Transcription Factor Atf1 Regulates Expression of Cellulase and Xylanase Genes during Solid-State Fermentation of Ascomycetes

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ABSTRACT Transcriptional regulation of cellulolytic and xylolytic genes in ascomycete fungi is controlled by specific carbon sources in different external environments. Here, comparative transcriptomic analyses of *Penicillium oxalicum* grown on wheat bran (WB), WB plus rice straw (WR), or WB plus Avicel (WA) as the sole carbon source under solid-state fermentation (SSF) revealed that most of the differentially expressed genes (DEGs) were involved in metabolism, specifically, carbohydrate metabolism. Of the DEGs, the basic core carbohydrate-active enzyme-encoding genes which responded to the plant biomass resources were identified in *P. oxalicum*, and their transcriptional levels changed to various extents depending on the different carbon sources. Moreover, this study found that three deletion mutants of genes encoding putative transcription factors showed significant alterations in filter paper cellulase production compared with that of a parental *P. oxalicum* strain with a deletion of *Ku70* (Δ PoxKu70 strain) when grown on WR under SSF. Importantly, the Δ PoxAtf1 mutant (with a deletion of *P. oxalicum* Atf1, also called POX03016) displayed 46.1 to 183.2% more cellulase and xylanase production than a Δ PoxKu70 mutant after 2 days of growth on WR. RNA sequencing and quantitative reverse transcription-PCR revealed that PoxAtf1 dynamically regulated the expression of major cellulase and xylanase genes under SSF. PoxAtf1 bound to the promoter regions of the key cellulase and xylanase genes *in vitro*. This study provides novel insights into the regulatory mechanism of fungal cellulase and xylanase gene expression under SSF.

IMPORTANCE The transition to a more environmentally friendly economy encourages studies involving the high-value-added utilization of lignocellulosic biomass. Solid-state fermentation (SSF), that simulates the natural habitat of soil microorganisms, is used for a variety of applications such as biomass biorefinery. Prior to the current study, our understanding of genome-wide gene expression and of the regulation of gene expression of lignocellulose-degrading enzymes in ascomycete fungi during SSF was limited. Here, we employed RNA sequencing and genetic analyses to investigate transcriptomes of *Penicillium oxalicum* strain EU2101 cultured on medium containing different carbon sources and to identify and characterize transcription factors for regulating the expression of cellulase and xylanase genes during SSF. The results generated will provide novel insights into genetic engineering of filamentous fungi to further increase enzyme production.

KEYWORDS gene expressional regulation, cellulase and xylanase, solid-state fermentation, *Penicillium oxalicum*

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As decomposers, soil microbes have a great influence on soil carbon cycling, essentially transferring carbon between environmental compartments for survival. The amount of global organic carbon stored in the soil is estimated to be equivalent to at least four times that in the atmosphere (1). Soil carbon comes mainly from above-ground plant litter and its leachates and is comprised of several polymers such as cellulose, hemicellulose, and lignin. In plant secondary cell walls, these polymers form a sandwich-like schematic architecture that is recalcitrant to enzymes of carbohydrate catabolism (2) and adapts to environmental stresses (3). In the process of natural decay in the soil, microbes produce enzymes such as carbohydrate-active enzymes (CAZymes) and proteinases, which contribute to the degradation of polysaccharides and the moving of lignin from plant to soil; these processes are effective but slow (3, 4). In the biotechnological industry, many soil fungi, such as *Trichoderma*, *Aspergillus*, and *Penicillium*, can produce CAZymes through solid-state fermentation (SSF) (5, 6).

SSF, simulating the natural habitat of soil microorganisms, is used for a variety of applications, from ancient biotechnologies, such as the production of Chinese liquor, rice wine, soy sauce, and vinegar, to the generation of biochemicals, such as enzymes, antibiotics, organic acids, and natural pigments, and to new uses involving alternative energy and environmental purposes, such as biomass biorefineries, production of biofuels (bioethanol, biodiesel, biobutanol, and biohydrogen) and biosurfactants, and the provision of bioremediation (5). SSF possesses particularly many advantages over its liquid counterpart (submerged fermentation [SmF]), such as lower levels of catabolite repression and the possibility of directly using agro-industrial residues as the substrate (5, 6).

Gene expression in a microorganism is strictly controlled by a complex regulatory network comprised of many transcription factors (TFs) and their target genes, the functions of which depend on the response to environmental factors. Gene expression levels under different culture conditions (e.g., SSF and SmF) differ to various degrees; for example, the glucoamylase gene and protease genes are highly expressed in *Aspergillus oryzae* during surface cultivation on wheat-based solid medium while they are poorly expressed in wheat-based liquid medium (7). Moreover, transcription of the laccase genes in the white rot basidiomycete *Pleurotus ostreatus* was upregulated in SmF but downregulated in SSF cultures (8). Recently, we compared transcriptional profiles of *Penicillium oxalicum* strain HP7-1 cultivated in liquid and solid media containing the same carbon source, and the results indicated that SSF elevated the expression of major cellulase genes and repressed the expression of genes involved in the citric acid cycle compared with levels in SmF cultures. Subsequently, a key regulator, *P. oxalicum* MBF1 (PoxMBF1), was identified, which negatively affected the production of cellulase and xylanase of *P. oxalicum* during SSF as well as SmF (9). However, this information is insufficient to elucidate the regulatory mechanism of cellulase and xylanase gene expression under SSF.

In the current study, *P. oxalicum* was used as a model to investigate its global transcriptomic profiling under SSF in response to the agricultural waste materials wheat bran (WB), WB plus rice straw (WR), or WB plus Avicel (WA). Subsequently, candidate regulatory genes of cellulase and/or xylanase gene expression were identified by screening. Knockout of candidate regulatory genes resulted in mutants that were tested for cellulase and xylanase production under SSF to identify regulatory genes required for the expression of cellulase and/or xylanase genes in *P. oxalicum* under SSF.

RESULTS

Global gene expression profiling during SSF of *P. oxalicum*. To systematically and comprehensively investigate how gene expression in *P. oxalicum* strain EU2101 is influenced by different carbon sources under SSF, the total RNAs of strain EU2101 grown on WA, WB, or WR for 48 h under SSF were extracted and sequenced. The results showed that approximately 25 million clean reads with a length of 100 bp were generated from each sample, and an average of 92.6% of the clean reads were mapped onto the genome of HP7-1 (10), using the Burrows-Wheeler Aligner (BWA) (11) and

FIG 1 Comparative analysis of the transcriptomes of *P. oxalicum* EU2101 cultured on solid medium containing wheat bran (WB), WB plus rice straw (WR), or WB plus Avicel (WA) as the sole carbon source under SSF. (a) Venn diagram showing the numbers of unique and shared differentially expressed genes (DEGs) from different transcriptomic comparisons. Numbers of total, upregulated, or downregulated genes are shown in black, red, or purple, respectively. Blue numbers represent the sum of the DEGs. In the overlapping areas, numbers revealed the results of different comparative transcriptomes. In the center of overlapping areas that were shared by the three groups, the left-hand, middle, and right-hand columns represent EU2101_WA versus EU2101_WB, EU2101_WB versus EU2101_WR, and EU2101_WA versus EU2101_WR, respectively. In each overlapping area shared by two compared transcriptomes, the left- and right-hand columns represent the comparison on the left or above the overlapping area and the comparison below the overlapping area, respectively. (b) DEGs annotated by KEGG. The threshold screening criteria for DEGs were $|\log_2$ fold change| of ≥ 1 and probability of ≥ 0.8 . (c) Heat map showing DEGs encoding carbohydrate-active enzymes. These enzyme genes appeared at least once in all comparisons of the transcriptomes expressed on the three carbon sources. (d) DEGs involved in the citric acid cycle (TCA cycle) of carbohydrate metabolism.

