Transcription Factor NsdD Regulates the Expression of Genes Involved in Plant Biomass-Degrading Enzymes, Conidiation, and Pigment Biosynthesis in *Penicillium oxalicum*

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ABSTRACT Soil fungi produce a wide range of chemical compounds and enzymes with potential for applications in medicine and biotechnology. Cellular processes in soil fungi are highly dependent on the regulation under environmentally induced stress, but most of the underlying mechanisms remain unclear. Previous work identified a key GATA-type transcription factor, *Penicillium oxalicum* NsdD (PoxNsdD; also called POX08415), that regulates the expression of cellulase and xylanase genes in *P. oxalicum*. PoxNsdD shares 57 to 64% identity with the key activator NsdD, involved in asexual development in *Aspergillus*. In the present study, the regulatory roles of PoxNsdD in *P. oxalicum* were further explored. Comparative transcriptomic profiling revealed that PoxNsdD regulates major genes involved in starch, cellulose, and hemicellulose degradation, as well as conidiation and pigment biosynthesis. Subsequent experiments confirmed that a ΔPoxNsdD strain lost 43.9 to 78.8% of starch-digesting enzyme activity when grown on soluble corn starch, and it produced 54.9 to 146.0% more conidia than the ΔPoxKu70 parental strain. During cultivation, ΔPoxNsdD cultures changed color, from pale orange to brick red, while the ΔPoxKu70 cultures remained bluish white. Real-time quantitative reverse transcription-PCR showed that PoxNsdD dynamically regulated the expression of a glucoamylase gene (POX01356/Amy15A), an α-amylase gene (POX09352/Amy13A), and a regulatory gene (POX03890/amyR), as well as a polyketide synthase gene (POX01430/alb1/wA) for yellow pigment biosynthesis and a conidiation-regulated gene (POX06534/brlA). Moreover, in vitro binding experiments showed that PoxNsdD bound the promoter regions of the above-described genes. This work provides novel insights into the regulatory mechanisms of fungal cellular processes and may assist in genetic engineering of *P. oxalicum* for potential industrial and medical applications.

IMPORTANCE Most filamentous fungi produce a vast number of extracellular enzymes that are used commercially for biorefineries of plant biomass to produce biofuels and value-added chemicals, which might promote the transition to a more environmentally friendly economy. The expression of these extracellular enzyme genes is tightly controlled at the transcriptional level, which limits their yields. Hitherto our understanding of the regulation of expression of plant biomass-degrading enzyme genes in filamentous fungi has been rather limited. In the present study, regulatory roles of a key regulator, PoxNsdD, were further explored in the soil fungus *Penicillium oxalicum*, contributing to the understanding of gene regulation in filamentous fungi and revealing the biotechnological potential of *P. oxalicum* for potential industrial and medical applications.

KEYWORDS *Penicillium oxalicum*, transcription factor, PoxNsdD, starch-degrading enzyme, conidiation, pigment biosynthesis

Soil-plant-atmosphere carbon cycling is an important feature of terrestrial ecosystems, in which soil microbes act as decomposers and play crucial roles in regulating carbon flux between the biosphere and the atmosphere (1, 2). Minor changes in the
balance between soil carbon storage and release might affect the global climate and contribute to the greenhouse effect. Among soil microbes, fungi efficiently consume soil organic carbon stored in polysaccharides from plant biomass by secreting carbohydrate-active enzymes (CAZymes) (3). However, this process operates at a relatively low level in native ecosystems and is limited by enzyme production and biomass architecture (4). Cellulase, xylanase, and amylase have been employed in industrial applications, including biorefining of plant biomass into biofuels and/or high-value-added chemicals that are otherwise expensive to produce.

Soil fungi produce a wide range of chemical compounds that have been utilized by humans for many years, such as monascorubrin and its derivatives from *Monascus* species, which serve as natural red colorants for food (5), and penicillin and derived β-lactam antibiotics produced by *Penicillium chrysogenum* (6). Soil fungi can also produce mycotoxins that contaminate human and animal food and that represent a threat to human and animal health (7). Genomic data on soil fungi have revealed numerous unexplored secondary metabolites, for example, 68 secondary metabolite gene clusters were identified in *Talaromyces pinophilus* 1-95 alone (8).

