The Different Roles of *Penicillium oxalicum* LaeA in the Production of Extracellular Cellulase and β-xylosidase

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Cellulolytic enzyme hydrolysis of lignocellulose biomass to release fermentable sugars is one of the key steps in biofuel refining. Gene expression of fungal cellulolytic enzymes is tightly controlled at the transcriptional level. Key transcription factors such as activator ClrB/CLR2 and XlnR/XYR1, as well as repressor CreA/CRE1 play crucial roles in this process. The putative protein methyltransferase LaeA/LAE1 has also been reported to regulate the gene expression of the cellulolytic enzyme. The formation and gene expression of the cellulolytic enzyme was compared among *Penicillium oxalicum* wild type (WT) and seven mutants, including ΔlaeA (deletion of laeA), OE clrB (clrB overexpression), OE clrB ΔlaeA (clrB overexpression with deletion of laeA), OE xlnR (xlnR overexpression), OE xlnR ΔlaeA (xlnR overexpression with deletion of laeA), ΔcreA (deletion of creA), and ΔcreA ΔlaeA (double deletion of creA and laeA). Results revealed that LaeA extensively affected the expression of glycoside hydrolase genes. The expression of genes that encoded the top 10 glycoside hydrolases assayed in secretome was remarkably downregulated especially in later phases of prolonged batch cultures by the deletion of laeA. Cellulase synthesis of four mutants ΔlaeA, OE clrB ΔlaeA, OE xlnR ΔlaeA, and ΔcreA ΔlaeA was repressed remarkably compared with their parent strains WT, OE clrB, OE xlnR, and ΔcreA, respectively. The overexpression of clrB or xlnR could not rescue the impairment of cellulolytic enzyme gene expression and cellulase synthesis when LaeA was absent, suggesting that LaeA was necessary for the expression of cellulolytic enzyme gene activated by ClrB or XlnR. In contrast to LaeA positive roles in regulating prominent cellulase and hemicellulase, the extracellular β-xylosidase formation was negatively regulated by LaeA. The extracellular β-xylosidase activities improved over 5-fold in the OE xlnR ΔlaeA mutant compared with that of WT, and the expression of prominent β-xylosidase gene *xyl3A* was activated remarkably. The cumulative effect of LaeA and transcription factor XlnR has potential applications in the production of more β-xylosidase.

**Keywords:** cellulase, β-xylosidase, LaeA, transcription factor, *Penicillium oxalicum***
INTRODUCTION

Biological conversion of cellulosic biomass to fuels and chemicals through enzymatic hydrolysis of cellulose allows the production of substantial amounts of biofuels and biochemicals to replace fossil fuels. The enzymatic hydrolysis of lignocellulose biomass to release fermentable sugars is one of the key steps in biofuel refining (Himmel and Bayer, 2009). Many filamentous fungi are able to degrade plant cell wall material by secreting large numbers of cell wall-degrading enzymes, including cellulase and hemicellulase (Lynd et al., 2002). Therefore, investigating the regulation of lignocellulosic enzyme gene expression is important to improve the production of these enzymes in biofuel refining.

Mechanisms of lignocellulosic enzyme gene regulation have been extensively studied in filamentous fungi such as Trichoderma reesei (teleomorph Hypocrea jecorina) (Seiboth et al., 2004; Kubicek et al., 2009), Aspergillus (Gielkens et al., 1999), and Neurospora crassa (Coradetti et al., 2012). Fungal cellulase and hemicellulase gene expression are tightly controlled at the transcriptional level (Ilmén et al., 1997). The expression of most cellulase and hemicellulase genes is transcriptionally co-regulated by the coordinated action of transcription factors. Several transcription factors have been identified to play essential roles in the expression of cellulase in T. reesei, Aspergillus nidulans, N. crassa, and Penicillium oxalicum, such as the repressor CRE1/CreA (Strauss et al., 1995) and ACE1 (Aro et al., 2003) and the positive regulators Xyr1/XlnR and CLR2/ClrB (Coradetti et al., 2012; Li et al., 2015).

CRE1 (ortholog in Aspergillus spp.) is the key transcription factor found in filamentous fungi. Increased cellulase production was observed in the CRE1 deletion/mutation strains of T. reesei (Nakari-Setälä et al., 2009) and N. crassa (Sun and Glass, 2011). In T. reesei, CRE1 binding sites were found in the prominent cellulase gene cbh1 and prominent hemicellulase gene xyn1 promoters, where mutations in the binding sequences led to the constitutive expression of these genes even in the presence of glucose (Ilmén et al., 1996; Mach et al., 1996). Xyr1 (ortholog XlnR in Aspergillus spp.) is a general activator of hydrolase formation in many fungi. In T. reesei and Aspergillus, Xyr1/XlnR has been shown to positively affect the transcription of major cellulase and hemicellulase genes (van Peij et al., 1998; Stricker et al., 2006). The overexpression of the xlnR gene in Aspergillus oryzae has resulted in elevated cellulolytic and xylanolytic activities in the culture supernatant (Noguchi et al., 2009). Recently, a zinc binuclear cluster transcription factor CLR-2 was reported to be an important activator of both cellulase and hemicellulase genes in N. crassa (Coradetti et al., 2012). CLR-2 is conserved in the genomes of most filamentous fungi. In A. nidulans, approximately 50% of the cellulose-responsive genes showed strict dependence on functional clr-2 homolog (clrB), and the cellulolytic activity on Avicel was significantly impaired as a result of clrB deletion (Coradetti et al., 2013).

In addition to the key transcription factors, other regulators such as LAE1 were also found to play important roles in regulating cellulase and hemicellulase gene expression. The lae1/laeA (loss of aflR expression) gene was originally identified in A. nidulans (Bok et al., 2005). This gene was later proven to affect morphological development and the biosynthesis of a large number of secondary metabolites in many filamentous fungi, such as Aspergillus (Kale et al., 2008; Oda et al., 2011; Chang et al., 2012), Penicillium chrysogenum (Kosalková et al., 2009), and Fusarium verticillioides (Butchko et al., 2012). LAE1 function is always associated to the epigenetic control by its putative protein methyltransferase function (specifically for histone tails), as LAE1 possesses S-adenosyl-methionine-binding (SAM) motifs. However, LAE1's direct methyl-accepting substrate has not been discovered yet, although it was shown to be self-methylated in Met 207 (Patananan et al., 2013). LAE1 somehow is assumed to counteract the trimethylation of H3K9 and the binding of heterochromatin protein to this repressive chromatin mark (Strauss and Reyes-Dominguez, 2011). LAE1 was also found to control glycoside hydrolase gene and transcriptional activator Xyr1 gene expression in T. reesei. A complete loss of expression of all seven cellulases and auxiliary factors for cellulose degradation and low xyr1 transcript levels was observed in T. reesei laeA deletion strain (Seiboth et al., 2012).

