr1* gene cultivated at various pH conditions. *T. reesei* QM9414 and PC-3-7 were cultivated in a medium containing 1% Avicel under pH 3.5 (black bars), 4.5 (white bars), and 5.5 (gray bars) using tartrate buffer for 3 days. Transcriptional analysis of cellulase genes (A) *cbh1*, (B) *cbh2*, (C) *egl1*, (D) *bgl1*, (E) *xyn1*, and (F) *xyn2*, and cellulase activator gene (G) *xyr1* was performed by qRT-PCR. All values were normalized to β-actin (*act1*) expression. Data represents average of three independent experiments. Error bars indicate standard deviations.

data and they were found to code protein ID tr124132, tr70560, and tr21415, respectively. The expression level of these genes and *palC*, *palF* (19), and *pac1* was determined by qRT-PCR. Both the expression amounts and the pattern of *palC*, *palH*, *palI*, and *pac1* expression (depending on culture pH) were similar between QM9414 and PC-3-7 (Fig. S2). However, higher expression level of *palA* and *palB* was observed at pH 5.5 in PC-3-7 compared to QM9414. The expression level of *palF* increased with increasing pH in PC-3-7 while it was maintained at a constant level from pH 3.5–5.5 in QM9414.

To further confirm whether the regulation of cellulase and hemicellulase genes by PACI was conserved in PC-3-7, the *pac1* deletant of PC-3-7, PCΔpac1, was constructed (Fig. S3) and its enzyme production was investigated. Secreted protein amounts were lower in PCΔpac1 than those in PC-3-7 at all culture pH conditions tested (Fig. 3A). Avicelase activity was similar between PC-3-7 and PCΔpac1 except at pH 4.0 where PCΔpac1 showed higher activity in comparison with all the other tested pH conditions (Fig. 3B). According to the enzyme assay, CMCase activity was hardly affected by *pac1* deletion (Fig. 3C). The activities derived from PCΔpac1 were higher than those from PC-3-7 for cellobiase at pH 3.5 and 4.0 and for xylanase at all pH conditions (Fig. 3D and E). However, there were no significant differences in the pattern of secretome between PC-3-7 and PCΔpac1 (Fig. S4).

To investigate the effect of *pac1* disruption on gene expression in PC-3-7, the expression levels of *cbh1*, *cbh2*, *egl1*, and *bgl1* as cellulase genes, *xyn1*, *xyn2*, and *xyn3* as hemicellulase genes and *xyr1*, *ace3*, *ace2*, *cre1*, and *ace1* as encoding transcription factors were measured by qRT-PCR. For *cbh1*, *cbh2*, and *egl1*, *pac1* disruption resulted in reduced expression at pH 4.5 and 5.5 (Fig. 4A). In addition, decreased expression of xylanase genes was observed not only at pH 4.5 and 5.5 but also at pH 3.5 in PCΔpac1 (Fig. 4B). Especially, the expression of *xyn2* and *xyn3* was lower in PCΔpac1 than in PC-3-7 at all the tested pH conditions (Fig. 4B). However, the expression pattern of cellulase and xylanase gene of PCΔpac1 was not consistent with that of enzyme activity (Fig. 3B–E). In case of transcription factors, the expression of *xyr1*, *ace3*, *ace2*, and *ace1* was similar at all the tested pH conditions in PCΔpac1 (Fig. 4C). In addition, lower expression levels of transcription factors at pH 4.5 and 5.5 were exhibited in PCΔpac1 than in PC-3-7 (Fig. 4C).