Specific transcription factors (TFs) tightly regulate the production of plant biomass-degrading enzymes and/or the biosynthesis of secondary metabolites in host cells at the transcriptional level. Most studies on the identification of such TFs and the exploration of their regulatory mechanisms have focused mainly on cellulolytic filamentous fungi, such as *Trichoderma*, *Aspergillus*, and *Penicillium* (9–11). Studies on such TFs mainly include the transcription activators CLR-2/ClrB of *Aspergillus nidulans* FGSC A4 (12) and *Penicillium oxalicum* 114-2 (13) and HP7-1 (14), XYR1/XLR-1/XlnR of *Trichoderma reesei* QM9136 (15) and *P. oxalicum* 114-2 (13), AmyR of *P. oxalicum* 114-2 (13) and *Aspergillus niger* CICC2462 (11), and LaeA of *P. oxalicum* 114-2 (16) and *T. reesei* QM9414 (17) and the carbon catabolite repressor CreA/CRE1/CRE-1 of *A. nidulans* FGSC A4 (18) and *T. reesei* QM9414 (19). Interestingly, expression of genes encoding plant biomass-degrading enzymes and genes involved in asexual reproduction is coregulated by some TFs, such as BrIA (10), ClrC (20), and FlbC (21) of *P. oxalicum* 114-2.

NsdD in *Aspergillus* spp. and its orthologs, such as Pro44 in *Sordaria macrospora*, Ltf1 in *Botrytis cinerea*, Csm1 in *Fusarium fujikuroi*, SUB-1 in *Neurospora crassa*, and SSNs1 in *Sclerotinia sclerotiorum*, regulate development and/or biosynthesis of secondary metabolites (22–28). Additional functions involving stress tolerance, light response, and virulence have been observed in the plant pathogens *B. cinerea*, *F. fujikuroi*, and/or *N. crassa* (23, 26, 27). NsdD is positioned between FLB (fluffy low BrIA expression) and BrIA and functions as a repressor by downregulating brIA in *Aspergillus* (25, 29). The *P. oxalicum NsdD* gene (*PoxNsdD*) was initially found to regulate the expression of cellulase and xylanase genes in *P. oxalicum* (30). However, the details of the regulatory roles of *PoxNsdD* in *P. oxalicum* merit further investigation.

In the present study, we investigated the regulatory roles of *PoxNsdD* in *P. oxalicum* through global RNA sequencing and biochemical and microbiological techniques.

**RESULTS**

Transcriptomic analyses reveal that *PoxNsdD* regulates the expression of genes involved in plant biomass-degrading enzymes, conidiation, and pigment biosynthesis in *P. oxalicum*. A previous study reported that *PoxNsdD* positively regulated the production of cellulase and xylanase when *P. oxalicum* was grown in medium containing *Avicel* as the sole carbon source (30). To further explore the regulatory roles of *PoxNsdD* in *P. oxalicum*, deep RNA sequencing was employed to analyze the transcriptomic profiling of a *PoxNsdD* deletion mutant (Δ*PoxNsdD*) grown on *Avicel*, with the parental strain Δ*PoxKu70* used as a control. In total, ~23 million clean reads with a length of 100 bp (accession number SRA493765 in the Sequence Read Archive [SRA] database) were generated from each sample, indicating an average of 76-fold coverage (see Table S1 in the supplemental material). Over 90% of clean reads were successfully mapped to the genome of the wild-type strain HP7-1 (14). Pearson’s correlation coefficients (r) for three biological replicates for each sample were subsequently
calculated, and the results displayed high r values (>0.90), which illustrated that these transcriptomic data were reliable (Fig. S1).

Using a probability of ≥0.8 and a log2 fold change of ≥1 as the threshold, transcripts of 761 genes were found to be altered significantly in the ΔPoxNsdD strain compared to those in the ΔPoxKu70 strain (Data Set S1), and these were defined as the PoxNsdD regulon. Among them, 308 were downregulated (-10.6 < log2 fold change < -1.0), and 453 were upregulated (1.0 < log2 fold change < 9.1). Eukaryotic orthologous group (KOG) annotation revealed that most of the 761 differentially expressed genes (DEGs) were mainly involved in primary and secondary metabolism (42%), specifically in category E (amino acid transport and metabolism) and category Q (secondary metabolite biosynthesis, transport, and catabolism) (Fig. 1A).