Penicillium oxalicum, previously called Penicillium decumbens, has been used for industrial-scale glycoside hydrolase production for more than 20 years in China (Qu et al., 1991). Data from the entire genome-sequencing analysis revealed that this fungus has a unique lignocellulose-degrading enzyme arsenal during evolution, including 18 predicted cellulase genes, 51 predicted hemicellulase genes, and 12 additional genes that encode accessory enzymes for the degradation of cellulose and hemicellulose (Liu et al., 2013a). The mechanism behind the regulation of lignocellulosic enzyme genes in P. oxalicum is similar to that in Aspergillus and Neurospora. Several transcription factors such as CreA (Liu et al., 2013a), ClrB, and XlnR (Li et al., 2015) have been found to play important roles in regulating cellulolytic enzyme gene expression. For instance, the overexpression of clrB, combined with both deletion of creA and an intracellular β-glucanase gene bgl2, remarkably activated the cellulase gene expression and improved filter paper activity (FPA) by over 20-fold (Li et al., 2015; Yao et al., 2015).

In this study, we investigated the roles of P. oxalicum LAE1 in regulating the expression of cellulase and β-xyllosidase gene, especially when transcription activator gene (clrB or xlnR) was overexpressed or transcription repressor gene (creA) was deleted. We found that LAE1 extensively affected P. oxalicum glycoside hydrolase gene expression, especially in later phases of prolonged batch cultures. The transcription repressor gene creA was also affected by LAE1. The overexpression of transcription activator gene clrB or xlnR could not rescue the impairment of cellulolytic enzyme gene expression and cellulase synthesis when LAE1 was absent. Interestingly, in contrast to the remarkable downregulation of prominent cellulase and hemicellulase by the deletion of laeA, the extracellular β-xyllosidase formation was an exception. The extracellular β-xyllosidase activities improved over 5-fold in the OExlnRDΔlaeA mutant compared with that of WT, and the expression of prominent β-xyllosidase gene xyl3A was activated remarkably.
MATERIALS AND METHODS

Strains and Culture Conditions
All the strains used in this study were listed in Table 1. Fungal strains were routinely grown on potato dextrose agar (PDA) or Vogel’s medium agar (Vogel, 1956), which were supplemented or modified as indicated. For conidial production, PDA plates were incubated at 30°C for 5 days and aerial conidia were harvested by flooding the plate with sterile distilled H2O containing 0.02% Tween 80. Conidial concentrations were determined by direct count using a hemocytometer.

Construction of Mutants in P. oxalicum
Standard techniques were used for nucleic acid manipulations. An overlap PCR method (Davidson et al., 2002) was used to create the fragments for targeted gene deletion. P. oxalicum 114-2 genomic DNA was used as the template for the PCR amplification of the 5’- and 3’-flanking regions of the laeA. The plasmid pME2892 (Krappmann et al., 2005) was used as the template for the PCR amplification of resistant gene pyrithiamine (PtrA). Primers UlaeA-F/UlaeA-R, ptrA-F/ptrA-R, and DlaeA-F/DlaeA-R were used to amplify the 5’ homologous flanking region (2296 bp), ptrA gene (2008 bp), and 3’ homologous flanking region (3072 bp) of laeA, respectively. The three resulting PCR fragments were fused and amplified by PCR using the nested primers ClaeA-F/ClaeA-R. The PCR products were directly transformed into OEClrB or OEInR to obtain OEClrBΔlaeA or OEInRΔlaeA. The primers hyg-F and hyg-R were used to amplify the hph gene (a hygromycin B phosphotransferase encoding gene (hphG, 2425 bp) from plasmid pSilenT1 (Nakayashiki et al., 2005). The primers gpdA-F and gpdA-LaeA-R were used to amplify the glyceraldehyde-3-phosphate dehydrogenase promoter (gpdA) from A. nidulans genome (1256 bp), whereas the primers LaeA-F/LaeA-R were used to amplify the intact P. oxalicum laeA open reading frame with its terminator (2179 bp). The three resulting PCR fragments were fused and transformed into WT to obtain OEΔlaeA.

The protoplast preparation, transformation, and screening of recombinant clones were performed as previously described by Qin et al. (2013). The genomic DNA of the transformants was isolated and used as the template to verify the integration events. Primers YlaeA-1/prtA-R and YlaeA-2/prtA-F were used to certify the deletion of laeA and obtained 2840 bp and 2413 bp fragments, respectively. Primers Yhhyg-gpdA-F/Yhhyg-gpdA-R and YgpdA-F/YgpdA-LaeA-R were used to certify the overexpression of laeA and obtained 2263 bp and 1299 bp fragments, respectively. Then, the mutants were further analyzed by Southern blot. Primers S-DlaeA-F/S-DlaeA-R and were used for PCR amplification to generate the probes for the examination of OEClrBΔlaeA and OEInRΔlaeA. Primers S-OfaeA-F and S-OfaeA-R were used for PCR amplification to generate the probes for the examination of OElaeA. The probes were labeled via PCR using a PCR DIG Probe Synthesis kit (Roche). The genomics DNA were digested with the restriction endonucleases and separated from 0.75% agarose gel electrophoresis. Then the DNA was transferred to the Hybond-N + nylon membranes (Amersham Biosciences/GE Healthcare, USA). Blots were visualized using a DIG DNA labeling system following the manufacturer’s protocol (Roche). The PCR verification and Southern blot results are shown in Figure S1. The primers used in this study were listed in Table S1.

Phenotypic Analysis of Wild Type and Mutants
One microliter conidial suspension (1 × 106 conidia ml−1) of each strain (1 μL hyphal fragment solution of ΔcreAΔlaeA strain was dropped onto the agar because it cannot generate conidia) were spotted onto various media on a 9-cm plate, including PDA or modified Vogel’s salts agar medium (Vogel, 1956) with various carbon sources supplemented with 2% (W/V) glucose, 2% lactose, 2% glycerol, or 2% ball-milled microcrystalline cellulose at 30°C for 4 days. Canon EOS 600D (Canon, Japan) was used for photographing. To test for conidiation, 106 conidia were spread on 9-cm Vogel’s + glucose plates and were cultivated at 30°C for 4 days. Afterward, a 5-mm diameter colony agar plug was removed from the plate. Three replications were conducted for each treatment. Conidia were then harvested by gently rubbing them off in an equal volume of physiological salt (0.2% w/v Tween 80 and 0.8% w/v NaCl). Conidial concentrations were determined by direct count using a hemocytometer.

