How an essential Zn2Cys6 transcription factor PoxCxrA regulates cellulase gene expression in ascomycete fungi?

Lu-Sheng Liao†, Cheng-Xi Li†, Feng-Fei Zhang†, Yu-Si Yan, Xue-Mei Luo, Shuai Zhao* and Jia-Xun Feng*

Abstract

Background: Soil ascomycete fungi produce plant-biomass-degrading enzymes to facilitate nutrient and energy uptake in response to exogenous stress. This is controlled by a complex signal network, but the regulatory mechanisms are poorly understood. An essential Zn2Cys6 transcription factor (TF) PoxCxrA was identified to be required for cellulase and xylanase production in *Penicillium oxalicum*. The genome-wide regulon and DNA binding sequences of PoxCxrA were further identified through RNA-Sequencing, DNase I footprinting experiments and in vitro electrophoretic mobility shift assays. Moreover, a minimal DNA-binding domain in PoxCxrA was recognised.

Results: A PoxCxrA regulon of 1970 members was identified in *P. oxalicum*, and it was displayed that PoxCxrA regulated the expression of genes encoding major plant cell wall-degrading enzymes, as well as important cellodextrin and/or glucose transporters. Interestingly, PoxCxrA positively regulated the expression of a known important TF Pox-ClrB. DNase I footprinting experiments and in vitro electrophoretic mobility shift assays further revealed that PoxCxrA directly bound the promoter regions of *PoxClrB* and a cellobiohydrolase gene *cbh1* (*POX05587/Cel7A*) at different nucleic acid sequences. Remarkably, PoxCxrA autoregulated its own *PoxCxrA* gene expression. Additionally, a minimal 42-amino-acid PoxCxrA DNA-binding domain was identified.

Conclusion: PoxCxrA could directly regulate the expression of cellulase genes and the regulatory gene *PoxClrB* via binding their promoters at different nucleic acid sequences. This work expands the diversity of DNA-binding motifs known to be recognised by Zn2Cys6 TFs, and demonstrates novel regulatory mechanisms of fungal cellulase gene expression.

Keywords: Zn2Cys6 transcription factor, PoxCxrA, DNA binding, Cellulase gene expression, *Penicillium oxalicum*
processes such as sporulation, autophagy, apoptosis or necrosis [4]. Fungal cells control CAZyme production by utilising complex nutrient-sensing pathways comprising numerous sensors and receptors such as kinases, transcription factors (TFs) and their targets [5]. However, the overall process remains poorly understood.

Penicillium oxalicum produces integrated cellulolytic enzymes that degrade insoluble cellulose, and displays a preference for particular carbon sources. When P. oxalicum grows in the presence of glucose, expression of cellulolytic enzyme-encoding genes is repressed via carbon catabolite repression (CCR). Among the TFs involved in CCR, the core zinc finger TF CreA/Cre-1 is the one best studied in filamentous fungi, including P. oxalicum. CreA directly or indirectly represses the expression of all major CAZyme genes and their regulatory genes that are involved in the degradation of plant cell walls in the presence of glucose [6, 7].

When cellulose is present as a sole carbon source, induction of cellulolytic genes in P. oxalicum is dependent on a few essential TFs including the Zn2Cys6 TFs PoxCxrA and PoxClrB. Individual deletion of PoxCxrA and PoxClrB results in almost no cellulase production by P. oxalicum [2, 8]. Clr2, a homolog of PoxClrB in Neurospora crassa, binds to a DNA sequence identical to that bound to by the Saccharomyces cerevisiae TF Gal4p (CGGN11CCG) [9]. Although PoxCxrA binds directly to the promoter regions of major cellulase and xylanase genes [8], the DNA element recognised by PoxCxrA remains unknown.

In the present study, we employed high-throughput sequencing of transcripts (RNA-seq) to analyse transcriptional levels of genes in P. oxalicum deletion mutant ΔPoxCxrA following exposure to Avicel in comparison with the parental strain ΔPoxKu70 to identify the regulon of PoxCxrA. Moreover, we identified two different DNA motifs recognised by PoxCxrA, as well as the minimal DNA-binding domain of PoxCxrA via in vitro DNase I footprinting and electrophoretic mobility shift assay (EMSA) experiments.

Results

**PoxCxrA positively regulates the expression of most genes encoding plant-cell-wall-degrading enzymes in P. oxalicum**

In previous work, an essential TF PoxCxrA was found to be required for cellulase production in P. oxalicum, when subjected to cellulose as a carbon source [2, 8]. However, its regulon is not yet fully understood. To comprehensively explore the regulatory roles of PoxCxrA in P. oxalicum, RNA-Seq was employed to analyse the transcriptomes of the P. oxalicum mutant strain ΔPoxCxrA and the parental strain ΔPoxKu70 cultured in medium containing Avicel as the sole carbon source for 24 h after a shift from glucose. A total of 21–23 million clean reads with a length of 100 bp were generated across all samples (Additional file 1: Table S1), > 90% of which were successfully mapped into the genome of P. oxalicum wild-type strain HP7-1 [2]. To evaluate the correlations among the three biological replicates for each strain, the Pearson’s correlation coefficient (r) was calculated. The resulting high r value (> 0.85; Additional file 2: Figure S1) suggests that the transcriptomic data were reliable and suitable for further analysis.