Bowtie (12), and shown to contain, on average, 8,719 predicted protein-encoding genes. Pearson's correlation coefficients calculated for three biological replicates of *P. oxalicum* EU2101 under each set of culture conditions were significant ($r \geq 0.77$) (see Fig. S1 in the supplemental material), indicating that the transcriptomic data were reliable. The expression levels of genes in the transcriptomes were calculated as the number of fragments per kilobase of exon per million mapped reads (FPKM), and their differences were analyzed with NOISeq in the R package (13).

Using a $|\log_2$ fold change| of ≥ 1.0 and a probability of ≥ 0.8 as the thresholds, 251, 765, and 472 genes were detected as differentially expressed genes (DEGs) between EU2101_WA and EU2101_WB, EU2101_WB and EU2101_WR, and EU2101_WA and EU2101_WR, respectively (Fig. 1a). Among these DEGs, 74, 180, and 312 genes were shared by two groups of cultures, with only 28 genes being shared by all three groups of cultures (Fig. 1a). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database analyses showed that most of the DEGs were involved in metabolism, mainly involving carbohydrate metabolism, energy metabolism, and amino acid metabolism (Fig. 1b).

A total of 950 DEGs were identified that appeared at least once in all comparisons of the transcriptomes expressed on the three carbon sources, and these were called the

basic core genes in the response of *P. oxalicum* to plant biomass (Data Set S1). Among them, a total of 119 distinct transcripts, corresponding to one-quarter of the total genes encoding CAZymes in the genome of the wild-type strain HP7-1 (10), were identified, which were called the basic core CAZyme-encoding genes (BCCGs) in response to plant biomass in *P. oxalicum* (Data Set S1). The 119 CAZymes encoded belonged to 46 families of glycoside hydrolases, 6 families of glycosyl transferases, 5 families of carbohydrate esterases, 7 families of auxiliary activities, 3 families of polysaccharide lyases, and 11 families of carbohydrate-binding modules. There were 70, 43, and 96 CAZyme DEGs found between genes of EU2101_WR and EU2101_WA, EU2101_WR and EU2101_WB, and EU2101_WA and EU2101_WB, respectively, of which 11 were common to all comparisons. Notably, approximately half of these genes were predicted to encode plant cell wall-degrading enzymes (CWDEs) (Data Set S1).

A heat map hierarchical clustering assay was employed to cluster these 119 CAZyme genes with similar expression patterns, and the results showed that eight major groups (A to H) were constructed, with 8, 13, 10, 3, 8, 46, 11, and 20 members, respectively (Fig. 1c). Most of the CWDE genes were clustered in three groups, E, F, and H.

In group E, seven of eight members encoding CWDEs consisted of a β -xylosidase gene, *POX07665*, an α -L-arabinofuranosidase gene, *POX07891*, a Δ -4,5-unsaturated β -glucuronidyl hydrolase gene, *POX06998*, an endo- β -1,4-xylanase (XYN) gene, *POX03430*, an exo- α -L-1,5-arabinanase gene, *POX00008*, a pectate lyase gene, *POX07890*, and an unsaturated rhamnogalacturonidyl hydrolase gene, *POX08875*. Group E genes displayed high (H) expression in WR culture, moderate (M) expression in WA culture, and low (L) expression in WB culture.

In group F, 27 of the 46 members encoded CWDEs, comprised of one β -glucosidase (BGL) gene (*POX07963*), two endo- β -1,4-glucanase (EG) genes (*POX06147* [*Cel5A*] and *POX07535* [*Cel12A*]), five XYN genes (*POX00063* [*Xyn10A*], *POX05916*, *POX06783* [*Xyn11A*], *POX08484* [*Xyn11B*], and *POX08990*), eight β -xylosidase/ α -L-arabinofuranosidase genes (*POX00006*, *POX00923*, *POX01914*, *POX01921*, *POX05540*, *POX06599*, *POX06600*, and *POX09154*), two acetyl xylan esterase genes (*POX01218* and *POX03711*), one α -galactosidase gene (*POX09085*), one lytic polysaccharide monooxygenase (LPMO) gene (*POX08897*), one endo- β -1,4-galactanase gene (*POX06689*), one feruloyl esterase gene (*POX09137*), one pectin lyase gene (*POX04920*), one pectin acetylesterase gene (*POX07373*), one rhamnogalacturonan acetylesterase gene (*POX08476*), one endo-polygalacturonase gene (*POX04815*), and one rhamnogalacturonan lyase gene (*POX08785*). In terms of expression, group F gene levels were H in WR culture, L in WA culture, and M in WB culture (Fig. 1c).

In group H, 15 of the 20 members encoded CWDEs, including two cellobiohydrolase (CBH) genes (*POX05587* [*Cel7A-2*] and *POX04786* [*Cel6A*]), five EG genes (*POX01166* [*Cel5B*], *POX01896* [*Cel5C*], *POX05570* [*Cel45A*], *POX05571* [*Cel7B*], and *POX06983*), two BGL genes (*POX03641* and *POX06079*), one swollenin-encoding gene (*POX08485*), one LPMO gene (*POX02308* [*Cel61A*]), one EG gene (*POX06601*), one polygalacturonase gene (*POX05580*), one β -xylosidase/ α -L-arabinofuranosidase gene (*POX01646*), and one β -1,4-mannanase gene (*POX01937*). Group F gene expression levels were M in WR culture, M in WA culture, and L in WB culture (Fig. 1c).