Cellulase and Xylosidase Activity Assay
The strains were first grown in 100 mL liquid glucose minimal medium (GMM, per liter: glucose 20.0 g, KH2PO4 3 g, (NH4)2SO4 2.0 g, MgSO4·7H2O 0.56 g, CaCl2 0.56 g, FeSO4·7H2O 7.5 mg, MnSO4·H2O 2.5 mg, ZnSO4·7H2O 3.6 mg, CoCl2·6H2O 3.7 mg, CuSO4 3.2 mg) with initial pH 5.5 at 200 rpm for 24 h in 30°C. Then, 0.3 g vegetative mycelia was collected through vacuum filtration and was added to 100 mL of Vogel’s salts medium supplemented with 20 g L−1 inducing carbon sources (10.0 g L−1 microcrystalline cellulose plus 10.0 g L−1 wheat bran) at 30°C and 200 rpm. Samples were collected at the time points indicated in the text. Microcrystalline cellulose (CB0279) was purchased from Sangon (Shanghai, China). Culture supernatants were diluted with sodium acetate buffer solution (0.2 M, pH 4.8). The filter paper activities (FPA)
of the culture supernatants were assayed against Whatman No. 1 filter paper using a DNS method (Sun et al., 2008). β-D-xylopyranoside (p-NPX) was used as the substrate for β-xylanase activity assay. The experiment was conducted by incubating 50 µL of 0.1 mg/mL p-NPX in a 0.1 M sodium citrate buffer (pH 4.8) with 100 µL enzyme elute for 30 min at 50°C. Then, 150 µL of 10% (w/v) Na2CO3 was added to terminate the reaction. One enzyme activity unit was defined as the amount of enzymes required to produce 1 µmol glucose or pNP per minute under the assayed conditions. Three biological triplicates were performed in all analyses.

SDS-PAGE and Biomass Analysis
Unconcentrated supernatants were added to loading buffer, boiled for 5 min for degeneration, and loaded onto a 12% Tris-HCl polyacrylamide gel. Coomassie blue stain reagent was used for staining. The biomasses of all strains were measured using the methods described by Yao et al. (2015). Briefly, the WT and mutants were pre-grown in 1 × Vogel’s medium with glucose for at 30, 200 rpm for 24 h. The same quantity of dehydrated mycelia from each strain was transferred to the same freshly prepared media for another 6, 12, 24, 36, 48, 60, 72, and 96 h. All sampled mycelia were dried at 65°C to achieve constant weight, and dry cell weight was estimated.

Gene Expression Analysis by qRT-PCR
The strains were cultivated following the method described in “Cellulase and xylanase activity assay.” Total RNA was extracted from frozen lyophilized mycelia using the RNAiso Plus reagent (TaKaRa Biotechnology). Then, RNA was treated with DNase I, and first- and second-strand cDNA synthesis was performed using a PrimeScript RT Reagent kit with gDNA Eraser (TaKaRa Biotechnology). qRT-PCR was performed by SYBR Premix Ex Taq (Perfect Real Time, TaKaRa Biotechnology) using a LightCycler 480 system with software version 4.0 (Roche, Mannheim, Germany). The primers that were used to examine gene expression included act-F/act-R (for the actin gene, EPS26156.1), cel7A-F/cel7A-R (for the cel7A/cbh1 gene), cel7B-F/cel7B-R (for the cel7B/eg1 gene), cel3A-F/cel3A-R (for cel1A/bgl1 gene), amy15A-F/amy15A-R (for the amy15A gene), xln10A-F/xln10A-R (for xln10A gene), xyl3A-F/xyl3A-R (for xyl3A gene), laeA-F/laeA-R (for the laeA gene, EPS25650), creA-F/creA-R (for the creA gene, EPS28222), chrB-F/chrB-R (for the chrB gene, EPS31045), and xlnR-F/xlnR-R (for the xlnR gene, EPS32714). The primers used are shown in Table S1. Three biological triplicates were performed, and qRT-PCR of each gene was performed in three triplicates. The expression of actin was chosen as the reference gene for data normalization. The number of gene expression copies was calculated using the standard curves constructed for each gene, and the data were normalized to the expression levels of the actin gene. The relative expression level was defined as follows: Rel. expression level (gene X) = copy number of gene X/copy number of gene actin. Statistical analysis was performed using Minitab, and P ≤ 0.05 were considered statistically significant. The primers used in this study were listed in Table S1.

Digital Gene Expression Profiling and Go Analysis
The culture method was the same as that described in the “Cellulase and xylosidase activity assay” section. Digital gene expression profiling was performed according the methods described by Zhang et al. (2016). Briefly, after 24 h or 60 h of cultivation, total RNA was extracted from frozen mycelia using the RNAiso Plus reagent (TaKaRa Biotechnology). Oligo (dT) beads were employed to purify mRNA and guide double-stranded cDNA synthesis. Digital gene expression profiling based on Illumina sequencing was performed by the Beijing Genomics Institute in Shenzhen, China. Each tunnel generated millions of raw reads with a sequencing length of 35 bp. The 30 adaptor sequences were first removed from the sequencing reads to produce 21-nt long tags. Afterward, low quality tags, one copy tags, and tags which were not 21-nt long were removed to generate clean tags. All clean tags were obtained after raw data processing was mapped to the reference tag database (predicted transcripts plus downstream 300-nucleotide sequences) of the corresponding strain. The copy number of unambiguous tags (tags mapped to a single gene) for each gene was normalized to TPM for differential expression analysis. Genes with significantly different expression levels were identified through a significance test with combined thresholds (FDR ≤ 0.001 and fold change ≥ 2) (Audic and Claverie, 1997). Blast2GO was used for the function enrichment analysis of gene sets with the threshold at FDR ≤ 0.05 (Conesa et al., 2005).

Yeast Two-Hybrid Assay
Yeast two-hybrid assay was performed according the protocols of Clontech (Clontech Laboratories, Inc.). Briefly, pGBKT7 derived prey laeA and pGADT7 derived bait creA, chrB or xlnR were co-transformed into the Saccharomyces cerevisiae strain AH109, which has four reporter genes including lacZ, mel1, ade2 and his3. Transformants were selected on SD/-Trp/-Leu/-His media containing 2.5 mM 3AT. To further confirm the interaction between LaeA and transcription factors, the transformants were tested on the SD/-Trp/-Leu/-His media containing 10 mg/ml X-α-gal. And the transforms were spotted in the SD/-Trp/-Leu/-His media containing 2.5 mM 3AT.

Accession Numbers
The Whole Genome Shotgun projects were deposited in DDBJ/EMBL/GenBank under the accession number AGIH00000000. The raw data of expression profiling were deposited in NCBI’s Gene Expression Omnibus (GEO) database under the accession number GSE71287.