Comparative transcriptomic profiling identified 1970 DEGs in deletion mutant ΔPoxCxrA, compared with the parental strain ΔPoxKu70, according to the |log2 fold change| ≥ 1 and p value ≤ 0.05 thresholds (Additional file 3: Table S2), which were defined as the PoxCxrA regulon. The PoxCxrA regulon included 1010 genes down-regulated compared with ΔPoxKu70. Functional annotation based on Eukaryotic Orthologous Group (KOG) classification revealed that most of these DEGs were involved in primary and secondary metabolism (category E, amino acid transport and metabolism; category Q, secondary metabolite biosynthesis, transport and catabolism), and fell into the general function prediction only category (category R) (Fig. 1).

Expression of genes encoding three key module signal carriers (i.e. sugar transporters), TFs, and functional proteins (i.e. CAZymes) formed a complex signalling network, and this was investigated in detail. Among the 1970 DEGs, annotation revealed that 154 DEGs encoded CAZymes, including seven auxiliary activity families, six carbohydrate-binding module families, eight carbohydrate esterase families, 42 glycoside hydrolase families and 14 glycosyltransferase families, as well as two polysaccharide lyase families. Among them, 46 genes encoding putative plant cell wall-degrading enzymes (CWDEs) were detected. Importantly, most
key cellulase and xylanase genes in *P. oxalicum* were included in this DEG set, such as three cellobiohydrolase (CBH) genes *POX05587/Cel7A-2* (also called *cbh1*), *POX02490/Cel7A-1* and *POX04786/Cel6A*, three endo-β-1,4-glucanase (EG) genes *POX06147/Cel5A*, *POX02740* and *POX06983*, four β-glucosidase (BGL) genes *POX00968, POX03062, POX07963* and *POX08882*, five xylanase (Xyn) genes *POX00063/xyn10A, POX06783/xyn11A, POX06601, POX08484/Xyn11B* and *POX08990*, two lytic polysaccharide monoxygenase *POX02308/aa9A* and *POX08897* (Additional file 3: Table S2), which accounted for approximately 70% of cellulase and xylanase genes in the whole genome of *P. oxalicum* wild-type strain HP7-1 (Fig. 2).

Of these 154 CAZyme-encoding genes, 95 were downregulated (−7.0 < log2 fold change < −1.0) compared with the parental strain Δ*PoxKu70*. Interestingly, expression of all 17 cellulase and xylanase genes described

![Fig. 2](image-url)
above were down-regulated in the ΔPoxCxrA, except for two genes (POX00968 and POX02490/Cel7A-1) (Fig. 2).

**PoxCxrA regulates the expression of genes encoding cellobextrin and glucose transporters**

In complex signal transduction pathways, transporters/sensors play important roles, as demonstrated for cellobextrin transporters PoxCdtC and PoxCdtD in cellulase production in *P. oxalicum* [10]. Among the 1970 DEGs, 34 were annotated as sugar/inositol transporters (IPR003663) and/or major facilitators, or sugar transporter-like (IPR005828). Remarkably, two known genes (POX06051/PoxCdtC and POX05915/PoxCdtD) encoding cellobextrin transporters PoxCdtC and PoxCdtD, three genes (POX07576, POX07227 and POX08783) encoding *N. crassa* glucose transporter RCO3-like [11], and GLT1 and HGT-2 proteins [12], sharing 51–76% sequence identity, were included.

The regulon of *PoxCxrA* included 23 down-regulated genes encoding cellobextrin and glucose transporters (−5.4 < log2 fold change < −1.2). Among them, transcriptional levels of POX06051/PoxCdtC, POX05916/PoxCdtD and POX07576 were down-regulated in the ΔPoxCxrA compared with ΔPoxKu70, with a log2 fold change from −4.4 to −1.3 (Fig. 3 and Additional file 3: Table S2). In contrast, POX07227 and POX08783 transcript levels increased 4.5–3.1-fold in ΔPoxCxrA (Fig. 3).