Notably, a total of 12 DEGs were found to be associated with the citric acid cycle (tricarboxylic acid [TCA] cycle) of carbohydrate metabolism, including *POX00147*, *POX00538*, *POX00835*, *POX01075*, *POX01760*, *POX04356*, *POX04450*, *POX04925*, *POX05565*, *POX05567*, *POX08044*, and *POX08673* (Fig. 1d), which encode aconitase, 2-oxoglutarate dehydrogenase, phosphoenolpyruvate carboxykinase, citrate synthase, succinate dehydrogenase cytochrome B subunit, citrate synthase, fumarase, succinate dehydrogenase iron-sulfur protein, succinyl-coenzyme A (CoA) synthetase α subunit, succinyl-CoA synthetase β subunit, succinate dehydrogenase flavoprotein subunit, and malate dehydrogenase, respectively. The transcriptional levels of all 12 genes, except for those of *POX05565* and *POX05567*, were significantly downregulated by 50.8 to 80.1% on WR culture compared with levels on WB culture, whereas only three genes (*POX00835*, *POX01075*, and *POX04356*) were downregulated by 59.2 to 69.3% on WA culture. In contrast, two genes, *POX05565* and *POX05567*,

FIG 2 Screening for and identification of novel regulators required for cellulase and xylanase production in *P. oxalicum* under SSF. (a) Venn diagram showing unique and shared candidate regulatory genes on the different carbon sources. The screening thresholds were $|\log_2$ fold change| of ≥ 1.0 and probability of ≥ 0.8 . Red numbers represent the sum of DEGs from each group. WB, wheat bran; WA, WB plus Avicel; WR, WB plus rice straw. (b) Filter paper cellulase (FPase) activities of *P. oxalicum* deletion mutants of candidate regulatory genes. Crude cellulase was collected from *P. oxalicum* strains grown on WR under SSF for 5 days. *, $P \leq 0.05$; **, $P \leq 0.01$ (for differences between the results for the deletion mutants and those for the parental $\Delta PoxKu70$ strain by Student's *t* test). The data points represent means $\pm$ standard deviations. gds, gram of dry substrate.

increased their transcript abundances by 193.0 to 320.2% on both WR and WA cultures compared to levels on WB culture (Fig. 1d).

In addition, DEGs encoding putative TFs were also screened for among these transcriptomics data, and 11, 21, and 6 were detected in comparisons between EU2101_WR and EU2101_WA, EU2101_WR and EU2101_WB, and EU2101_WA and EU2101_WB, respectively. Notably, three genes, eight genes, and one gene were detected specifically in comparisons between EU2101_WR and EU2101_WA, EU2101_WR and EU2101_WB, and EU2101_WA and EU2101_WB, respectively, with no gene being differently expressed in all three comparisons (Fig. 2a). In total, 25 DEGs encoding TFs were detected in *P. oxalicum* EU2101 with various degrees of expression, including upregulation and downregulation between a $\log_2$ fold change of -2.1 and $\log_2$ fold change of 2.9 , which appeared at least once in all of the comparisons of the transcriptomes expressed on the different carbon sources under SSF (Data Set S1).

The 25 putative TFs detected contained eight types of InterPro (IPR)-annotated domains, including the Zn₂Cys₆ fungus-type DNA-binding domain (IPR001138) and the winged-helix repressor DNA-binding domain (IPR011991). Of these 25 genes, 5 were regulatory genes known to be required for cellulase production in filamentous fungi under SmF conditions, including *POX01184*, *POX02768* (*PacC*), *POX04860* (*PDE_07199*),

POX05726, and *POX06534* (*BrlA*) (14–17). Additionally, previous work also reported six genes, *POX01183*, *POX04860*, *POX05726*, *POX06377*, *POX09124*, and *POX09469*, that were involved in the production of cellulase in *P. oxalicum* under SSF (9).

Construction and characterization of deletion mutants of candidate regulatory genes. To investigate the regulation of cellulase and xylanase genes in *P. oxalicum* under SSF, attempts were made to generate knockouts for each of the 25 DEGs encoding putative TFs described above. The *P. oxalicum* parental strain with a deletion of *Ku70* (Δ *PoxKu70* strain) allows high-frequency gene deletion through homologous recombination (10). In previous work, 11 genes, including *POX01183*, *POX01184*, *POX04193*, *POX04590*, *POX04860*, *POX05726*, *POX06377*, *POX07099*, *POX07938*, *POX09124*, and *POX09469*, were deleted in the Δ *PoxKu70* mutant (9, 14). Ultimately, an additional eight deletion mutants were constructed and confirmed by PCR (Fig. S2a) with specific primer pairs (Table S1). Of the 10 deletion mutants, as shown in Fig. 2b, 3 showed significant changes in production of filter paper cellulase (FPase), ranging from 75.3% to 156.1% of the level of the parental Δ *PoxKu70* strain ($P \leq 0.05$, Student's *t* test) when cultivated on WR for 5 days. These three genes (*POX03016*, *POX04193*, and *POX07099*) were reported to be involved in cellulase production in filamentous fungi under SSF. Importantly, the Δ *POX03016* mutant showed an average 50.0% increase in FPase activity, which ranked as the top one in the hierarchy of FPase activity of all three mutants, and was selected for further study. The identity of the Δ *POX03016* mutant was further confirmed by Southern hybridization analysis (Fig. S2b) with specific probes (Table S1) to exclude the possibility that the deletion cassette had been inserted into wrong sites in the Δ *PoxKu70* strain genome.

Bioinformatics analysis of POX03016. *POX03016* is comprised of 464 amino acids. Assays with the Simple Modular Architecture Research Tool (SMART) revealed that *POX03016* contained four conserved structural domains including Aft1_OSA (Aft1 osmotic stress response domain; E value, $9.5e-22$), Aft1_HRA (E value, $4.5e-25$), Aft1_HRR (E value, $2.4e-19$), and the basic region leucine zipper (BRLZ; E value, $3.5e-18$) (Fig. 3a). Further alignment using blastP at the National Center for Biotechnology Information (NCBI) indicated that *POX03016* shared high identity (64 to 98%) with proteins from *Penicillium*, *Aspergillus*, and *Talaromyces* and identities below 60% with those of other fungi, such as 49% with TRIREDRAFT_103372 (EGR52603.1) in *Trichoderma reesei* QM6a and 44% with Atf1 in *Schizosaccharomyces pombe* 972h-. Moreover, a neighbor-joining phylogenetic tree also showed that *POX03016* of *P. oxalicum* HP7-1 was highly conserved among industrial filamentous fungi including *Penicillium*, *Aspergillus*, and *Talaromyces*, but not in *T. reesei* or *Neurospora crassa* (Fig. 3b). To facilitate further study, *POX03016* was redesignated *PoxAtf1* (*Penicillium oxalicum* Atf1).

Cell growth and enzyme production of Δ *PoxAtf1* mutant under SSF on WR. To quantitatively investigate the effect of *PoxAtf1* on mycelial growth of *P. oxalicum* in SSF, the growth curves of both the Δ *PoxAtf1* and Δ *PoxKu70* mutants were investigated. As shown in Fig. 4a, mycelial growth of the Δ *PoxAtf1* mutant was similar to that of the Δ *PoxKu70* mutant up to 36 h after inoculation, whereas the growth of Δ *PoxAtf1* mutant was significantly decreased during the later stages of culture.