RESULTS
All the Mutants with the Deletion of laeA Showed Less Conidiation
The fungal strains used in this study are listed in Table 1. Equivalent fresh spores of the WT and mutants were inoculated on PDA plates or on 1 × Vogel’s salts (Vogel, 1956) with 2% (w/v) glucose, 2% lactose, 2% glycerol, or 2% cellulose as the sole carbon source for 4 days at 30°C (Figure 1A). Then, the colony morphology and the conidiation were observed. The
colonies of ΔlaeA, OEclrBΔlaeA, and OEExnRΔlaeA appeared greenish-brown compared with the dark-green colonies observed in WT, OEclrB, and OEExnR. ΔcreAΔlaeA even formed a white colony (Figure 1A). Conidiation was quantified by plating conidia on top of agar layers, and conidiospore production was subsequently analyzed. The conidiation levels in all laeA deletion mutants were dramatically reduced; the conidiation of ΔlaeA, OEclrBΔlaeA, and OEExnRΔlaeA was ~4.1, ~13.2, and ~9.0% of WT, respectively (Figure 1B). No conidium was observed in ΔcreAΔlaeA, consistent with previous report (Zhang et al., 2016). This observation indicated that LaeA positively regulated asexual development.

**All the Mutants with the Deletion of laeA Showed Reduced Cellulase Formation**

When the strains were grown on cellulose agar (1 × Vogel’s salts plus with 2% cellulose as the sole carbon), the cellulolytic halo of all the four mutants ΔlaeA, OEclrBΔlaeA, OEExnRΔlaeA, and ΔcreAΔlaeA decreased remarkably compared with their parent strains WT, OEclrB, OEExnR, and ΔcreA, respectively. No cellulolytic halo was observed around the ΔlaeA colony, whereas a clear cellulolytic halo was found around the WT colony (Figure 2). ClrB and XlnR were transcription activators for the cellulase gene (Stricker et al., 2006; Coradetti et al., 2012); thus, it was expected their overexpression strain (OEclrB and OEExnR) showed increased cellulolytic ability with a more pronounced cellulolytic halo than that of the WT; the result was consistent with the previous report described by Li et al. (2015). When laeA was deleted, however, the overexpression of clrB (OEclrBΔlaeA) or xlnR (OEExnRΔlaeA) did not result in the formation of a cellulolytic halo (Figure 2). CreA suppressed the expression of most cellulase and hemicellulase genes through carbon catabolite repression mechanism (Nakari-Setälä et al., 2009). As a result, the deletion of creA (ΔcreA) was observed, exhibiting a large and clear cellulolytic halo around its colony compared with that of the WT. The result was consistent with the previous report described by Liu et al. (2013a). However, when laeA was absent, the diameter of the cellulolytic halo around ΔcreAΔlaeA was reduced (Figure 2).

For the agar, pure cellulose was used as the sole carbon source. Complex carbon sources from plant materials were more efficient than pure cellulose in promoting the expression of lignocellulose-degrading enzymes. Therefore, we cultivated the WT and the mutants in a submerged medium with wheat bran and microcrystalline cellulose. Then, the levels of FPA compared with WT. However, in the absence of LaeA, the FPA of all the four mutants ΔlaeA, OEclrBΔlaeA, OEExnRΔlaeA, and ΔcreAΔlaeA decreased remarkably compared with their
parent strains WT, OEclrB, OExlnR, and ΔcreA, respectively, after 2 days cultivation. On the third day, the FPA of WT, ΔlaeA, OEclrB, OExlnRΔlaeA, OExlnR, OExlnRΔlaeA, and ΔcreA, 1 µL of conidia solution was dropped onto the agar at a density of 10⁵ conidia ml⁻¹. For ΔlaeAΔcreA, 1 µL of hypochlorite fragment solution was dropped onto the agar because it cannot generate conidia.

The difference between the mutants and WT increased along with prolonged culture time; the FPA of WT, ΔlaeA, OEclrB, OExlnRΔlaeA, OExlnR, OExlnRΔlaeA, and ΔcreA was only 32.1, 16.4, 41.4, and 58.9%, respectively, compared with that of 21.4, 56.8, and 73.2% WT, respectively. The growth kinetics of their parent strain WT ([Figure 3A], OEclrB ([Figure 3B]), OExlnR ([Figure 3C]), and ΔcreA ([Figure 3D]) strains. The FPA of OEclrBΔlaeA, OExlnRΔlaeA, and ΔcreAΔlaeA also decreased compared with that of 21.4, 56.8, and 73.2% WT, respectively.

The difference between the mutants and WT increased along with prolonged culture time; the FPA of WT, ΔlaeA, OEclrBΔlaeA, OExlnRΔlaeA, and ΔcreAΔlaeA was only 5.0, 8.1%, 4.7%, and 8.4%, respectively, compared with that of WT on the fifth day ([Figure 3]).

Then, the supernatants from the WT and the mutants were profiled by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) ([Figure S2]). Equal volumes of supernatants were loaded. Significantly less protein bands were detected in ΔlaeA, OEclrBΔlaeA, OExlnRΔlaeA, and ΔcreAΔlaeA than in WT, especially in the range of 40–116 kDa. Previous reports have shown that this range is an area for aggregation of glycoside hydrolases; in particular, most glucoamylases, cellulbiohydrolases, endoglucanases, and β-glucosidases were located in the area (Liu et al., 2013b).

Were the changes in cellulase synthesis in the mutants caused by their different biomass levels? The growth kinetics of WT and all the mutants were examined ([Figure S3]). Except that ΔcreA and ΔcreAΔlaeA exhibited lower biomass formation than WT, the similar growth kinetic curve of biomass level was observed among WT, ΔlaeA, OEclrB, OExlnR, OEclrBΔlaeA, and OExlnRΔlaeA, showing that the reduced cellulolytic activities in ΔlaeA, OEclrBΔlaeA, and OExlnRΔlaeA or increased cellulolytic enzyme activities in OEclrB and OExlnR did not correlate with the biomass level. The introduction of a wild type copy of laeA at the laeA locus (RlaeA) restored the growth defects and the cellulolytic enzyme synthesis defects of the ΔlaeA mutant ([Figure S4]). No evident difference was observed in the conidiation and cellulolytic enzyme synthesis in the mutant of overexpressed laeA (OElaeA) in P. oxalicum (Figure S5).