In the PoxNsdD regulon, 95 DEGs were annotated to encode CAZymes, including enzymes from 35 glycoside hydrolase families, 2 glycosyltransferase families, 7 carbohydrate esterase families, 6 auxiliary activity families, and 7 carbohydrate-binding module families, as well as 1 polysaccharide lyase. Among them, 63 DEGs were downregulated with log2 fold changes between -6.4 and -1.1, including 47 genes encoding plant cell wall-degrading enzymes (CWDEs) and two major amylase genes, POX01356/Amy15A and POX09352/Amy13A (Fig. 1B). Notably, the major CWDE genes in the P. oxalicum genome were detected in the PoxNsdD regulon, including two cellobiohydrolase genes (cbh genes; POX04786/Cel6A and POX05587/Cel7A-2), nine endo-β-1,4-glucanase genes (eg genes; POX01166/Cel5B, POX01896/Cel5C, POX02740, POX04137, POX05570/Cel45A, POX05571/Cel7B, POX06147/Cel5A, POX06983, and POX07535/Cel12A), four β-1,4-glucosidase genes (bgl genes; POX00968, POX03062, POX03641, and POX06079), three xylanase genes (xyn genes; POX00063/Xyn10A, POX06601, and POX06783/Xyn11A), and two genes (POX02308/Cel61A and POX08897) encoding lytic polysaccharide monooxygenases. Conversely, 32 DEGs were upregulated with log2 fold changes between 1.1 and 7.0, including five chitinase genes (POX00889, POX01447, POX03021, POX06241, and POX08171) involved in the degradation of fungal cell walls, a xyn gene (POX05916), and a polygalacturonase gene (POX01225) (Fig. 1B; Data Set S1).

Interestingly, 22 DEGs encoding putative TFs were also detected in the PoxNsdD regulon, among which 16 were upregulated, with log2 fold changes of 1.02 to 6.43, and...
6 were downregulated ($-10.6 < \log_2 \text{fold change} < -1.07$). Five regulatory genes (POX00972/cIcC, POX04420/POxCxrB, POX04860/PDE_07199, POX05726, and POX06534/brlA) were known to regulate the expression of cellulase genes in *P. oxalicum* (10, 20, 30). In addition, the POX06534/brlA, POX07025/abaA, and POX07099/flbD genes were known to positively regulate conidiation in filamentous fungi (10, 25, 29).

In addition, in the PoxNsdD regulon, 16 DEGs (POX06534/brlA, POX07025/abaA, POX07099/flbD, POX01390/agyA, POX01391/abrA, POX01430/abrBl/aWA, POX01431/abrBl/yA, POX03412/arpA, POX03413/arpB, POX01651/rodA-like gene, POX01764/rodA, POX06595/rodB, POX03398/axl2, POX05090/tmaP, POX00872/nimX, and POX09181/vosA) were predicted to be involved in conidiogenesis (10), including 15 that were upregulated (1.1 < \log_2 \text{fold change} < 6.6) and one (POX03389/axl2) that was downregulated ($\log_2 \text{fold change} = -2.65$) (Fig. 1C), which indicated that PoxNsdD also affected conidiation of *P. oxalicum*.

We next determined the number of DEGs involved in secondary metabolism of *P. oxalicum*. A total of 230 DEGs covering 69 putative biosynthetic gene clusters (BGCs) was identified (Data Set S1), of which 90 were downregulated ($-4.4 < \log_2 \text{fold change} < -1.0$) and 140 were upregulated (1.0 < \log_2 \text{fold change} < 9.0) in the ΔPoxNsdD strain. Sixty-four of the predicted BGCs were classified into 13 different types, as follows: 34 cf putative gene clusters, 7 terpene gene clusters, 5 nonribosomal peptide synthase (Nrps) gene clusters, 4 T1 polyketide synthase (T1pks)-Nrps gene clusters, 3 T1pks gene clusters, 2 cf fatty acid gene clusters, 2 cf_saccharide gene clusters, and 2 indole gene clusters, as well as single representatives of cf_fatty acid-Nrps, T3pks, indole-terpene-Nrps, indole-Nrps, and cf_fatty acid-T1pks-Nrps gene clusters. The remaining five gene clusters appeared to synthesize other, unknown secondary metabolites. Furthermore, compared to known secondary metabolite gene clusters, 11 were predicted to produce the following, with gene similarities ranging from 10 to 100%: aspyridone (clusters 10 and 28), emericellin (cluster 11), citrinin (cluster 17), leucinostatins (cluster 29), roquefortine C/meleagrin (cluster 33), beauvericin (cluster 49), cytochalasin (clusters 51 and 54), malbrancheamide (cluster 72), and viridicatumtoxin (cluster 88).