![Fig. 3 Regulation of putative transporter genes by PoxCxrA. Values in heatmaps were calculated by log2 (Gene_FPKM in ΔPoxCxrA/Gene_FPKM in ΔPoxKu70). FPKM fragments per kilobase of exon per million fragments mapped](image-url)
**PoxCxrA regulates the expression of genes encoding TFs controlling cellulase gene expression**

Genes encoding putative TFs among the 1970 DEGs were explored, and 88 candidates were identified, mostly encoding zinc finger proteins such as Zn2Cys6, C2H2 and CCHC families. Among the 88 TFs, 14 were known to regulate cellulase production in filamentous fungi, including eight activators (POX00331/FlbC, POX01184, POX01960/PoxClrB, POX02484, POX04420/PoxCxrB, POX05726, POX08415/NsdD and POX08910) and six repressors (POX00864, POX04860/PDE_07199, POX06534/BrIa, POX06759, POX07254/CreA and POX08375/Ace1) [2, 8, 13–17]. Comparative transcriptomics indicated that 45 down-regulated genes encoding putative TFs (−9.6<log2 fold change < −1.0). Interestingly, deletion of PoxCxrA resulted in down-regulation of 11 known regulatory genes (POX00331/FlbC, POX01184, POX01960/PoxClrB, POX02484, POX04420/PoxCxrB, POX04860/PDE_07199, POX05726, POX06534/BrIa, POX06759, POX07254/CreA and POX08415/NsdD; −6.8<log2 fold change < −1.1), and up-regulation of known regulatory genes POX08910, POX00864 and POX08375/Ace1 (1.1<log2 fold change <1.3), compared with the ΔPoxKu70 transcriptome (Fig. 2 and Additional file 3: Table S2).

**PoxCxrA and PoxClrB dynamically regulates the expression of one another**

Interestingly, PoxCxrA regulated the expression of a key regulatory gene PoxClrB through RNA-Seq. To further elucidate this regulation, RT-qPCR was employed. When ΔPoxKu70 was exposed to Avicel, transcription of PoxCxrA was down-regulated to some extent (2.7- to 8.2-fold) after 4 h (p ≤ 0.05, Student’s t test) compared with that in ΔPoxKu70. In contrast, PoxCxrA expression increased by 1.5- to 2.1-fold in ΔPoxCxrA during the latter stages of cultivation (24–48 h after induction; Fig. 4a).

**Expression of both PoxCxrA and PoxClrB is induced by cellulose**

When *P. oxalicum* strain ΔPoxKu70 was transferred into medium containing Avicel (induced state), PoxCxrA and PoxClrB exhibited similar transcriptional levels to those without a carbon source (de-repressed state) during the early induction stage (0–12 h), but only the transcriptional level of PoxClrB was higher than that without a carbon source during later stage (48 h). The expression of both PoxCxrA and PoxClrB on Avicel were higher than that on glucose (repressed state). Surprisingly, PoxCxrA expression on Avicel increased by ~70% compared with its expression without a carbon source, but only at 24 h. Expression level of PoxClrB in ΔPoxKu70 was higher than those of PoxCxrA during all states (induced, repressed and de-repressed). PoxCxrA expression under Avicel induction increased before 24 h, but reduced after 24 h (Fig. 4b).

**PoxCxrA directly binds to the promoter regions of PoxClrB and PoxCxrA, and genes encoding celldextrin and glucose transporters**

To further confirm whether PoxCxrA directly or indirectly regulates PoxClrB expression, in vitro EMSA experiments were employed. The putative DNA-binding domain PoxCxrA17–150 was recombinantly expressed and purified by fusing with thioredoxin
(Trx), His and S-tags. A 300-bp DNA fragment from the promoter region of PoxClrB tagged with 6-carboxyfluorescein (6-FAM) was amplified using specific primers (Additional file 4: Table S3) and used as the probe for EMSA experiments. A DNA fragment from the promoter region of the β-tubulin gene POX05989 was used as a control. The results revealed that a complex was formed between PoxCxrA17–150 and the promoter region of PoxClrB, and its concentration increased with an increasing amount of fusion protein (1.0–2.0 µg). Bovine serum albumin (BSA) and Trx-His-S did not interact with the PoxClrB probe, and there was no interaction between PoxCxrA17–150 and the control POX05989 promoter region. Competitive EMSA experiments revealed that the concentration of the complex decreased gradually with an increasing amount of protein-binding DNA fragment without 6-FAM (Fig. 5), suggesting that PoxCxrA17–150 specifically bound the promoter region of PoxClrB.

Each EMSA experiment comprised different DNA-binding domains (PoxCxrA17–150, PoxClrB1–120 or both; 0–2 µg) and 50 ng of the target probe labelled with 6-FAM. Probes without 6-FAM, and the β-tubulin gene POX05989, were used as competitive probes and negative controls, respectively. The fusion protein Trx–His–S purified from Escherichia coli cells harbouring the empty plasmid pET32a (+), and BSA alone, were used as protein controls.