LaeA Extensively Affected Glycoside Hydrolase Gene Expression, Especially in Later Phases of Prolonged Batch Cultures

We noticed that the deletion of laeA resulted in a massive reduction of FPA, especially in prolonged batch cultures ([Figure 3]). To obtain a global view of the effect of laeA deletion on glycoside hydrolase expression, the expression profiles of WT and ΔlaeA were assayed. Glycoside hydrolases were characteristically produced after the shift from exponential growth to stationary phase. Therefore, two time points for gene expression profiling were selected. In particular, “24 h time point” indicates the exponential growth phase (earlier phases of cultures), whereas “60 h time point” indicates a stationary phase and exhibits a cellulase formation peak (later phases of prolonged batch cultures). WT and ΔlaeA were cultivated in cellulose and wheat bran medium, which is an optimum medium for cellulolytic enzyme formation. The mRNA from WT and ΔlaeA at the cultivation time of 24 h and 60 h, respectively, was collected and was subjected to high-throughput Illumina sequencing. More than 3 million tags were obtained for each sample. The copy number of unambiguous tags (tags mapped to one single gene) for each gene was normalized to the number of transcripts per million (TPM) clean tags. A genome-wide expression profiling analysis revealed the extensive expression of the entire P. oxalicum genome. Of the 10021 protein-coding genes in the genome database, 8192 (81.7%), 8292 (82.7%), 8351 (83.3%), and 8229 (82.1%) genes were expressed in WT-24 h, ΔlaeA-24 h, WT-60 h, and ΔlaeA-60 h, respectively. Genes of significantly differential expression levels were identified through a significance test with combined thresholds (FDR ≤ 0.001 and fold change ≥ 2). Blast2GO was used for function enrichment analysis of gene sets with a threshold of FDR ≤ 0.05.

The ΔlaeA mutant was compared with WT at 24 h, showing that the expression levels of 1657 genes exhibited significant differences (2-fold or greater, FDR < 0.001) between ΔlaeA and WT. Of these, 719 were regulated 4-fold or greater (FDR < 0.001), with 399 genes downregulated and 320 genes upregulated. GO enrichment analysis revealed that downregulated genes (4-fold or greater, FDR < 0.05) were mainly involved in heme binding, monooxygenase activity, catalytic activity, hydrolase activity, acting on glycosyl bonds, and binding of ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process ([Figure S6A]). The upregulated genes were mainly involved in substrate-specific transmembrane transporter activity ([Figure S6B]). The downregulated and upregulated genes (GO category: molecular function), along with their predicted functions, are listed in Tables S2, S3.

For the ΔlaeA mutant compared with WT at 60 h, the expression levels of 2488 genes exhibited significant differences (2-fold or greater, FDR < 0.001) between ΔlaeA and WT. Of these, 1145 were regulated 4-fold or greater (FDR < 0.001),...
FIGURE 3 | Cellulolytic activity assay of WT and various mutants. The strains were cultivated in liquid Vogel’s salts medium supplemented with 1% wheat bran and 1% microcrystalline cellulose as carbon resources to induce cellulase gene expression. The strains were cultivated at 30°C for 5 days. FPA, a measure of the combined activities of both endo- and exo-type cellulases, was determined by hydrolyzing a strip of filter paper (Whatman #1, 50 mg, 1 cm × 6 cm) for 1 h at 50°C.

(A) FPA of WT and ΔlaeA. (B) FPA of OEcloB and OEcloBΔlaeA. (C) FPA of OEinhR and OEinhRΔlaeA. (D) FPA of ΔcreA and ΔcreAΔlaeA. The values show the mean of three biological replicates, and the error bar indicates the standard deviation.

with 703 downregulated genes and 442 upregulated genes. GO enrichment analysis revealed that the downregulated genes (4-fold or greater, FDR < 0.05) in ΔlaeA compared with that in WT were mainly involved in cellulose binding, aspartic-type endopeptidase activity, cellulase activity, monooxygenase activity, heme binding, electron carrier activity, and cellulose 1,4-beta-cellobiosidase activity (Figure S6A). GO enrichment analysis revealed no statistically significant results for the upregulated genes with this cutoff. The downregulated genes (GO category: molecular function) and their predicted functions are listed in Table S4.

Of the downregulated genes, many secondary metabolism hallmark genes and glycoside hydrolase genes were present. As expected, many genes involved in secondary metabolism were downregulated by laeA, which agreed with previous reports (Palmer and Keller, 2010). We focused on the glycoside hydrolase gene expression. Among the 129 predicted glycosidase hydrolase genes expressed in the four samples, the expression levels of 61 genes (P < 0.001) were significantly different between ΔlaeA and WT at both 24 h and 60 h (Figure 4A). Moreover, the gene expression of 40 (65.6%) glycosidase hydrolase was downregulated in ΔlaeA compared with that in WT at 24 h time point. Furthermore, the laeA exhibited a strong and wide-ranging effect on glycoside hydrolase transcriptional regulation observed at 60 h. The gene expression of 54 (88.5%) glycosidase hydrolase was significantly downregulated in ΔlaeA compared with that in WT (Figure 4A).

When P. oxalicum was cultivated on cellulose and wheat-bran-containing media, the secretome data revealed that the top 10 extracellular proteins were glycoside hydrolases, including Amy15A (PDE_09417, Genbank No. EPS34453.1), Amy13A (PDE_01201, Genbank No. EPS26265.1), Cel7A/CBH1 (PDE_07945, Genbank No. EPS32984.1), Cel61A (PDE_05633, Genbank No. EPS30681.1), Chi18A (PDE_08122, Genbank No. EPS33160.1), Cel3A/BGL1 (PDE_02736, Genbank No. EPS329792.1), Xyn10A (PDE_08094, Genbank No. EPS33132.1), Cel7B/EGI (PDE_07929, Genbank No. EPS32968.1), Cel5B/EGII (PDE_09226, Genbank No. EPS34262.1), and Cel6A/CBHII (PDE_07124, Genbank No. EPS32164.1). Their products account for 28.9, 11.0, 9.6, 5.6, 4.9, 3.4, 3.3, 2.5, 1.9, and 1.5% of the total extracellular protein of P. oxalicum, respectively (Liu et al., 2013b). All top 10 extracellular protein-encoding genes [Figure 4A; numbers (1) to (10) indicate the amount of secretion from highest to the lowest] were detected with significant regulation in ΔlaeA compared with that in WT. laeA exhibited a strong effect on the expression at 60 h. The transcripts of amy15A, amy13A, cel7A/cbh1, cel61A, chi18A, cel3A/bgl1, xyn10A, cel7B/eg1, cel5B/eg2, and cel6A/cbh2 detected in ΔlaeA were only 43.2, 34.3, 13.8, 6.8, 0, 28.3, 9.5, 2.2, 32.5, and 30.5% of WT at 60 h, respectively (Figure 4B).
FIGURE 4 | Expression profiling analysis of glycoside hydrolase genes in ΔlaeA compared with WT. WT and ΔlaeA were cultivated in cellulose and wheat bran medium, which are optimum media for glycoside hydrolase formation. mRNA from WT and ΔlaeA at cultivation time of 24 and 60 h, respectively, was collected and subjected to high-throughput Illumina sequencing. (A) Expression analysis of 61 glycoside hydrolase genes in ΔlaeA compared with WT at 24 and 60 h. The color of each block represents the log2 (fold change) in gene expression. The blue numbers from (1) to (10) (from the highest secretion amount to the lowest) represent the top 10 extracellular proteins found in *P. oxalicum* secretome (Liu et al., 2013b). The blue dots indicate seven genes, the expression levels of which are upregulated at 60 h. The blue dot with a pink circle indicates a β-xylosidase-encoding gene *xyl3A*. (B) Expression levels of the top 10 extracellular protein-encoding genes. The numbers (1) to (10) correspond to the numbers in (A). The copy number of unambiguous tags (tags mapped to a single gene) for each gene was normalized to TPM (number of transcripts per million clean tags).
LaeA Not Only Modulates the Expression of Glycoside Hydrolase Genes But Also the Expression of Glycoside Hydrolase-Related Transcription Factor Genes