In addition, the effects of *PoxAtf1* on cellulase and xylanase production when the Δ *PoxAtf1* strain was grown on WR after transfer from glucose were also investigated in detail. The Δ *PoxAtf1* and Δ *PoxKu70* strains were first precultured for 24 h in glucose medium, and their mycelia were subsequently transferred to solid medium containing WR and cultured under SSF conditions for 2 to 4 days. The results showed that the Δ *PoxAtf1* mutant increased the activities of cellulases, including FPase, carboxymethylcellulase (CMCase), *p*-nitrophenyl-cellobiosidase (pNPCase), *p*-nitrophenyl- α -D-glucosidase (pNPGase), and xylanase, by 25.1 to 209.3% ($P \leq 0.05$, Student's *t* test) in comparison with activities of the Δ *PoxKu70* strain. Intriguingly, increasing cellulase and xylanase activity of *P. oxalicum* caused by deletion of *PoxAtf1* occurred mainly early in the induction stage, with,

FIG 3 Characterization of POX03016 from *P. oxalicum*. (a) Modular architecture. (b) Unrooted phylogenetic tree of POX03016 and its orthologs. The phylogenetic tree was constructed using MEGA, version 7.0, software with the neighbor-joining method and a Poisson model. The bootstrap values at the nodes were calculated by setting 1,000 replicates as a parameter.

e.g., a 46.1% increase in CMCase activity at 2 days in contrast to a 25.1% increase at 4 days (Fig. 4b to f).

To further confirm that the changes in enzymatic activity in the $\Delta PoxAtf1$ mutant were caused by *PoxAtf1* deletion, a complementary strain, the *CPoxAtf1* strain, was constructed (Fig. S3). Tests of enzyme activity indicated that the *CPoxAtf1* strain restored cellulase and xylanase production to levels similar to those of the parental $\Delta PoxKu70$ strain (Fig. 4).

Global regulation of *PoxAtf1* in *P. oxalicum* under SSF. To better understand the effects of *PoxAtf1* at the transcriptional level in *P. oxalicum*, RNA sequencing of transcriptomes from both the $\Delta PoxAtf1$ and $\Delta PoxKu70$ mutants grown on WR under SSF for 24 h after transfer from a glucose-containing medium was performed on an Illumina HiSeq 2000 platform. A total of 22 million clean reads with a length of 100 bp were generated from each sample, and an average of 89.02% clean reads were mapped onto the genome of the wild-type strain HP7-1 (10), corresponding to 8,677 predicted protein-encoding genes. The three biological replicates of each *P. oxalicum* strain exhibited a high Pearson's correlation coefficient ($r > 0.6$) (Fig. S4), suggesting that the transcriptomic data were reliable.

DEGs between the transcriptomes of the $\Delta PoxAtf1$ and $\Delta PoxKu70$ strains were screened for and identified using DESeq2 (18) with a $|\log_2$ fold change| of ≥ 1.0 and P value of ≤ 0.05 as the thresholds. A total of 519 DEGs were detected in the $\Delta PoxAtf1$ mutant in comparison with genes of the $\Delta PoxKu70$ mutant and termed the *PoxAtf1* regulon (Data Set S2), including 253 upregulated and 266 downregulated genes. KEGG database analyses showed that 85% of the DEGs in the *PoxAtf1* regulon were involved in metabolism, mainly involving carbohydrate metabolism and amino acid metabolism (Fig. 5a).

FIG 4 Growth profiles of the *P. oxalicum* $\Delta PoxAtf1$ mutant and the parental $\Delta PoxKu70$ strain (a) and enzymatic activities (b to f) of crude enzyme of the *P. oxalicum* $\Delta PoxAtf1$ mutant, the parental $\Delta PoxKu70$ strain, and the complementary $CPoxAtf1$ strain. For growth profiles, *P. oxalicum* strains were inoculated into liquid medium containing glucose as the sole carbon source, and the inoculated medium was incubated at 28°C for 72 h. Mycelia were harvested every 12 h, dried, and weighed. For enzyme activity measurement, crude enzyme solution was collected from *P. oxalicum* strains grown on solid medium containing wheat bran plus rice straw (WR) as the sole carbon source under SSF for 2 to 4 days after a transfer from the glucose medium. *, $P \leq 0.05$; **, $P \leq 0.01$ (for differences between the results for the $\Delta PoxAtf1$ mutant and the $\Delta PoxKu70$ strain, and between the results for the $\Delta PoxAtf1$ mutant and the complementary $CPoxAtf1$ strain, by Student's *t* test). The data points represent means $\pm$ standard deviations. gds, gram of dry substrate.

Among the *PoxAtf1* regulon, 62 DEGs encoding CAZymes were found, namely, 47 upregulated and 15 downregulated genes, with $\log_2$ fold changes from -3.1 to 2.5 . Interestingly, approximately 68% of the 47 upregulated genes encoded CWDEs, including a CBH gene (*POX04786* [*Cel6A*]), six EG genes (*POX01166* [*Cel5B*], *POX01896* [*Cel5C*], *POX05570* [*Cel45A*], *POX06147* [*Cel5A*], *POX06983*, and *POX07535* [*Cel12A*]), two BGL genes (*POX03641* and *POX06079*), and five XYN genes (*POX03430*, *POX05916*, *POX06601*, *POX06783* [*Xyn11A*], and *POX08484* [*Xyn11B*]). Moreover, a swollenin gene, *POX08485*, an LPMO gene, *POX08897*, and a chitinase gene, *POX09448* (*ChiB1*), were also included. Conversely, of the 15 downregulated DEGs, only three CWDEs were identified, namely, one CBH gene, *POX02490* (*Cel7A-1*), a β -xylosidase/ α -L-arabinofuranosidase gene, *POX09154*, and one polygalacturonase gene, *POX01225* (Fig. 5b).

In addition to the CAZyme genes, 17 DEGs encoding putative TFs were also identified, consisting of 12 downregulated and 5 upregulated genes, with a $\log_2$ fold change from -3.9 to 1.8 . These TFs could be classified into six types, such as zinc finger type (58.8%; i.e., Zn2Cys6, C2H2, CCHC, and GATA) and winged-helix type (11.8%). Of

FIG 5 Comparative analysis of the transcriptomes from *P. oxalicum* $\Delta PoxAtf1$ mutants and the parental $\Delta PoxKu70$ strain cultured in medium containing wheat bran plus rice straw (WR) under SSF. (a) Differentially expressed genes (DEGs) in the $\Delta PoxAtf1$ strain annotated by KEGG. The screening criteria for DEGs were $|\log_2$ fold change| of ≥ 1 and a P value of < 0.05 . (b) DEGs encoding carbohydrate-active enzymes (CAZymes); CWDEs, plant cell wall-degrading enzymes. (c) DEGs involved in conidiation of *P. oxalicum*. In panel b, the values with color represent $\log_2$ fold changes (FPKM $\Delta PoxAtf1$ /FPKM $\Delta PoxKu70$) of DEGs in the $\Delta PoxAtf1$ mutant versus expression in the parental $\Delta PoxKu70$ strain. FPKM, fragments per kilobase of exon per million mapped reads.

these 17 DEGs, three regulatory genes (*POX05726*, *POX06534* [*BrlA*], and *POX09469*) are known to regulate cellulase production in filamentous fungi (14, 16; see also Data Set S2).