The RT-qPCR data described above revealed that PoxClrB negatively regulated PoxCxrA expression during the latter stages of induction, but the regulatory mode was unclear. In vitro binding experiments showed that the putative DNA-binding domain of PoxClrB1–120 was unable to bind to the promoter region of the PoxCxrA gene (Fig. 5), indicating that PoxClrB indirectly regulates transcription of PoxCxrA in P. oxalicum. EMSA experiments also showed that PoxCxrA17–150 bound to the promoter region of its own gene, but PoxClrB1–120 did not, suggesting the autoregulation of PoxCxrA. When a mixture of PoxCxrA17–150 and PoxClrB1–120 was treated with probe POX05587/Cel7A-2, a band representing a larger protein–DNA complex than that formed by either PoxCxrA17–150 or PoxClrB1–120 individually was observed (Fig. 5), indicating that the binding motifs recognised by PoxCxrA17–150 and PoxClrB1–120 are distinct.

Intriguingly, genes encoding cellobextrin and glucose transporters were included in the PoxCxrA regulon. EMSA experiments revealed that PoxCxrA17–150 could bind to the promoter regions of genes encoding cellobextrin transporters PoxCdtC and PoxCdtD, and putative glucose transporters POX07576/RCO-3, POX08783/HGT-2, POX07209 and POX04369 (Fig. 6).

Different PoxCxrA-binding motifs are present in the promoter regions of target genes POX05587/Cel7A-2 and PoxClrB

Based on the above results and those of previous work [8], PoxCxrA appears to regulate the expression of cbh gene POX05587/Cel7A-2 and TF gene PoxClrB by directly binding to their promoters. In vitro EMSA and DNase I footprinting experiments were, therefore, performed to identify protein-binding motifs (PBMs) in the promoters of the target genes. An initial DNase I footprinting experiment was performed using a 100-bp DNA fragment corresponding to the upstream flanking sequence (starting from the transcription initiation ATG codon) labelled with 6-FAM at the 3′-terminus to identify the PBM of PoxCxrA17–150 in the promoter region of POX05587/Cel7A-2. The results revealed that two putative PBMs (PBM1, 5′-ATCGA-TCCTCAGA-3′; PBM2, 5′-TCATCTCCTCCCA-3′) were protected by PoxCxrA17–150 to various
Fig. 6 EMSA analysis of the interactions between the DNA-binding domain PoxCxrA17–150 and the promoter regions of genes encoding putative sugar transporters. Each EMSA experiment comprised PoxCxrA17–150 (0–2 µg) and 50 ng of target probes labelled with 6-carboxyfluorescein (6-FAM). Probes without 6-FAM, and the promoter of β-tubulin gene POX05989, were used as competitive probes and negative control, respectively. The fusion protein Trx–His–S purified from E. coli cells harbouring the empty plasmid pET32a (+), and BSA alone, were used as protein controls.

Fig. 7 The PoxCxrA17–150-binding sequence in the promoter region of cellobiohydrolase gene POX05587/Cel7A-2. a DNase I footprinting experiment results. b EMSA confirmation of PBM_POX05587. c Schematic diagram of PBM_POX05587 truncation. d Key nucleotides in PBM_POX05587 confirmed via EMSA experiments. Each EMSA experiment comprised 2 µg of PoxCxrA17–150 and 50 ng of truncated PBM_POX05587 as probe labelled with 6-carboxyfluorescein. The promoter of β-tubulin gene POX05989 was used as a probe control. The fusion protein Trx–His–S purified from E. coli cells harbouring the empty plasmid pET32a (+), and BSA alone, were used as protein controls. '-' and '+' represent EMSA experiments without and with DBD PoxCxrA17–150, respectively.
degrees (Fig. 7a). Further EMSA experiments confirmed that PoxCxrA\textsubscript{17–150} bound to probes containing PBM1 or PBM1 plus PBM2, but not PBM2 (Fig. 7b), suggesting that PBM1 contains the binding motif of PoxCxrA in the POX05587/Cel7A-2 promoter region (PBM\textsubscript{POX05587}).

Meanwhile, a series of PoxClrB probes with different lengths (PoxClrB\textsubscript{−375}, PoxClrB\textsubscript{−355}, PoxClrB\textsubscript{−330}, PoxClrB\textsubscript{−310}, PoxClrB\textsubscript{−295}, PoxClrB\textsubscript{−284} and PoxClrB\textsubscript{−270} labelled with 6-FAM at the 5′-terminus) were amplified using specific primer pairs (Additional file 4: Table S3; Fig. 8a). EMSA experimental results revealed protein–DNA complexes between PoxCxrA\textsubscript{17–150} and PoxClrB\textsubscript{−375}, PoxClrB\textsubscript{−355}, PoxClrB\textsubscript{−330}, PoxClrB\textsubscript{−310} and PoxClrB\textsubscript{−295}, but not with the other probes or negative controls (Fig. 8b), suggesting that PoxCxrA binds the PBM\textsubscript{PoxClrB} in the promoter region of PoxClrB from positions 285–295 (5′-GCT GAG TCCTT-3′) (Fig. 8c).

Compared with PBM\textsubscript{POX05587}, three conserved sites were identified (i.e. GCTGAGTCCTT or ATCAGATCCTCAAGA).