Then, we detected the expression patterns of five out of the above 10 genes in WT and seven mutants at different culture times. These genes include the prominent glucoamylase gene amy15A, cellbiohydrolase gene cel7A/chb1, endogluccanase gene cel7B/eg1, β-glucosidase gene cel3A/bgl1, and xylanase gene xyn10A. The strains were pre-cultured on glucose medium for 20 h, starved for 4 h under no carbon source conditions, and transferred to cellulose and wheat bran medium for further cultivation. The transcript levels of the five genes were quantified in cells grown from 12, 24, 36, 48, and 60 h (Figure 5). The expression levels of amy15A (Figure 5A), cel7A/chb1 (Figure 5B), cel7B/eg1 (Figure 5C), and xyn10A (Figure 5E) in OEcreB, OEXnr, and ΔcreA dramatically increased from 12 to 60 h. For example, amy15A expression in the OEcreB, OEXnr, and ΔcreA increased respectively by 167, 232, and 173% at the 36 h time point compared with WT. The expression level of cel7A/chb1 in the OEcreB, OEXnr, and ΔcreA increased respectively by 37, 56, and 23% compared with that of WT. However, the expression of amy15A, cel7A/chb1, cel7B/eg1, and xyn10A in ΔlaeA, OEcreBΔlaeA, OEXnrΔlaeA, and ΔlaeAΔcreA decreased remarkably, especially in the later phases of prolonged batch cultures. At the 48 h time point, amy15A expression in the ΔlaeA, OEcreBΔlaeA, OEXnrΔlaeA, and ΔcreAΔlaeA decreased to 8.93, 23.2, 17.3, and 46.4% of WT, respectively; cel7A/chb1 expression in the ΔlaeA, OEcreBΔlaeA, OEXnrΔlaeA, and ΔcreAΔlaeA decreased to 44.0, 58.2, 91.3, and 64.7% of WT, respectively (Figure 5).

As transcription factors creA, clrB, and xlnR have been identified to play essential roles in the expression of cellulase, and Lae1 was reported controlling transcriptional activator xyr1 gene expression in T. reesei (Seiboth et al., 2012), does LaeA affect creA, clrB, or xlnR expression? Then, the transcription levels of genes creA, clrB, and xlnR were quantified in WT and the mutants grown from 12, 24, 36, 48, and 60 h (Figure 6). The expression levels of creA was considerably increased in ΔlaeA compared with that in WT (Figure 6A), suggesting LaeA negative roles in regulating creA expression. The expression of clrB or xlnR was higher than that of in WT before 24 h-point culture time, but lower than that of in WT along with prolonged culture time (Figures 6B,C). It was expected that overexpression of clrB in WT (OEclrB) upregulate clrB expression remarkably; and overexpression of xlnR in WT (OE xlnR) upregulate xlnR expression remarkably. However, when LaeA was absent, the clrB expression in OEcreBΔlaeA or xlnR expression in OEXnrΔlaeA decreased. The result was unexpected, because the over-expression of clrB or xlnR was driven from the gpdA promoter, but not the native promoters. Meanwhile, the relatively high expression of clrB or xlnR in OEcreBΔlaeA or OEXnrΔlaeA (compared with WT) cannot rescue the impairment of cellulolytic enzyme gene expression by the deletion of laeA (Figure 3A), suggesting LaeA was necessary in the expression of cellulolytic enzyme gene activated by ClrB and XlnR. To investigate why laeA overexpression did not affect the cellulolytic enzyme production, the expression level of the key transcription factor gene (clrB, xlnR, and creA) in the OELaeA was compared with that of WT (Figure 5D). In contrast with that the expression of transcription repressor gene creA increased remarkably in ΔlaeA, the expression levels of creA in OELaeA was identical with that of in WT. The expression of transcription activator gene xlnR was also identical with that of in WT.

FIGURE 5 | Expression levels of prominent cellulolytic enzyme genes determined using real-time quantitative PCR (qPCR). (A) Glucoamylase gene amy15A. (B) Cellbiohydrolase gene cel7A/chb1. (C) Endoglucanase gene cel7B/eg1. (D) β-glucosidase gene cel3A/bgl1. (E) Xylanase gene xyn10A.
Interestingly, the expression of transcription activator gene \( \text{clrB} \) had a small increase (about 2-folds) compared with that of WT. Obviously, this insignificant change in \( \text{clrB} \) expression had no effect on cellulolytic enzyme production (Figure S5C). The results showed, normal expression of \( \text{laeA} \) was enough to trigger proper expression of the key transcription factor gene; excessive \( \text{laeA} \) expression was not needed.

**Combination of \( \text{laeA} \) Deletion and \( \text{xlnR} \) Overexpression Activated Extracellular \( \beta \)-xylosidase Synthesis**

Of the 61 glycosidase hydrolase genes, only seven genes were upregulated at 60 h (Figure 5A, blue dots). Of the 7 genes, only PDE_00049 (a \( \beta \)-xylosidase-encoding gene) product was detected in \( P. \) oxalicum secretome (Liu et al., 2013a). Thus, the extracellular \( \beta \)-xylosidase activity and PDE_00049 (\( \text{xyl3A} \)) expression levels were assayed. Although, most cellulase and hemicellulase formation activities were reduced by the deletion of \( \text{laeA} \), this deletion activated \( \beta \)-xylosidase synthesis, especially when \( \text{laeA} \) deletion and \( \text{xlnR} \) overexpression was combined. At 48 h, the extracellular \( \beta \)-xylosidase activity in OExlnR\( \Delta \text{laeA} \) was 1.5 IU/mL, which was 5.8-fold greater than that in WT (Figure 7A). The results of qPCR for \( \text{xyl3A} \) showed that the transcription level of OExlnR\( \Delta \text{laeA} \) was 28.5-fold greater than that of WT at 48 h. This result suggests that \( \beta \)-xylosidase gene expression was regulated at the transcriptional level (Figure 7B).