Interestingly, 39 DEGs encoding putative transporters were found in the *PoxAtf1* regulon, including 25 upregulated genes ($1.0 < \log_2$ fold change < 2.0) and 14 downregulated genes ($-2.5 < \log_2$ -fold change < -1.0). These putative transporters were annotated as amino acid/polyamine transporter I (IPR002293), bicarbonate transporter (IPR003020), ABC transporter-like (IPR003439), sugar/inositol transporter (IPR003663), oligopeptide transporter (IPR004813), major facilitator, sugar transporter-like (IPR005828), major facilitator superfamily (IPR011701), and amino acid transporter, transmembrane domain (IPR013057). Among them, two important DEGs encoding cellobiohydrolase transporters, *POX06051* (CdtC) and *POX05915* (CdtD), were included, which increased their transcript abundance by 2.1- and 1.4-fold, respectively, in the $\Delta PoxAtf1$ mutant compared to the levels of the $\Delta PoxKu70$ mutant.

Comparative transcriptional profiling also revealed that *PoxAtf1* affected conidiation of *P. oxalicum*. A total of eight DEGs involved in conidiogenesis were identified, including seven downregulated DEGs (*POX00014* [*ChsC*], *POX01390* [*AygA*], *POX02234*

FIG 6 Transcriptional regulation of major cellulase and xylanase genes in *P. oxalicum* cultured in medium containing wheat bran plus rice straw (WR) as the sole carbon source under SSF revealed by RT-qPCR at three different time points (12, 24, and 48 h) after a shift. All of the gene expression levels in the $\Delta PoxAtf1$ mutant were normalized against the level of the $\Delta PoxKu70$ mutant. *, $P \leq 0.01$; **, $P \leq 0.05$ (for differences between results for the $\Delta PoxAtf1$ and $\Delta PoxKu70$ mutants by Student's *t* test). The data points represent means $\pm$ standard deviations.

[CatA], POX03412 [ArpA], POX03413 [ArpB], POX06534 [BrlA], and POX09195 [PpoA]), encoding chitin synthase C, pigment synthesis protein AygA, catalase, scytalone dehydratase, HN-reductase, regulatory protein BrlA, and fatty acid oxygenase PpoA, respectively ($-2.8 < \log_2$ fold change < -1.2), and one upregulated gene (POX03389 [Axl2]), encoding IgE-binding protein ($\log_2$ fold change of 1.1) (Fig. 5c) in the $\Delta PoxAtf1$ strain.

Dynamic regulation of cellulase and xylanase gene expression by *PoxAtf1* under SSF. To dynamically investigate the regulation of cellulase and xylanase gene expression by *PoxAtf1* under SSF in *P. oxalicum*, both the $\Delta PoxAtf1$ and $\Delta PoxKu70$ mutants were subjected to reverse transcription-quantitative PCR (RT-qPCR) analysis. The cellulase and xylanase genes tested for included POX05587 (*Cel7A-2*), POX04786 (*Cel6A*), POX01166 (*Cel5B*), POX06147 (*Cel5A*), POX06983, POX07535 (*Cel12A*), POX03641, POX06079, POX05916, POX06783 (*Xyn11A*), and POX08484 (*Xyn11B*). The results revealed that the abundances of transcripts of all tested genes in the $\Delta PoxAtf1$ mutant increased by 36.2 to 458.6% before 24 h of culture compared to levels in the $\Delta PoxKu70$ mutant, with the exception that POX05587 (*Cel7A-2*) at 24 h had no significant difference in abundance. In contrast, five genes, POX07535 (*Cel12A*), POX03641, POX06079, POX06983, and POX08484 (*Xyn11B*), had significantly increased transcriptional levels by 20.0 to 127.5% at 48 h, whereas three genes, POX04786 (*Cel6A*), POX06147 (*Cel5A*), and POX06783 (*Xyn11A*), had significantly reduced transcript abundances by 14.2 to 42.7% at 48 h (Fig. 6).

***PoxAtf1* binds to the promoter regions of genes encoding major cellulases and xylanases *in vitro*.** *In vitro* binding experiments were carried out with an electrophoretic mobility shift assay (EMSA) in order to confirm how *PoxAtf1* regulates the expression of cellulase and xylanase genes under SSF. The putative DNA-binding domain of *PoxAtf1*, namely, amino acids 316 to 433, was recombinantly expressed (rPoxAtf1_{316–433}) in *Escherichia coli* and then purified. Approximately 1 kb of the promoter region of each target gene selected as the EMSA probe was amplified by PCR with specific primer pairs (Table S1). The tested target genes included two CBH genes (POX05587 [*Cel7A-2*] and POX04786 [*Cel6B*]), three EG genes (POX01166 [*Cel5B*], POX06147 [*Cel5A*], and POX07535 [*Cel12A*]) two BGL genes (POX03641 and POX06079), and two XYN genes (POX06783 [*Xyn11A*] and POX08484 [*Xyn11B*]). Differential amounts of the purified fusion protein rPoxAtf1_{316–433} with thioredoxin (Trx)-His-S as the tag were mixed with 40 ng of each EMSA probe and subjected to native polyacrylamide gel electrophoresis. The results indicated that the bands with altered mobilities, representing rPoxAtf1_{316–433}-DNA complexes, appeared on all gels on which the mixture of the rPoxAtf1_{316–433} and the tested probes was loaded, while no band was detected for the promoter region of the gene POX05989 encoding β -tubulin, which was used as a negative control. As expected, the brightness of the shifted bands gradually became

FIG 7 *In vitro* binding experiments between PoxAtf1 and the promoter regions of target genes. Each binding reaction mixture contained 0 to 2.5 μg of Trx-His-S-tagged rPoxAtf1₃₁₆₋₄₃₃ and ~50 ng of each candidate probe. The purified Trx-His-S fusion protein, bovine serum albumin (BSA), or the promoter sequence of the β-tubulin gene was used as a negative control.

stronger with the increase in the protein mass added. The rPoxAtf1₃₁₆₋₄₃₃-DNA complexes were not found with Trx-His-S or bovine serum albumin (BSA) protein alone. Subsequently, competitive experiments further showed that the concentration of the shifted bands gradually decreased with an increasing amount of the competitive probes without 6-carboxyfluorescein (Fig. 7), suggesting that PoxAtf1 bound specifically to the promoters of *POX05587* (*Cel7A-2*), *POX04786* (*Cel6B*), *POX01166* (*Cel5B*),

POX06147 (Cel5A), *POX07535 (Cel12A)*, *POX03641*, *POX06079*, *POX06783 (Xyn11A)*, and *POX08484 (Xyn11B)*.