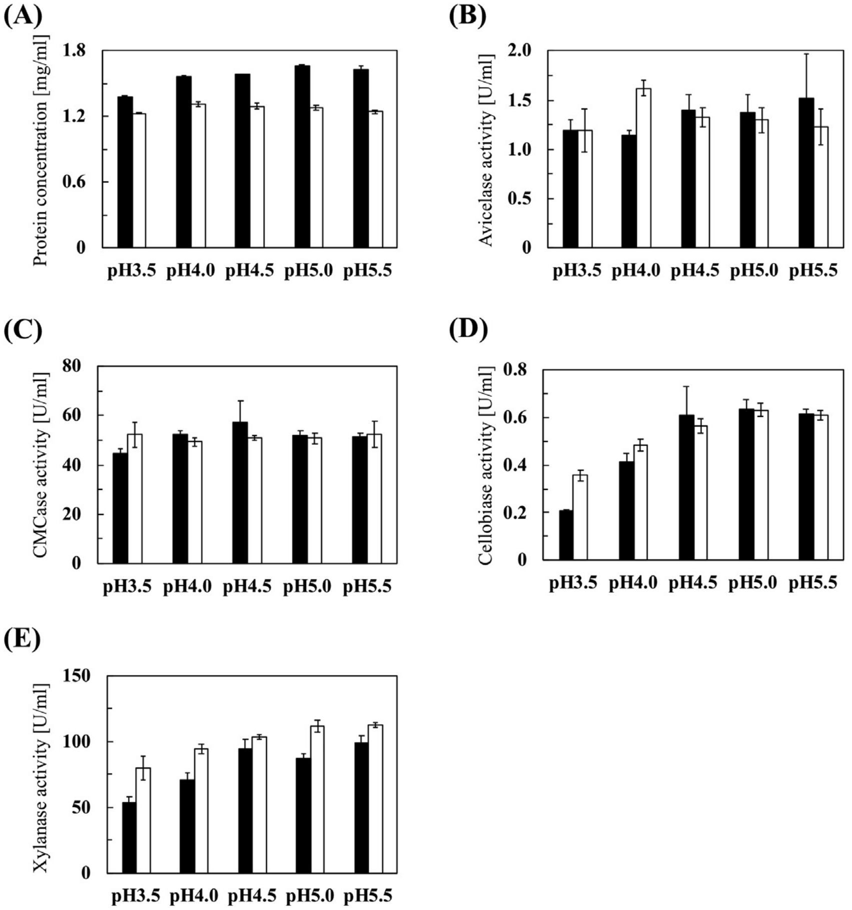

FIG. 3. Cellulase productivity of PC-3-7 and PCΔpac1 at various pH conditions. *T. reesei* PC-3-7 (black bars) and PCΔpac1 (white bars) strains were cultivated in a medium containing 1% Avicel under pH 3.5, 4.0, 4.5, 5.0, and 5.5 using tartrate buffer for 5 days. (A) Protein concentrations (B), and Avicelase (C), CMCase (D), cellobiase, and xylanase (E) activities were measured from supernatants. Values represent mean of triplicate experiments. Error bars indicate standard deviations.