**No Interaction Was Found between \( \text{laeA} \) and Transcription Activators In vitro**

Originally, we thought that transcription activators (\( \text{ClrB} \) and \( \text{XlnR} \)) might recruit \( \text{laeA} \) playing a common regulating function, as their co-existence was required for proper cellulolytic enzyme gene expression. So, yeast two-hybrid assay was done to determine if \( \text{laeA} \) had physical interaction with transcription factors. Yeast two-hybrid experiments did not reveal any physically interaction between \( \text{laeA} \) and \( \text{ClrB} \) (or \( \text{XlnR} \)). On the SD/-Trp/-Leu media with X-\( \alpha \)-gal, the strain with \( \text{laeA} \) and \( \text{clrB} \) (or \( \text{xlnR} \)) was not colored (Figure S7A). And They did not grow on the SD/-Trp/-Leu/-His media with 2.5 mM 3AT (Figure S7B). No interaction was observed between \( \text{laeA} \) and \( \text{CreA} \) either.

**DISCUSSION**

\( \text{laeA} \) is a highly conserved protein in filamentous fungi. Studies on \( \text{LaA} \) orthologs have established significant diversity in its impact on fungal secondary metabolism and development and have been well documented in numerous fungi species (Palmer and Keller, 2010; Bayram and Braus, 2012). However, only two papers have so far reported that \( \text{laeA} \) also participates in the expression of cellulolytic enzyme-encoding genes. \( \text{laeA} \) is required to activate the expression of cellulase and hemicellulase gene and promote the expression of transcriptional activator \( \text{Xyr1} \) in \( T. \) reesei (Seiboth et al., 2012). Moreover, the deletion of \( \text{laeA} \) downregulated the glucoamylase gene expression in \( Aspergillus \) \( \text{flavus} \) (Duran et al., 2014).

Some reports suggested asexual sporulation may be associated with glycoside hydrolase gene transcription based on the finding that the deletion of \( \text{lae1} \) impaired asexual sporulation and glycoside hydrolase gene transcription simultaneously; moreover, asexual sporulation triggers the expression of massive glycoside hydrolase genes (Metz et al., 2011; Seiboth et al., 2012). In the present study, we also noticed the reduced conidiation in all mutants with the deletion of \( \text{laeA} \). However, sporulation...
and cellulase gene expression were not always coherent and sometimes occurred oppositely. Our research also indicated that the P. oxaliciyum ΔcreA mutant with reduced conidia can form more cellulases, suggesting that the two processes are linked but not dependent on each other.

Our data showed that the deletion of laeA resulted in a massive reduction of glycoside hydrolase production, especially in prolonged batch cultures. The expression profiling results also showed that LaeA exhibited a stronger and wider effect on glycoside hydrolase transcriptional regulation during the stationary phase (later phases of prolonged batch cultures) than during the exponential growth phase (earlier phases of cultures). Indeed, the growth-rate dependency of LaeA function in P. chrysogenum has been reported. A significant downregulation and several morphological changes in the penicillin-biosynthesizing genes were observed in prolonged batch cultures of two different P. chrysogenum strains (Kosalková et al., 2009; Butchko et al., 2012). When the laeA deletion mutant was grown in chemostat cultures, only moderate transcriptional response and penicillin production was observed even at glucose-limited conditions (Veiga et al., 2012). Interestingly, Fekete et al. observed that the growth-rate dependence of LaeA function in T. reesei was in the opposite direction. The loss of function of LAE1 was more dominant at high growth rates (Fekete et al., 2014). The Velvet protein complex formed by LaeA, VeA, and VeB (Bayram et al., 2008) was assumed to play considerable roles in this process. Moreover, LAE1 was required for the expression of vel1 (ortholog of A. nidulans VeA) (Karimi-Aghcheh et al., 2013). The deletion of T. reesei lae1 or vel1 impaired the expression of cellulases and cellulase regulator XyR1 on lactose, which suggested that cellulase expression might be regulated by the Velvet complex (Seiboth et al., 2012; Karimi-Aghcheh et al., 2014). Similarly, Hoff reported that the role of Velvet complex in P. chrysogenum was more pronounced after prolonged incubation (Hoff et al., 2010). So, as direct protein interaction has been found between P. oxaliciyum LaeA and VeA (self-communication), it was assumed the Velvet complex in P. oxaliciyum played important roles in this process.

LaeA has been assumed to modulate transcription through histone methyltransferase functions (Reyes-Dominguez et al., 2008). However, the precise molecular function of LaeA remains unknown because its direct methyl-accepting substrate has not been identified (Patananan et al., 2013). The study on T. reesei showed that the LAE1-regulated expression of genes did not correspond to the changes in histone methylation; no enrichment with any of the histone marks was tested at the cellulolytic gene region or xyr1 gene locus (Seiboth et al., 2012). The precise function of LaeA toward the formation of glycoside hydrolase remained to be identified. According out research, a new found was that creA expression was upregulated remarkably by the deletion of laeA, whereas the expression clrB or xlnR was repressed by the deletion of laeA especially after 24 h culture time. xyr1/xlnR gene was activated with most of the cellulolytic enzyme genes in the presence of cellulose (Portnoy et al., 2011), whereas its repression was mediated by CreA (de Vries et al., 1999; Tamayo et al., 2008). The upregulation of creA expression by the deletion of laeA may have caused the repression of xlnR expression in the ΔlaeA mutant. Originally, we thought the repressed expression of clrB and xlnR resulted in the downregulation of cellulolytic enzyme gene expression in ΔlaeA, because cellulolytic enzyme gene repression also occurred in later phases of prolonged batch cultures, consistent with the tendency of clrB and xlnR. Transcription activators (ClrB and XlnR) might recruit LaeA playing a common regulating function, as their co-existence was required for proper cellulolytic enzyme gene expression. However, no physical interaction was observed between LaeA and ClrB, or XlnR, showing that LaeA and transcription activators have genetic, but physically separate functions in cellulolytic enzyme gene expression. Furthermore, the overexpression of clrB or xlnR could not rescue the impairment of cellulase gene expression caused by lack of LaeA, suggesting clrB or xlnR repression caused by laeA deletion was not the main reason that resulted in cellulolytic enzyme gene repression. Transcription factors ClrB, XlnR, and CreA have been identified as critical dose-dependent regulators; dose effect of transcriptional abundance is important for the high expression for cellulases (Li et al., 2015). So, we assumed that transcription repressor gene creA activation together with transcription activator gene clrB and xlnR repression, played synergistic and dose-controlled regulation mechanisms, which was the most important reason for cellulolytic enzyme gene repression in ΔlaeA.