DISCUSSION

In the present study, we comprehensively and systematically investigated global gene expression profiling under SSF conditions of the soil fungus *P. oxalicum*, which is a natural decomposer of plant biomass through secretion of considerable amounts of plant-biomass degradation enzymes (PBDEs). Most genes involved in carbohydrate metabolism, energy metabolism, amino acid metabolism, and translation were affected at a transcriptional level to various extents in response to different plant biomass sources. Importantly, deletion of *PoxAtf1* resulted in an increase of cellulase and xylanase production of *P. oxalicum* under SSF, suggesting that *PoxAtf1* might be a target in genetic engineering to improve fungal enzyme production in the future.

Moreover, the BCCGs consisted of 119 members, which mainly included known key CBH genes such as *POX05587 (Cel7A-2)* and *POX04786 (Cel6A)*, EG genes such as *POX05571 (Cel7B)*, *POX06147 (Cel5A)*, *POX07535 (Cel12A)*, and *POX05570 (Cel45A)*, BGL genes such as *POX03641* and *POX06079*, and XYN genes such as *POX06783 (Xyn11A)* and *POX00063 (Xyn10A)*, with the addition of some genes encoding assisting enzymes such as LPMOs, xylosidases, β -mannanases, and α -galactosidases. Moreover, their transcriptional levels were activated to various extents by substrates with different chemical compositions. For example, the transcript abundances of 29 CWDE genes in the BCCGs induced by WR, such as EG genes *POX06147 (Cel5A)* and *POX07535 (Cel12A)*, XYN genes *POX06783 (Xyn11A)* and *POX08484 (Xyn11B)*, and xylosidase genes *POX06600* and *POX06599* as well as the α -galactosidase gene *POX09085* and acetyl-xylan esterase gene *POX01218*, were significantly greater than those induced by WA, and these transcript abundances determine extracellular enzyme activity of *P. oxalicum* (data not shown). Notably, synergistic regulation of the response of many TFs to different carbon sources contributed to the specific expression of the BCCGs in *P. oxalicum* under SSF, based on the identification of TFs that regulated cellulase activity.

Here, the TF *PoxAtf1* of *P. oxalicum*, an ortholog of *AtfA/Atf1* in *S. pombe* 972h-, could regulate the expression of cellulase and xylanase genes. *AtfA/Atf1* is a member of the ATF/CREB family, which functions as an essential downstream component of the mitogen-activated protein kinase (MAPK) *Styl/Hog1p* pathway activated by several environmental stresses (19). *AtfA/Atf1* orthologs have been extensively studied in fungi since *Atf1* was identified as a regulator, promoting sexual differentiation and entry into the stationary phase in *S. pombe* (20). Results have shown that *AtfA/Atf1* and its orthologs are quite highly conserved and that they are involved in multiple cellular processes. In *S. pombe*, *Atf1* regulates the transcription of genes involved in recombination at some hot spots and in the initiation of the mating and meiosis processes, survival at stationary phase (20), heterochromatin assembly, and responses to osmotic, heat, starvation, and nutritive stress (19, 21, 22). In filamentous fungi, *AtfA/Atf1* regulates the expression of genes related to conidial stress in *Aspergillus fumigatus*, *Aspergillus nidulans*, and *Aspergillus oryzae* (23–26), genes related to vegetative hyphae and/or secondary metabolism in *A. nidulans* (27, 28), *Botrytis cinerea* (29), *Claviceps purpurea* (30), *Fusarium graminearum* (31), *Fusarium oxysporum* (32), and *Magnaporthe oryzae* (33), and fungal virulence in *B. cinerea*, *C. purpurea*, *F. graminearum*, and *M. oryzae* (29, 30, 32, 33). In the present study, we have been able to further explain biological functions of *AtfA/Atf1* in filamentous fungi.

In *S. pombe*, under environmental stress conditions, the MAPK *Styl/Hog1p* has to be phosphorylated and to accumulate at the cell nucleus, thereby promoting *Atf1* activation and the gene regulation mediated by it. Phosphorylation of *Atf1* enables transcription of the stress promoter through recruitment of RNA polymerase II, the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex, the TATA-binding proteins, and heterochromatin assembly factors (19, 34). *Atf1* achieves gene regulation dependent on its interacting TFs, such as *Pcr1* and/or *Pap1*, in response to environmental stresses (19, 35). Interestingly, the subunits of the SAGA complex, such as *Gcn5* and *Ada2* and the heterochromatin

protein HP1 (a homolog of Swi6 of *S. pombe*), are required for cellulase production in *T. reesei* and *P. oxalicum*, respectively (36–38). In addition, in the *PoxAtf1* regulon, a number of cellulase and xylanase genes and their regulatory genes, such as *POX05726* and *POX06534* (*BrlA*), were also found (7, 14, 16), indicating that *PoxAtf1* could indirectly and/or directly regulate the expression of cellulase and xylanase genes in *P. oxalicum* under SSF. However, how *PoxAtf1* might inactivate and/or activate transcriptional initiation of cellulase and xylanase genes or their regulatory genes through the recruitment of the SAGA complex, heterochromatin factors, or the repressors, including TFs instead of Pcr1 and/or Pap1, in response to plant biomass composition is unclear and needs to be studied further.

As well as the known regulatory roles of AtfA reported in *Aspergillus* (25, 39), *PoxAtf1* positively regulated hyphal growth and conidiation of *P. oxalicum* under environmental stress as identified by phenotypic and transcriptomic profiling analyses. Surprisingly, deletion of *PoxAtf1* inhibited the growth of vegetative hyphae of *P. oxalicum* only at the late culture period, which might result in the decrease in cellulase and xylanase production at this period compared with that at the early period, which might be related to the recruitment of the SAGA complex and heterochromatin proteins by Atf1 because they were also required for hyphal growth and conidiation in *T. reesei* and *P. oxalicum* (36–38).

In conclusion, the research described in this paper carried out a comprehensive and systematic study of global gene expression of *P. oxalicum* under SSF conditions and identified the BCCGs of the *P. oxalicum* response to plant biomass composition. Transcriptional expression of the BCCGs differed to various extents, depending on the different chemical compositions of the plant biomass used as the substrate. As a consequence, we also identified a key transcription factor, *PoxAtf1*, which played essential roles in the regulation of BCCG expression. This study provides novel insights into expressional regulation of fungal cellulase and xylanase genes under SSF and identified new targets for genetic engineering to improve fungal cellulase and xylanase production.

MATERIALS AND METHODS

Strains and culture conditions. *P. oxalicum* strains, including EU2101 (12769; China General Microbiological Culture Collection Center [CGMCC], Beijing, China) and the $\Delta PoxKu70$ mutant (3.15650; CGMCC), were cultured under SmF and maintained for passage and subculture on potato dextrose agar (PDA) plates as previously reported (14). *P. oxalicum* strain EU2101 was isolated and identified as a cellulase hyper-producing mutant after multiple-round mutagenesis programs, including treatment with ^{60}Co gamma ray irradiation and ethyl methane sulfonate/UV light (data not shown), of the wild-type strain HP7-1, which had been isolated from Huaping National Nature Reserve in Guilin, China (40).