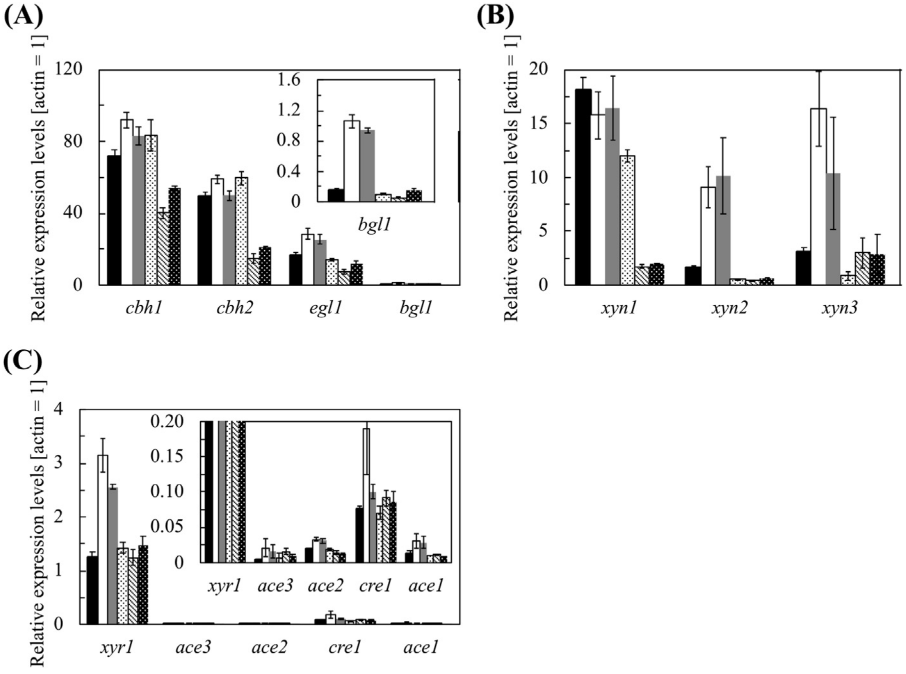

FIG. 4. Expression profile of *T. reesei* glycosyl hydrolase genes and transcription factor genes cultivated at various pH conditions. *T. reesei* PC-3-7 (black bars, white bars, and gray bars) and PCΔpac1 (dotted black bars, hatched bars, and dotted white bars) strains were cultivated in a medium containing 1% Avicel under pH 3.5 (black bars and dotted black bars), 4.5 (white bars and hatched bars), and 5.5 (gray bars and dotted white bars) using tartrate buffer for 3 days. Transcriptional analysis of cellulase genes *cbh1*, *cbh2*, *egl1*, and *bgl1* (A), xylanase genes *xyn1*, *xyn2*, and *xyn3* (B), and transcription factor genes *xyr1*, *ace3*, *ace2*, *cre1*, and *ace1* (C) was performed by qRT-PCR. All values were normalized to β-actin (*act1*) expression. Data represents average of three independent experiments. Error bars indicate standard deviations.

Cellulase and xylanase gene expression patterns of PC-3-7 were similar to that of *xyr1* expression while *pac1* disruption showed different expression patterns between cellulase and xylanase genes and *xyr1* at only pH 3.5 (Fig. 4). On the other hand, the *pac1* deletion in QM9414 showed similar expression patterns between cellulase and xylanase genes and *xyr1* (Fig. S5). Since PC-3-7 and QM9414 showed different effects as a response to *pac1* deletion at only pH 3.5 (Fig. 4), it appears that a pH-responsive mechanism that is different from that of the PACI-related mechanism might cause the PC-3-7 phenotype.

Enzyme production at high pH had been acquired at an early stage of mutant development In the process of constructing hyper-cellulolytic mutants by random mutagenesis there were five generations between QM9414 and PC-3-7. Since many genes have been mutated in PC-3-7 genome (8), it is very difficult to clarify which mutation affects its pH response. Thus, it was important to identify strains with a change in pH response during the process leading to PC-3-7 development. To identify which mutant acquired high pH-dependent cellulase productivity, we examined cellulolytic enzymes production of a series of *T. reesei* mutant strains — wild type QM6a, standard strain QM9414, and Japanese mutants N-25, K-14, KDG-12, and PC-3-7 (Figs. 5 and S1) (33) — at different pH conditions. SDS-PAGE results showed enhanced cellulase production at high pH in N-25 and its descendants (Fig. 5A). For Avicelase and CMCase, the pattern of enzyme activity in mutants was similar to SDS-PAGE and protein concentration (Fig. 5A–D). However, high cellobiase activity was observed at pH 4.5 in QM9414, whereas the highest activity was detected at pH 5.5 in N-25 and its descendants (Fig. 5E). Interestingly, N-25 strain had low cellulase production at pH 3.5. This phenotype was restored at K-14, which was the next strain of N-25 (Fig. 5).

Comparative genome analysis between QM9414 and N-25 The pattern of protein production in N-25 suggested that the mutations generated in it were related to pH responsive cellulase production at both lower and higher pH conditions. Thus, we performed genome sequencing of QM9414 and N-25 using the Illumina sequencer to identify the mutations generated in N-25. A total of 6.2 million Illumina MiSeq reads with an average coverage depth of 45 folds for QM9414 and 5.5 million reads with 40-fold coverage depth for N-25 were mapped to the wild-type (QM6a) reference genome. From detected single nucleotide variations (SNVs), filtering was carried out and SNVs occurring on exon and affecting its translation product were extracted (Table 1).