The positive effect of LaeA on the gene expression of most cellulolytic enzymes has been demonstrated clearly. However, the significant increase of xyllosidase gene expression and xyllosidase synthesis was unexpectedly observed in OExlnRAlaeA. Among the xylanolytic enzymes, endo-xylanases and β-xidosides are important because they complete the breakdown of hemicellulose fraction (Kulkarni et al., 1999). In our study, the expression of the prominent endo-xylanase gene (xyn10A) and prominent β-xidoside gene (xyl3A) showed distinct patterns from one another in the absence of LaeA. Although, nine xyllosidase-encoding genes were predicted in the P. oxaliciyum genome (Liu et al., 2013a), PDE_00049 (xyl3A) product was the only extracellular β-xidoside found in the secretome (Liu et al., 2013b), suggesting that PDE_00049 (xyl3A) is the most prominent and important extracellular β-xidoside gene in P. oxaliciyum. Xyl3A belongs to glycosyl hydrolase family 3, which includes the extensively studied extracellular β-xidoside. The deduced amino acid sequence of P. oxaliciyum xyl3A exhibited high similarities to that of A. nidulans XlnD (67%) (Pérez-González et al., 1998), A. niger XlnD (63%) (van Peij et al., 1997), and Talaromyces emersonii Bxl1 (68%) (Reen et al., 2003). These β-xidosides have been verified as potentially efficient candidates to facilitate the hydrolysis of hemicellulose applications in industrial processes.

Out of the various fungi studied, Xyr1/XlnR has been extensively reported to regulate the expression of xylanolytic enzymes, such as xylanase, β-xidoside, and D-xyllose reductase (van Peij et al., 1998; Stricker et al., 2006). The constitutive expression of xyr1/xlnR can cause elevated mRNA levels of xylanase gene and β-xidoside gene bxlII in T. reesei and A. oryzae (Mach-Aigner et al., 2008; Noguchi et al., 2009). Thus, the overexpression of xlnR was expected to upregulate the expression of xyl3A. However, the deletion of laeA unexpectedly did not downregulate the xyl3A expression and β-xidoside secretion.
Indeed, Duran et al. reported that the Velvet protein complex regulated various types of glycoside hydrolase expression in different patterns; the amount of glucoamylase protein was reduced, whereas the production of alpha-amylase was increased in the veA mutant (Duran et al., 2014), suggesting the flexibility of LaeA in regulating glycoside hydrolase gene expression. According to our previous research, ten physically linked regions of coregulated genes were found in laeA deletion strain, which were distributed on 5 chromosomes (Zhang et al., 2016). Among them, 8 regions were silenced and 2 regions were activated. The gene expression in these regions showed position effect. As position effect is shown to regulate expression of transgenes in A. nidulans (Palmer et al., 2010) due to insertion into different regions of a genome, overexpression of β-xilosidase gene by inserting it into one of the activated regions in ΔlaeA might be an effective way to improve β-xilosidase synthesis. Furthermore, the OEExnRΔlaeA mutant unexpectedly induced a remarkable increase in the expression of xyl3A and extracellular β-xilosidase synthesis. The robust induction of β-xilosidase expression and increased β-xilosidase activity in OEExnRΔlaeA mutant was remarkably greater than that in each solo gene mutant, confirming the cumulative effects of both genes. This study confirmed that the regulation of xyl3A by LaeA was different from the gene regulation pathways of most cellulase or hemicellulase genes. The OEExnRΔlaeA mutant has potential applications in the production of extracellular β-xilosidase.

Indeed, the OEExnRΔlaeA mutant has been used as the parent strain to overexpress β-xilosidase, which improved by over 20-fold compared with that of the WT (self-communication). Thus, the production of highly efficient enzymes that assist in the conversion of plant biomass is facilitated.

AUTHOR CONTRIBUTIONS

YL, XZheng, XZhang, LB, and YZ performed the experiments. YQs, and JZ analyzed the data and revised the manuscript. YQin designed the work and drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02091/full#supplementary-material

Figure S1 | Strategy and results for verification of OElaeA, OEcrBΔlaeA and OEExnRΔlaeA. (A) PCR verification. Lane 1 and Lane 2, the upstream fragments amplified by ptrA-F/YlaeA-2 and downstream fragments amplified by YlaeA-1/ptrA-R to verification of OEExnRΔlaeA, lane 3 and lane 4, the upstream fragments amplified by ptrA-F/YlaeA-2 and downstream fragments amplified by YlaeA-1/ptrA-R to verification of OEExnRΔlaeA; lane 5 and lane 6, the upstream fragments amplified by Ynhy-gpdA-F/Ynhy-gpdA-R to verification of OElaeA; lane 7 and lane 8, the downstream fragments amplified by YgpdA-F/YgpdA-ΔlaeA-ΔR to verification of OEExnRΔlaeA. (B) Southern blot verification of OElaeA, OEcrBΔlaeA, and OEExnRΔlaeA.

Figure S2 | Growth kinetics of WT and the mutants.

Figure S3 | SDS–PAGE of extracellular protein for WT and the mutants.

Figure S4 | Characteristics of laeA recombined strain (RlaeA) compared with WT and ΔlaeA. (A) Colony morphology. (B) SDS–PAGE of extracellular protein. (C) FPA assay.

Figure S5 | Characteristics of laeA overexpression strain (OElaeA) compared with WT. (A) Colony morphology. (B) SDS–PAGE of extracellular protein. (C) FPA assay. (D) Expression levels of creA, clrB, and xlnR determined using real-time quantitative PCR (qPCR).

Figure S6 | GO enrichment analysis between ΔlaeA and WT at 24 and 60 h.

Figure S7 | Interaction analysis between LaeA and transcription factors (CreA, ClrB and XlnR) using yeast two-hybrid assay. The open reading frames (ORFs) of the transcription factors CreA, ClrB and XlnR were amplified using cDNA from P. oxalicum WT as the templates and cloned into plasmid pGBK7T7 which containing GAL4 activation domain (AD). The open reading frames of LaeA were cloned into plasmid pGBK7 containing GAL4 DNA binding domain (DNA-BD). The plasmids with AD and BD (AD-creA, AD-clrB, AD-xlnR, and BD-laeA) were co-transformed into yeast AH109. (A) The transformants were tested on double dropout medium SD/-Trp/-Leu/ containing 10 mg/ml X-a-gal, where the positive control colony turned blue and the negative control colony keep white. The results demonstrated no direct interaction between LaeA and CreA, OrlB, or XlnR. (B) The transformants were tested on triple dropout medium SD/-Trp/-Leu/-His media with 2.5 mM 3AT, where the positive control and fusion proteins with interaction could grow. The results demonstrated no direct interaction between LaeA and CreA, ClrB, or XlnR. The yeast liquid was diluted multiples into gradient (10^{-1}–10^{-6}).

Table S1 | Primers used in construction of mutants and real-time quantitative PCR.

Table S2 | List of downregulated genes (≥4-fold, FDR < 0.05) in ΔlaeA when cultivated for 24 h compared with WT with significantly enriched GO terms (GO category: molecular function).

Table S3 | List of upregulated genes (≥4-fold, FDR < 0.05) in ΔlaeA when cultivated for 24 h compared with WT with significantly enriched GO terms (GO category: molecular function).

Table S4 | List of downregulated genes (≥4-fold, FDR < 0.05) in ΔlaeA when cultivated for 60 h compared with WT with significantly enriched GO terms (GO category: molecular function).
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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