Subsequently, to characterise the key nucleotides in PBM\textsubscript{PoxClrB} and PBM\textsubscript{POX05587}, we generated a series of truncated PBM\textsubscript{PoxClrB} and PBM\textsubscript{POX05587} fragments as EMSA probes (Figs. 7c, 8c). The results revealed that protein–DNA complexes were formed between PoxCxrA\textsubscript{17–150} and PBM\textsubscript{POX05587−3}, PBM\textsubscript{POX05587−6}, PBM\textsubscript{POX05587−3′}, PBM\textsubscript{POX05587−6′}, PBM\textsubscript{PoxClrB−3′}, PBM\textsubscript{PoxClrB−6′} and PBM\textsubscript{PoxClrB−9′}, and the concentrations of binding bands gradually decreased with the truncation of probe length (Figs. 7d, 8d). These results showed that the core nucleotides in PBM\textsubscript{POX05587} and PBM\textsubscript{PoxClrB} were TCCT and GC, respectively, whereas their flanking sequences were also required for PoxCxrA binding.

Identification of a minimal DNA-binding domain of PoxCxrA

The putative DNA-binding domain (DBD) of PoxCxrA used above (residues 17–150) was serially truncated from the C-terminus to identify a minimal functional DBD. To facilitate the purification of the expressed recombinant protein in E. coli, we first fixed at 11th amino acid of N-terminus. Recombinant proteins PoxCxrA\textsubscript{11–150}, PoxCxrA\textsubscript{11–114}, PoxCxrA\textsubscript{11–87}, PoxCxrA\textsubscript{11–58} and PoxCxrA\textsubscript{11–31} were produced in E.

![Fig. 8](image-url)
coli cells and purified (Fig. 9a, b), and in vitro EMSA experiments were carried out to investigate their ability to bind a 6-FAM-labelled 300-bp DNA fragment of the POX05587/Cel7A-2 promoter region comprising the PoxCxrA binding site as EMSA probe. The results showed that each truncated protein bound the probe to form a protein–DNA complex that retarded electrophoretic mobility in gels, except for PoxCxrA11–31. The concentration of the protein–DNA complexes gradually increased with increasing protein loading (1.0–3.0 µg). Competitive EMSA experiments were simultaneously performed, and the results indicated that the amount of complex tended to reduce with increasing amounts of competitive probe without 6-FAM. BSA and Trx–His–S control proteins did not bind the POX05587/Cel7A-2 promoter region (Fig. 9b). PoxCxrA11–58 was confirmed to bind to PMB_POX05587 via in vitro EMSA experiments (Fig. 9c). Subsequently, the PoxCxrA17–58 was also expressed and purified. EMSA binding experiments indicated a clear band comprised of PoxCxrA17–58 and PMB_POX05587 on the gel (Fig. 9d). Thus, the minimal

![Image](image-url)
42-amino-acid segment of PoxCxrA_{17–58} suffices for DNA binding.

Subsequently, an alignment analysis of PoxCxrA_{17–38} with the DBDs in known Zn2Cys6-containing TFs including PoxClrB, Pho7, XlnR, Clr1 and Gal4 that came from P. oxalicum HP7-1 [2], Schizosaccharomyces pombe [18], P. oxalicum strain 114–2 [7], N. crassa strain OR74A [19] and Saccharomyces cerevisiae S288C [9], was respectively performed. The results found three pairs of highly conserved zinc-coordinating cysteines that are essential for protein binding [18] and relatively conserved flanking amino acids such as arginine (R), lysine (K), aspartic acid (D) and proline (P; Fig. 9e). The retained amino acids showed high diversity. Several relatively conserved amino acids (18th R, 19th R, 27th Q, 30th K, 32th K and 38th P) were respectively replaced by alanine to generate a mutated PoxCxrA_{17–58}. In vitro EMSA further displayed that all the mutated PoxCxrA_{17–58} lost the ability to bind the PM_POX05587 (Fig. 9f).

**Discussion**

PoxCxrA is known to be a critical transcriptional activator in P. oxalicum exposed to cellulose as a carbon source, but its regulatory mechanism is unclear. Herein, we found that PoxCxrA regulates cellulase production in P. oxalicum by controlling the expression of PoxClrB, and further elucidated their regulatory network. PoxCxrA autoregulated the transcription of its own PoxCxrA gene, but PoxClrB did not. Moreover, the DNA-binding domain of PoxCxrA_{17–150} bound to the promoter regions of PoxClrB and POX05587/Cel7A-2 at different binding sites (5'-ATCAGATCCTCAAAGA-3' and 5'-GCTGAG TCCTTT-3', respectively) according to in vitro DNase I footprinting and EMSA experiments. A minimal functional DBD (residues 17–58) of PoxCxrA was identified. These findings provide novel insights into the regulatory mechanism governing cellulase gene expression in P. oxalicum.