A comparative analysis of the QM9414 and N-25 revealed 29 SNVs (10 SNVs in the promoter (1 kbp upstream of the start codon), 5 SNVs in terminator (500 bp downstream of the stop codon), and 14 SNVs in exons (Table 1). Among the 10 SNVs found in coding regions, two genes, *tr75418* and *tr22911*, encoded transcription factors. *tr75418* encoded ACEI, the repressor of cellulase expression

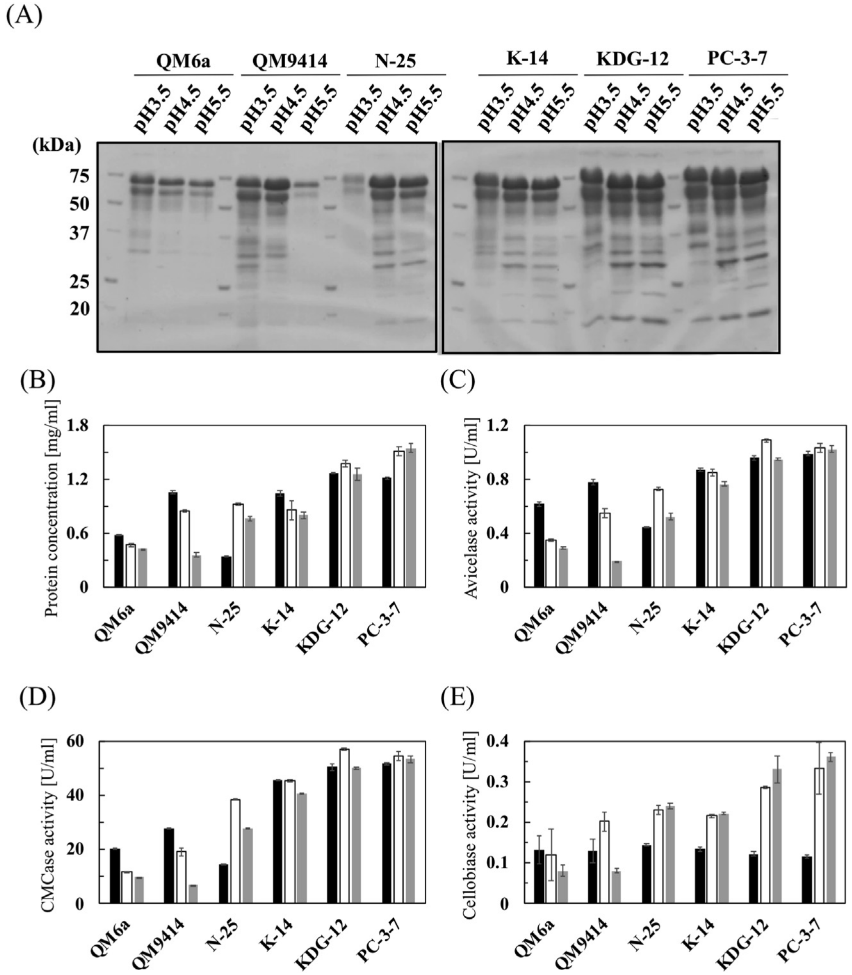

FIG. 5. Cellulase productivity of *T. reesei* Japanese mutants at various pH conditions. *T. reesei* mutants - QM6a, QM9414, N-25, K-14, KDG-12, and PC-3-7 - were cultivated in a medium containing 1% Avicel under pH 3.5 (black bars), 4.5 (white bars) and 5.5 (gray bars) using tartrate buffer for 5 days. (A) Supernatants (16 µl) were analyzed by 12.5% SDS-PAGE. Gels were stained by Coomassie Brilliant Blue. Protein concentrations (B), and avicelase (C), CMCase (D), and cellobiase activity (E) were measured from supernatants. Values represent mean of triplicate experiments. Error bars indicate standard deviations.

TABLE 1. List of SNVs identified between strains QM9414 and N-25.