P. oxalicum strains were cultivated under SSF as described by Su et al. (41). One hundred milliliters of SSF medium contained 20 ml of mineral salt solution (5.0 g/liter yeast extract, 2.5 g/liter KH_2PO_4 , 2.5 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% Tween 80, 5.0 mg/ml $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mg/liter $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4 mg/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 mg/liter CoCl_2 , pH 5.0) and 10 g of carbon sources (WR, WA, or WB). The raw plant materials WB and rice straw were purchased from a local farmer's market in Nanning, China. All plant materials were milled to 40-mesh particle size after being dried at 50°C to constant weight. SSF conditions were static at 28°C for 5 days.

To provide the mycelium for RNA sequencing of *P. oxalicum* strain EU2101, 1 ml of spore suspension at a concentration of 1.0×10^8 spores/ml was inoculated into SSF medium containing WA, WR, or WB and statically cultured at 28°C for 48 h, at which point mycelia were collected and used for total RNA extraction.

An aliquot (1 ml) of spore suspension (1.0×10^8 spores/ml) of a *P. oxalicum* mutant of each candidate regulatory gene was inoculated onto WR SSF medium, and the inoculated medium was statically incubated at 28°C for 5 days. Crude cellulase was extracted from the cultivated solid medium and tested for FPase activity (41).

To investigate mycelial growth profiles, a fixed number of *P. oxalicum* $\Delta PoxAtf1$ mutant spores were inoculated separately into glucose liquid medium containing 4.0 g/liter $(\text{NH}_4)_2\text{SO}_4$, 4.0 g/liter KH_2PO_4 , 0.6 g/liter CaCl_2 , 0.60 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g/liter $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0016 g/liter MnSO_4 , 0.0017 g/liter ZnCl_2 , 0.002 g/liter CoCl_2 , 1 ml of Tween 80, and 1.0 g of glucose, with the parental $\Delta PoxKu70$ strain being used as a control. The inoculated medium was incubated at 28°C, with shaking at 180 rpm. The mycelia were collected every 12 h until 72 h by centrifugation at $11,300 \times g$ for 10 min, dried at 50°C, and weighed. For RNA sequencing and RT-qPCR of the $\Delta PoxAtf1$ and $\Delta PoxKu70$ strains under SSF, 1×10^8 spores were first inoculated into the glucose medium at 28°C, with shaking at 180 rpm for 24 h. One gram of the harvested mycelial samples was subsequently transferred into fresh WR SSF medium. After culture for 12, 24, or 48 h, the mycelia were collected and used for extraction of total RNA for RT-qPCR. Of these samples, the RNA at 24 h was also sent for RNA sequencing.

For enzymatic activity measurements of the $\Delta PoxAtf1$ and $\Delta PoxKu70$ mutants under SSF conditions, the precultured mycelia were cultivated for 2 to 4 days in WR SSF medium as described above.

Extraction of total DNA and RNA. The total DNA and RNA of *P. oxalicum* strains were extracted using chemical methods, as previously reported (10). Briefly, total DNA was extracted from the ground mycelium in liquid nitrogen using lysis reagent containing 20 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate, and 40 mM Tris-HCl, pH 8.0. Total RNA was extracted using a TRIzol RNA kit (Life Technologies, Carlsbad, CA). For extracted DNA and RNA, the quality and quantity were assessed by electrophoresis on 1% agarose gel and by spectrophotometric analysis at A_{260} and A_{280} values, respectively.

RNA sequencing. Total RNA of *P. oxalicum* strains was assayed and sequenced with the methods reported by Zhao et al. (10). A cDNA library with inserts of 100-bp length was constructed using the extracted total RNA and assessed with an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The eligible cDNA library was sequenced via an Illumina HiSeq 2000 system. The generated raw reads were filtered using SOAPnuke (parameters: -l 15, -q 0.2, and -n 0.05) and trimmomatic (parameters: ILLUMINACLIP, 2:30:10; LEADING, 3; TRAILING, 3; SLIDINGWINDOW, 4:15; MINLEN, 50) (42, 43) to remove reads demonstrating low quality, adaptor pollution, and high content of unknown bases (N) in order to obtain clean reads. The clean reads were mapped onto the genome of *P. oxalicum* wild-type strain HP7-1 to achieve functional annotations, using the software BWA, version 0.7.10-r789 (12), and Bowtie2, version 2.1.0 (12). The numbers of fragments per kilobase of exon per million mapped reads (FPKM), representing relative gene expression levels, were calculated using the software RSEM, version 1.2.12 (44). DEGs were screened with NOISeq (13) or DESeq2 (18) tools. Pearson's correlation coefficient was used to assess the reliability of transcriptomes of three biological replicates of each sample. Heat map analysis was performed using the software Heml, version 1.0 (45).

Gene deletion mutant construction. The construction of the deletion mutants of candidate TF genes was carried out in the *P. oxalicum* parental $\Delta PoxKu70$ strain as described by Zhao et al. (10). The $\Delta PoxKu70$ mutant spores were inoculated into CM medium containing 6 g/liter NaNO_3 , 0.52 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52 g/liter KCl, 1.52 g/liter KH_2PO_4 , 2.0 g/liter peptone, 10.0 g/liter glucose, 1.0 g/liter yeast extract, and 1.0 g/liter acid-hydrolyzed casein at pH 6.5 and then cultured at 28°C and 180 rpm for 8 h. The harvested mycelia were lysed by incubation in buffer containing 10 mM NaH_2PO_4 , 1.2 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g/liter lysozyme, 6 g/liter lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, MO), and 6 g/liter snailase at pH 5.8 for 2.5 h. Subsequently, the trapping buffer (0.1 M Tris-HCl and 0.4 M sorbitol, pH 7.0) was added to the lysed mycelia, and the protoplasts were separated by centrifugation for further use.

The knockout cassette of each candidate gene included 2 kb of each upstream and downstream DNA fragment flanking the candidate gene and a 1.8-kb DNA fragment encoding the G418 resistance gene and was constructed by recombinant PCR. Approximately 10 μg of the knockout cassette and $\Delta PoxKu70$ mutant protoplasts were mixed in 0.1 M spermidine for 30 min on ice. The mixture was subsequently transferred to a 1 $\times$ PTC solution (40% [wt/vol] polyethylene glycol 3350, 100 mM Tris-HCl [pH 8.0], 100 mM CaCl_2) and incubated at room temperature for 25 min. The PTC solution containing protoplasts and DNA was mixed with OCM medium in petri dishes and incubated at 50°C for 30 min. OCM medium contains 1.0 g/liter yeast extract, 1.0 g/liter casein enzymatic hydrolysate, and 10.0 g/liter agar. After incubation, PDA medium containing 250 $\mu\text{g}/\text{ml}$ hygromycin B and 500 $\mu\text{g}/\text{ml}$ G418 was used to select the transformants.