The PoxCxrA regulon was identified, which included a number of members involved in primary and secondary metabolism. To withstand starvation caused by insoluble cellulose as the sole carbon source, expression of major cellulase genes in P. oxalicum, including genes encoding CBH1 (GH7) and EGs (GH5 and GH12), was rapidly up-regulated, resulting in abundant intra- and extracellular cellobextrin production in fungal cells [20–22]. Accumulated intracellular cellobextrin triggered signaling cascades that activated the expression of PoxCxrA and PoxClrB, subsequently resulting in activation of the expression of cellulase genes such as POX05587/Cel7A-2.

Intriguingly, PoxCxrA also directly activated PoxClrB expression, whereas PoxClrB indirectly repressed the transcription of PoxCxrA in P. oxalicum. Regrettably, exactly how PoxCxrA expression is repressed by PoxClrB remains unknown (Fig. 10). In the early phase of P. oxalicum exposed to Avicel, both PoxCxrA and PoxClrB were transcribed at a background level. Expression of PoxCxrA was first up-regulated at the middle of the culture period, and then reduced in the latter stages due to autoinhibition or repression by PoxClrB (Fig. 10). In contrast, PoxClrB expression gradually increased in the middle and later stages.

Moreover, PoxCxrA up-regulated the expression of genes involved in cellobextrin transportation, such as POX06051/PoxCdtC and POX05915/PoxCdtD [10] and retarded the expression of genes involved in glucose transportation such as POX07576/GLT-1 and POX08783/HGT-2 [12] that caused CCR at high concentration. PoxCxrA stimulated cellulase gene expression via two pathways; direct binding, and through key TF mediators such as PoxClrB (Fig. 10), PoxCxrB and/or PoxNsdD [8].

Differences in regulatory networks occurred in different host cells. In N. crassa, cellobiose-activated CLR1 was necessary for the increased expression of clr2, a homolog of PoxClrB. CLR1 also induced most cellulase genes, thereby positively affecting enzyme production [19]. In the present study, we found that PoxCxrA was required for PoxClrB expression, but not POX03837, which encodes a homolog of CLR1 in P. oxalicum. Knockout of POX03837 had no effect on cellulase production in P. oxalicum HP7-1 cultured on Avicel (data not shown).

The PoxCxrA DBD resembles those of Gal4-like TFs (e.g. Gal4 in S. cerevisiae, CLR1 and XlnR in N. crassa, and PoxClrB). PoxCxrA_{17–58} was found to be the minimal functional DBD, and it comprises three pairs of zinc-coordinating cysteines and several conserved amino acids that are essential for protein binding [18]. The amino acids flanking these cysteines display high diversity, which might determine the binding motifs. In the literature, many binding motifs of Gal4-like proteins have been characterised, including CGGN5CGGNCCG (CLR1), CGGN11CCG (Gal4 or CLR2), GGNTAAA (XlnR) [9], TCG(G/C)(A/T)NTTNA (Pho7) [18] and 5'-ATCAGATCCTCAAAGA-3' or 5'-GCTGAG TCCTTT-3' determined in this study. This suggests that the amino acids flanking the cysteines are also required for binding.

Moreover, screening PoxCxrA-binding sequences in other target genes confirmed by in vitro EMSA experiments using PBM_POX05587 and PBM_PoxClrB revealed that the binding sequences were highly diverse, which supports the enormous regulon of PoxCxrA that includes 1970 members, accounting for almost a quarter of the entire P. oxalicum strain HP7-1 genome. However, identification and analysis of all PoxCxrA-binding sequences in the genome requires further study.
In summary, the present study determined regulon for the essential TF PoxCxrA that is required for cellulase production of *P. oxalicum*. PoxCxrA regulates cellulase gene expression via two mechanisms: regulating key TF mediator PoxClrB, and directly binding cellulase genes with diverse binding motifs. This work provides novel insights into the regulatory mechanisms of fungal cellulase gene expression.

**Methods**

**Strains and culture conditions**

*Penicillium oxalicum* mutant strains ΔPoxCxrA (no. 12965) and ΔPoxClrB (no. 3.15649), and the parental strain ΔPoxKu70 (no. 3.15650) were obtained from the China General Microbiological Culture Collection (CGMCC) [8]. Spores were collected after maintaining strains on potato dextrose agar (PDA) plates at 28 °C for 6 days with buffer containing 0.1% Tween-80. In general, an aliquot of conidial suspension (1 × 10^8 conidia per milliliter) was inoculated into 100 mL of modified culture medium (MMM; pH 5.5) containing (g/L) (NH₄)₂SO₄ (4.0), KH₂PO₄ (4.0), CaCl₂ (0.6), MgSO₄·7H₂O (0.60), FeSO₄·7H₂O (0.005), MnSO₄ (0.0016), ZnCl₂ (0.0017), CoCl₂ (0.002), and 1 mL of Tween-80, containing either 1.0% glucose or 2.0% Avicel (Sigma-Aldrich, St. Louis, MO, USA) as the sole carbon source. Mixtures were incubated on an orbital shaker at 28 °C at 180 rpm for 6 days.