Protein ID	Mutation	Element[a]	Amino acid change	Annotation/function
4860	C > T	Promotor		Siroheme synthase
5991	T > C	Exon	F158S	Importin 13
21120	T > G	Promotor		Alpha-methylacyl-CoA racemase
21937	C > T	Promotor		Mitochondrial protein import protein Mas5
22009	T > A	Exon	F341L	Tetracycline transporter
22911	C > G	Exon	P154A	Glucose-repressible alcohol dehydrogenase transcriptional effector Ccr4
48058	C > T	Promotor		Unknown
50212	T > C	Terminator		Unknown
52165	T > A	Terminator		Fk506 suppressor Sfk1
59100	A > T C > T	Promotor		Glycoprotease family protein
60498	A > T	Promotor		Female-specific protein transformer
61550	C > A	Exon	T275N	ATP-dependent RNA helicase DRS1
62938	C > G	Exon	L323V	Testis protein TEX11
63744	T > C	Terminator		Nuclear protein
65292	G > T	Exon	A264A	Short-chain-fatty-acid-CoA ligase
69384	A > T	Terminator		Uncharacterized protein Ymr031c homolog
70933	A > C	Promotor		Major facilitator superfamily protein
70943	C > T	Exon	G1162G	Putative histidine kinase HHK5p
72183	T > G	Promotor		Mannan-binding lectin
75418	G > T	Exon	G330V	Zinc finger transcription factor Ace1
77138	A > T	Terminator		Unknown
78582	A > C	Exon	Q323P	Ferrochelatase
82599	C > A	Promotor		Transcription factor Btf3
102411	T > G	Promotor		C6 transcription factor
108519	C > A	Promotor		Ubiquitin-conjugating enzyme
110122	C > T	Exon	N555N	Unknown
111593	T > A	Exon	H95Q	Unknown
120879	A > T	Exon	N285I	LuxR family transcriptional regulator
121495	A > T	Exon	V378V	Extracellular serine protease Tvsp1
121960	A > T	Exon	N194I	Unknown
122431	G > T	Promotor		Pex19 family protein

[a] Elements were classified into three categories; exon, promoter (1 kbp upstream of the start codon), and terminator (500 bp downstream of the stop codon).

on cellulase-inducing condition, and TR22911 had high homology to CCR4 involved in carbon catabolite repression in *Neurospora crassa* (34,35). Because we speculated that these transcription factors would correlate with cellulase production depending on the pH, the effect of deletion of these genes in QM9414 was applied to serve as a functional analysis of the mutated gene. The integration of the ORF deletion DNA fragment into the locus of the genome in which the genes encoding TR75418 and TR22911 were replaced with the *amdS* selective marker and *pyr4* selective marker, respectively, was confirmed by Southern analysis (data not shown). However, significant effect of gene disruption was not observed from the analysis of the secretome and enzyme activity of culture supernatants (Fig. S6).

DISCUSSION

In this study, a comparative analysis of enzyme production in *T. reesei* mutants maintained under different pH conditions was conducted to identify the pH-dependent enzyme production mechanism in this filamentous fungus. Researchers have long studied the inducer responsibility, gene regulation, genome analysis, and morphology of cellulase hyperproducing PC-3-7 [reviewed by Shida et al. (36)]. PC-3-7 produces large amount of cellulase using even a soluble cellulosic carbon source such as cellobiose (7). In addition, unlike QM9414, PC-3-7 can produce GH10 xylanase XYNIII (37). Because the importance of XYNIII in saccharification has been documented (11), PC-3-7 and its derivative strain are highly valuable for industrial application. The phenotypic differences in pH response between QM9414 and PC-3-7 have given us novel insights into the gene regulation mechanisms of cellulase and hemicellulases. A comparison between PC-3-7 and its parent strain QM9414 in terms of enzyme production and gene expression profiles of glycoside hydrolase genes indicated that PC-3-7 could produce significant quantities of enzymes at high pH (Fig. 1).

In fungi, Pal proteins transduce pH signals in neutral-to-alkaline conditions and activate the transcription factor PacC by pH-dependent and pH-independent proteolysis [reviewed by Peñalva et al. (38)]. In *T. reesei*, the PacC homolog, PACI, is a negative regulator of cellulase gene expression at high pH and enzyme production of its disruptant mimics acidic conditions (18). In addition, it is suggested that this repression is via reduction in expression of the key cellulase activator XYRI (18). Therefore, the high cellulase productivity at high pH phenotype of PC-3-7 led us to suspect that this could be due to a mutation in PAL proteins or PACI resulting in alteration or loss of their function in this strain. Such alterations in function have been reported previously. A truncation of the transcriptional repressor protein CREI in the cellulase hyperproducing mutant Rut C-30 was recently reported to give it an activating function (39). However, our hypothesis was disproved by a previous comparative genomic analysis (8) showing that genes encoding PAL homologs or PACI were not among the mutated genes in PC-3-7. In filamentous fungi, the expression of *pac1*/*pacC* is activated by PACI/PacC itself (38). The activation of PACI is caused by PAL proteins. We speculated that *pac1* expression will be altered if the expression of *pal* genes is different between QM9414 and PC-3-7. But, because the *pac1* expression level and pattern were similar between QM9414 and PC-3-7 (Fig. S2), we surmise that the difference in expression of *palA*, *palB* and *palF* between QM9414 and PC-3-7 was not a physiologically effective alteration. Additionally, because *pal* gene expression levels are quite low, it might be difficult to distinguish minor differences.