Construction of complementary strain. The complementary *CpoxAtf1* strain was constructed according to a previous description (14). In brief, a cDNA cassette composed of upstream and downstream sequence (~1.5 kb) of the integrative locus *POX05007* and the coding sequences of the *PoxAtf1* gene and the bleomycin resistance gene was constructed by PCR and homologous recombination. The complementary cassette was introduced into the fresh $\Delta PoxAtf1$ strain protoplast suspension, and the transformants were selected using the corresponding antibiotics (250 $\mu\text{g}/\text{ml}$ hygromycin B, 500 $\mu\text{g}/\text{ml}$ G418, 100 $\mu\text{g}/\text{ml}$ bleomycin) and confirmed by PCR.

Southern hybridization analysis. The genomic DNA of *P. oxalicum* $\Delta PoxAtf1$ mutant was digested with EcoRI (TaKaRa Bio, Inc., Dalian, China), and the fragments were then separated on 0.8% agarose gel. The short DNA fragments were subsequently transferred to Hybond-N⁺ nylon membrane (GE Healthcare Limited, Amersham, United Kingdom). A DIG-High Prime DNA Labeling and Detection Starter kit (Life Technologies, Carlsbad, CA) was used to label the probes and to detect the hybridization bands between the probes and enzyme-digested genomic DNA fragments. The probe was amplified by PCR using specific primers (see Table S1 in the supplemental material) and total DNA of the $\Delta PoxKu70$ mutant as the template.

Preparation of crude cellulase under SSF. The crude cellulase solution from *P. oxalicum* mutants was prepared as described by Su et al. (41). Briefly, *P. oxalicum* mutants were cultured on WR SSF medium for 2 to 4 days after transfer from glucose medium. To the cultured WR SSF medium was added 20 ml of sterile water per gram of fermentation substrate, and the mixture was then incubated at 28°C with shaking at 180 rpm for 1 h. The filtrate was separated through eight layers of muslin cloth, followed by centrifugation at 11,300 $\times g$ for 15 min, and the supernatant was used as crude cellulase.

Enzyme activity assays. Cellulase (FPase, CMCase, pNPCase, and pNPGase) and xylanase activities of *P. oxalicum* strains were measured using methods described previously (10). FPase, CMCase, and xylanase activities were tested through the 3,5-dinitro salicylic acid method (46) using Whatman no. 1 filter paper (50 mg, 1.0 by 6.0 cm^2), 1.0% CMC-Na (Sigma-Aldrich, Darmstadt, Germany), and 1.0% xylan from beechwood (Megazyme International Ireland, Wicklow, Ireland) as the substrate, respectively. The

reaction buffer was 100 mM citrate buffer, pH 5.0, and the reaction mixture was incubated at 55°C for 1 h, 30 min and 10 min depending on the different substrates (filter paper, CMC-Na, and xylan, respectively). The concentrations of reducing sugars generated were measured at 540 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μ mol of reducing sugar per min from the reaction substrates at pH 5.0 and 55°C. Each sample contained at least three independent biological replicates.

The pNPCase and pNPGase activities were assayed with *p*-nitrophenyl- β -D-cellobioside (pNPC) and *p*-nitrophenyl β -D-glucopyranoside (pNPG) (Sigma-Aldrich) used as the substrates, respectively. The reaction system contained 10 μ l of diluted crude cellulase solution, 116 μ l of 100 mM citrate buffer, and 14 μ l of 25 mM substrate, and 70 μ l of 0.4 M sodium carbonate was used to stop the reaction. The reaction mixture was incubated for 15 min at pH 5.0 and 50°C. The *p*-nitrophenol concentration generated was quantified by measuring the A_{410} by spectrometry. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 μ mol of *p*-nitrophenol per min from the 25 mM substrate at pH 5.0 and 50°C. Each sample consisted of at least three biological replicates.

RT-qPCR assay. The RT-qPCR assay was carried out as described by Zhao et al. (10). The first-strand cDNA was synthesized using a PrimeScript RT reagent kit (TakaRa Bio Inc.), using total RNA as the template. The RT-qPCR system contained 2 μ l of first-strand cDNA, 10 μ l of SYBR Premix Ex Taq II (TakaRa Bio, Inc.), 0.8 μ l of each primer at 10 μ M (Table S1), and 9 μ l of double-distilled H₂O (ddH₂O). The reaction parameters were set at 40 cycles of 95°C for 5 s and 60°C for 34 s. The fluorescent signal was detected at the end of each extension step at 80°C. The relative expression of the tested genes was achieved by normalizing all expression levels to the level of the parental Δ PoxKu70 strain and by using the actin gene as a control. Three independent RT-qPCR replicates were carried out for each sample.

EMSA and competitive EMSAs. EMSA and competitive EMSAs were carried out as previously reported by Yan et al. (14). Briefly, DNA probes, labeled with 6-carboxyfluorescein (FAM) at the 3' terminus, and unlabeled competitive DNA probes were amplified via PCR using specific primers (Table S1). The DNA fragment encoding the putative DNA-binding domain of PoxAtf1 was cloned into the expression vector pET-32a(+), recombinationally expressed in *Escherichia coli* Rosetta cells, and subsequently purified to generate the recombinant protein rPoxAtf1_{316–433}. The purification was performed with affinity chromatography on Talon metal affinity resin (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

Different amounts (0 to 2.5 μ g) of rPoxAtf1_{316–433} and ~50 ng of each DNA probe were mixed for 30 min on ice and then separated by 4% polyacrylamide-Tris-acetic acid-EDTA (TAE) gel electrophoresis. For competitive EMSAs, different amounts (0.05 to 5.0 μ g) of competitive DNA probes were mixed with 2.5 μ g of rPoxAtf1_{316–433} and ~50 ng of each DNA probe for 30 min on ice before TAE gel electrophoresis.

Phylogenetic analysis. The amino acid sequences of PoxAtf1 orthologs were obtained via NCBI blastP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). MEGA, version 7.0 (47), software was used to construct the phylogenetic tree of PoxAtf1 and its homologs, using the neighbor-joining method and a Poisson correction model, and the bootstrap values were calculated by setting 1,000 replicates.

Statistical analysis. The statistical analysis of the data was performed using Student's *t* test (two-tailed) in Microsoft Excel (Office 2016; Microsoft, Redmond, WA).

Data availability. All transcriptomic data are available from Gene Expression Omnibus in the NCBI database under accession number [GSE131173](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131173). DNA sequences are available from the GenBank database under accession numbers [MK988443](https://www.ncbi.nlm.nih.gov/nuccore/MK988443) to [MK988445](https://www.ncbi.nlm.nih.gov/nuccore/MK988445).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01226-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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