For transfer cultivation, *P. oxalicum* strains were first pre-cultured for 24 h on MMM supplemented with 1.0% glucose as the carbon source at 28 °C with shaking at 180 rpm. Mycelia of pre-cultured *P. oxalicum* strains were transferred into MMM containing 2.0% Avicel and cultured for 4–24 h at 28 °C with shaking at 180 rpm. Mycelia were harvested and total RNA was extracted for RNA-Seq and real-time quantitative reverse transcription-PCR (RT-qPCR) assays.

**RNA extraction**

For total RNA extraction, mycelia harvested from three replicate independent cultures at each time point were frozen, ground under liquid nitrogen using a pestle and mortar, and RNA was purified using a TRizol RNA Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA concentration and quality were determined from the ratio of the absorbance at 260 nm/280 nm measured using a Nanodrop ND-2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).
RNA-Seq
Total RNA was sequenced as described previously by Zhao et al. [2], and a cDNA library for each total RNA sample was constructed and assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). After confirming eligibility, cDNA libraries were sequenced using an Illumina HiSeq 4000 system. After quality control, clean reads were mapped onto the genome of the wild-type *P. oxalicum* HP7-1 strain to assess sequence homology and functional annotation using BWA v0.7.10-r789 [23] and Bowtie2 v2.1.0 [24]. RSEM v1.2.12 software [25] was used to analyse gene expression levels using the fragments per kilobase of exon per million mapped reads (FPKM) method. Differentially expressed genes (DEGs) were screened using the DESeq tool [26] with |log2 fold change| ≥ 1 and p value ≤ 0.05 as thresholds. The reliability of RNA-Seq data was assessed by Pearson’s correlation coefficients for three biological replicates for each sample. BLAST v2.2.26 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence homology and functional annotation analyses. DEGs detected by functional assays were functionally analysed based on Eukaryotic Orthologous Group (KOG) annotation to the *P. oxalicum* HP7-1 genome [2].

Expression and purification of truncated PoxCxrA constructs
Recombinant expression in *Escherichia coli* and protein purification were carried out as described previously by Yan et al. [8]. DNA sequences encoding a series of truncated PoxCxrA constructs (PoxCxrA11–150, PoxCxrA11–114, PoxCxrA11–87, PoxCxrA11–58, PoxCxrA17–58, PoxCxrA11–50 and PoxCxrA11–31) were separately amplified by PCR using specific primer pairs (Additional file 4: Table S3) and digested using appropriate restriction endonucleases. Digested DNA fragments were inserted into the expression vector pET32a (+) digested with the corresponding restriction endonucleases to generate recombinant plasmids that were subsequently introduced into competent *E. coli* cells by chemical transformation. After confirmation, cells harbouring constructs were pre-cultured for 12 h at 37 °C, then induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside, with culturing being continued for 20 h at 28 °C to produce fusion proteins possessing thioredoxin (TRX), His and S tags. Fusion proteins were purified by affinity chromatography on TALON Metal Affinity Resin (Clontech, Palo Alto, CA, USA). Cells harbouring empty pET32a (+) vector were cultured as described above and the resulting Trx–His–S protein was used as a negative control.

In vitro EMSA assays
In vitro EMSA experiments were performed as described previously by He et al. [17]. Briefly, DNA fragments of different lengths harbouring the putative promoter regions of *PoxClrB* and *POX05578/Cel7A-2*, labelled with 6-carboxyfluorescein (6-FAM) at the 3′ terminus, were amplified by PCR using specific primer pairs (Additional file 4: Table S3), and used as probes for in vitro EMSA experiments. Meanwhile, the same DNA fragments without 6-FAM and a 300-bp DNA fragment from the promoter region of the β-tubulin gene *POX05989* served as competitive and negative probes, respectively.

For EMSA experiments, mixtures containing ~50 ng EMSA probes and various amounts (0–2.0 µg) of fusion proteins in binding buffer consisting of 20 mM TRIS–HCl (pH 8.0), 0.1 mg/mL bovine serum albumin (BSA), 5% (v/v) glycerol, 50 mM KCl, 1 mM dithiothreitol and 1.0 µg sheared salmon sperm DNA) were cultured for 30 min at 30 °C, then separated by 4% polyacrylamide–TRIS–acetic acid–ethylene diamine tetraacetic acid (EDTA) gel electrophoresis. Gels were observed using a Bio-Rad ChemiDoc MP imaging system (BIO-RAD, Hercules, CA, USA) at an excitation wavelength range of 489–506 nm. Competitive binding experiments were performed as described above, except that probes were replaced with competitive probes.