In our study, the *pac1* disruption in PC-3-7 led to reduced expression of cellulase and xylanase genes at high pH (Fig. 4). Thus, PACI certainly appeared to function in PC-3-7. However, the expression pattern of cellulase and xylanase genes and *xyr1* was not similar in PCΔpac1 (Fig. 4) while QMΔpac1 showed similar expression pattern between these genes (Fig. S5). Furthermore, the *pac1* disruption in PC-3-7 did not significantly affect cellulase and xylanase activity and did not mimic acidic conditions, as described in a previous report (18). These facts suggest that there is another mechanism(s) responsible for ambient pH that masks the effect of the disruption of *pac1* on *T. reesei*. Hakkinën et al. (19) mentioned in their 2015 report another mechanism wherein a variety of glycoside hydrolase genes respond to changes in pH but only a few were under PACI regulation. However, their result contradicts the findings from our research and those of He et al. (18). The contradiction between PACI investigations might be due to various factors, such as differences in cultivation conditions, experimental settings, or detection principles of gene expression. In our investigation, PACI seems to act as a transcription activator in PC-3-7. However, our result was consistent with that of He et al. (18) investigating PACI disruption in QM9414 where cellulase expression of the disruptant mimicked acidic conditions (Fig. S5). Interestingly, our results also showed that enzyme production was not affected by *pac1* disruption (Fig. 3) although gene expression was reduced at pH 4.5 and 5.5 (Fig. 4). Enzymes produced by *T. reesei* are accumulated in the supernatants during cultivation, while the expression of cellulase gene shows an instantaneous response of the mycelia. Thus, there may be differences between enzyme production and gene expression in *T. reesei*. In addition, the enzyme preparation produced by *T. reesei* is a complex mixture containing many kinds of cellulase and hemicellulases, and they synergistically degrade the substrate. Therefore, the decrease in transcription might be masked in *T. reesei*. However, as it is currently difficult to explain this phenomenon, further molecular biological analysis of PC-3-7 is necessary.

In the case of xylanase production, regulation of expression seemed to differ depending on the cultivation pH, as has been described previously (19,40). In PC-3-7, although the expression level was significantly enhanced, the expression profile of the xylanase genes was similar to that of QM9414 (Fig. 2), which implies that the regulatory mechanism between QM9414 and PC-3-7 was not different. The *xyn1* gene was highly expressed at pH 3.5, whereas a large amount of expression was detected for *xyn2* and *xyn3* at pH 4.5 and 5.5 (Fig. 2). Some investigations have reported that *T. reesei* regulates cellulase production according to changes in environmental pH so that each enzyme can function at its optimal pH (13,14,19,41).

On the other hand, β-glucosidase gene expression responded differently to pH unlike the other cellulase and xylanase genes (Fig. 2). The expression pattern of cellulase gene was consistent with the expression of *xyr1* gene in both QM9414 and PC-3-7. However, *bgl1* expression was the highest at pH 4.5 in both strains, indicating that β-glucosidase gene expression is conserved in another mechanism, unlike cellulase gene expression. In fact, BGLR reportedly controls the expression of *bgls*, but not cellulase gene expression (7). In addition, the *pac1* deletion resulted in reduction of *bgl1* expression at pH 4.5 and 5.5, but not in cellulase gene expression in PC-3-7 (Fig. 4). Therefore, *bgl1* expression might be strongly controlled by PACI.

Our enzyme activity analysis of secreted enzymes revealed different enzyme ratios between lower and higher pH conditions (Fig. 1). Similar pH-dependent change in enzyme ratio was reported for Rut C-30 strain in which efficient production for xylanases was at high pH and for cellulases it was at low pH (13). Compared with Rut C-30, the optimal pH for each enzyme secretion was higher in PC-3-7. Almost in all cases, enzyme productions were enhanced at higher pH 5.0, but xylanase activity was quite high at pH more than 4.5 (Fig. 1). Thus, the enzyme component was increased with the ratio of xylanase in high pH cultivation in PC-3-7. This fact might indicate that the enzyme produced on high pH condition was suitable for hemicellulose containing pretreated biomass, and the enzyme produced at low pH condition was suitable for only cellulose containing pretreated biomass (11,42). In fact, the industrial strains X3AB1, E1AB1, and X3-2RB_AB1 were cultured at different pH conditions and the results concur with our expectations (data not shown). The saccharification ability against hydrothermal pretreated eucalyptus was improved by the enzyme preparation produced by the industrial strains at pH 3.5 compared to that at pH 5.5 (Fig. S7). Therefore, the application of pH-dependent cellulase productivity of PC-3-7 might improve the saccharification ability of JN series because the JN series strain was constructed from PC-3-7.

In the mutant development leading from QM9414 to PC-3-7, N-25 acquired cellulase hyperproducing characteristics at high pH. Indeed, the PACI deletion at N-25 did not affect cellulase production at high pH as was the case for PC-3-7 (Fig. S8). This meant that the mutation(s) generated in N-25 caused higher pH-dependent cellulase production (Fig. 5). Because our result revealed that the pH-dependent cellulase production acquired from N-25 was caused at the transcriptional level, we speculated that the gene encoding the transcriptional regulatory protein mutated in the N-25 strain. Comparative genome analysis between QM9414 and N-25 revealed that 10 genes underwent the mutation leading to amino acid substitution of their translation product in the N-25 genome (Table 1). Additionally, the transcriptional pattern of the 10 mutated genes was similar between QM9414 and N-25 (Fig. S9). After studying the genome database and BLAST analysis results, two transcription factor encoding genes, *tr75418* and *tr22911*, were selected for deletion analysis. *tr75418* encodes cellulase repressor ACEI, which acts as the repressor for cellulase under cellulase-inducing conditions in *T reesei* (34) and possesses a high global similarity to *A. nidulans* StzA, a related transcription factor in stress response (43) and to Ccr4 protein involved in carbon catabolite repression in *Saccharomyces cerevisiae* (44). Because the two mutated genes encode the transcriptional repressor related to carbon catabolism, we speculated that they are involved in different pH responses in N-25. However, each disruptant of these mutated genes in QM9414 showed similar pattern of cellulase production compared to the parent strain (Fig. S9). Therefore, it is possible that mutations of other candidate genes are responsible for the enhanced cellulase production at high pH in N-25. The mutations in genes encoding TR22009, TR61550, TR78582, and TR120879 occurred as amino acid substitutions on their functional domains (data not shown). These genes were related to homeostatic mechanisms such as membrane transport or metal ion concentration. In particular, the expression of *tr78582* (annotated to ferrochelatase gene) was high with cultivation at higher pH (Fig. S9). Ferrochelatase is the terminal enzyme of the heme biosynthetic pathway in all cells. Therefore, iron might be involved in cellulase production in *T. reesei*. Currently, we are analyzing the relationship between the mutated genes and cellulase productivity. It is also possible that the mutation occurring in N-25 might be responsible for the weakened cellulase production at low pH (Fig. 5). This phenotype was restored in the next mutant, K-14, suggesting that the suppression by the mutation occurred in K-14.

In summary, we identified characteristic responses of *T. reesei* Japanese mutants to varying pH through a comparative study. We found that the enzyme component of cellulase and xylanase differed depending on the culture pH condition in the Japanese mutant PC-3-7. In addition, we also suggested a novel pH response mechanism for cellulase production. We surmise that lower pH-dependent and higher pH-dependent cellulase production might be caused by different mechanisms in *T. reesei*. Further analysis of these mutations could unravel a new cellulase production mechanism against ambient pH.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.03.005.

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