DNase I footprinting
DNase I footprinting experiments were carried out as reported by Wang et al. [27] with minor modifications. For each assay, 350 ng of each probe (100-bp DNA fragments corresponding to the region upstream from the start codon ATG of *POX05587/Cel7A-2*) were separately incubated with 6.6 µg of recombinant PoxCxrA11–150 protein for 20 min at 30 °C. Subsequently, 0.015 U DNase I (Promega, Beijing, China) and 100 nM CaCl2 were added and the reaction was incubated for 1 min at 30 °C. DNase I stop solution, containing 200 mM unbuffered sodium acetate, 30 mM EDTA and 0.15% sodium dodecyl sulphate (SDS), was used to stop the enzymatic reaction, and DNA was extracted and sequenced.

RT-qPCR assays
RT-qPCR assays were carried out according to a previously reported method [8]. Briefly, total RNA was extracted from *P. oxalicum* deletion mutant ∆PoxCxrA grown on Avicel, and from the parental strain ∆PoxKu70 that served as a control. First-strand cDNA was synthesized from the extracted RNA as template using a PrimeScript RT Reagent Kit (TaKaRa Bio Inc., Dalian, China) according to the manufacturer’s instructions. Each target gene was subjected to PCR amplification using the first-strand cDNA as template in a 20 µL
reaction mixture containing 10 µM of each primer (0.8 µL; Additional file 4: Table S3), first-strand cDNA (0.2 µL) and SYBR Premix Ex Taq II (10 µL; TaKaRa Bio Inc.). Thermal cycling included 35 cycles at 95 °C for 3 s and 60 °C for 30 s. Fluorescent signals were measured at the end of each extension step at 80 °C. Transcriptional levels of each gene in deletion mutants ∆PoxCxrA and ∆PoxClrB were calculated relative to that of the control gene POX09428 encoding actin, and relative expression levels were normalised against levels in the ∆PoxKu70 strain. All RT-qPCR assays were performed independently in at least three replicates.

Network construction
Network was constructed using Cytoscape v.3.6.1 software [28].

Statistical analysis
Experimental data were statistically analysed by Student’s t tests using Microsoft Excel within Office 2016 (Microsoft, Redmond, WA, USA).

Accession number
Transcriptomic data have been deposited in the Sequence Read Archive database under Accession Numbers SRR8377263–SRR8377265 for the ∆PoxCxrA and SRR8377258, SRR8377259 and SRR8377266.

Additional files
Additional file 1: Table S1. Summary of transcriptomic data generated from Penicillium oxalicum.
Additional file 2: Figure S1. Pearson’s correlation analysis of transcriptomes from Penicillium oxalicum deletion mutant ∆PoxCxrA and the parental strain ∆PoxKu70. Total RNA was extracted from P. oxalicum strains cultivated in medium containing Avicel as the sole carbon source for 24 h after a shift from glucose, then sequenced.
Additional file 3: Table S2. PoxCxrA regulon in Penicillium oxalicum when subjected to Avicel as the sole carbon source.
Additional file 4: Table S3. Primers used in this study.

Abbreviations
6-FAM: 6-carboxyfluorescein; BSA: bovine serum albumin; BGL: β-glucosidase; CAZymes: carbohydrate-active enzymes; CBH: cellobiohydrolase; CCR: carbon catabolite repression; CMCase: carboxymethylcellulose; CWDEs: plant cell wall-degrading enzymes; DBD: DNA-binding domain; DEGs: differentially expressed genes; EG: endo-β-1,4-glucanase; EMSA: electrophoretic mobility shift assay; FPase: filter paper cellulase; FPKM: fragments per kilobase of exon per million fragment mapped; MMM: modified culture medium; PBMs: protein-binding motifs; PDA: potato dextrose agar; pNPCase: p-nitrophenyl-β-glucosidase; pNPGase: p-nitrophenyl-β-glucopyranosidase; RT-qPCR: real-time quantitative reverse transcription PCR; TFs: transcriptional factors; Xyn: xylanase.

Authors’ contributions
JXF conceived, supervised this study, wrote and revised the manuscript. SZ codesigned and co-supervised this study, and was involved in the data analysis and manuscript revision. LSL conducted in vitro binding experiments, identification of the binding sequences of PoxCxrA in POX05587 and its DNA-binding domain. CXL carried out bioinformatic analyses. FFZ performed identification of the binding sequences of PoxCxrA in PoxClB. YSY conducted RT-qPCR experiments. XML were involved in preparation of experimental materials and the analysis of experimental data. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
All authors consent for publication.

Ethics approval and consent to participate
Not applicable.

Funding
This work was financially supported by the Grants from the National Natural Science Foundation of China (Grant Nos. 31760023 and 31660305), and the ‘One Hundred Person’ Project of Guangxi.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 10 March 2019   Accepted: 16 April 2019
Published online: 03 May 2019

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