Among these clusters, a total of 33 core biosynthetic genes distributed among 22 BGCs were detected, including seven known BGCs in clusters 11, 29, 33, 49, 51, 72, and 88 (Fig. 2; Data Set S1). All the core biosynthetic genes in known BGCs were upregulated (1.6 < \log_2 \text{fold change} < 6.4) in the ΔPoxNsdD mutant in comparison to the ΔPoxKu70 strain (Fig. 2).

**Deletion of PoxNsdD from *P. oxalicum* results in significant reduction of production of enzymes degrading raw cassava starch and soluble starch.** Transcriptional assays revealed that PoxNsdD positively regulates the expression of two important amylase genes, POX01356/Amy1SA and POX09352/Amy13A, indicating that PoxNsdD might affect amylase production of *P. oxalicum*. A mycelial shift experiment from medium containing glucose to medium containing soluble corn starch over 2 to 4 days was performed, and the results showed that the ΔPoxNsdD deletion mutant lost 52.6 to 78.8% and 43.9 to 68.8% of raw cassava starch-degrading enzyme (RCSDE) activity and soluble starch-degrading enzyme (SSDE) activity compared to those of the ΔPoxKu70 parental strain (*P < 0.05; Student’s t test*). Complementation of the ΔPoxNsdD mutant (30) mostly restored the RCSDE and SSDE activities to the levels of the ΔPoxKu70 strain under the induction conditions with soluble corn starch (Fig. 3).

**PoxNsdD is involved in mycelial growth and conidiation in *P. oxalicum*.** The ΔPoxNsdD mutant was inoculated onto solid medium plates containing glucose, Avicel, soluble corn starch, or potato dextrose agar (PDA) for 2 to 4 days. Three days after inoculation, ΔPoxNsdD colonies were bigger and greener than those of the ΔPoxKu70 parental strain on PDA plates, whereas the mycelial growth of the ΔPoxNsdD mutant was significantly retarded on all plates containing glucose, Avicel, or starch (Fig. 4A and B). Quantitative analysis of asexual spores revealed that the ΔPoxNsdD mutant had a significantly increased number of asexual spores compared to that for the ΔPoxKu70 strain on all plates (*P < 0.05; Student’s t test*) (Fig. 4C). Furthermore, microscopy revealed that the
ΔPoxNsdD mutant promoted phialide development to a greater degree than that with the ΔPoxKu70 strain (Fig. 5). The complemented strain, CPoxNsdD, showed physiological features, such as colony color, number of asexual spores, and phialide development, similar to those of the ΔPoxKu70 parental strain (Fig. 4 and 5).

PoxNsdD is involved in pigment biosynthesis in P. oxalicum. During measurement of the cellulase and xylanase activities of the ΔPoxNsdD mutant cultivated in medium containing Avicel after a transfer from glucose, the ΔPoxNsdD mutant biosynthesized colorful pigments, but the ΔPoxKu70 parental strain did not (data not shown). When the ΔPoxNsdD mutant was directly inoculated into Avicel medium for 1 to 6 days, the culture color of the ΔPoxNsdD mutant changed to pale orange, in contrast to that of the ΔPoxKu70 strain. Additionally, a similar phenomenon was observed for the ΔPoxNsdD mutant when it was directly inoculated into medium containing glucose or soluble corn starch and cultured for 1 to 6 days. Complementation of the ΔPoxNsdD mutant changed the pigment biosynthesis of the ΔPoxNsdD mutant to that of the ΔPoxKu70 parental strain (Fig. 6).

Kinetics of PoxNsdD regulation of genes involved in starch degradation and pigment biosynthesis as well as TF-encoding genes in P. oxalicum. To further confirm the regulatory roles of PoxNsdD in P. oxalicum, real-time quantitative reverse transcription-PCR (RT-qPCR) was employed to measure the expression of target genes 4, 12, 24, and 48 h after a shift from glucose to soluble corn starch. Target genes included the glucoamylase gene POX01356/Amy15A, the α-amylase gene POX09352/Amy13A, and their transcriptional activator gene POX03890/amyR, as well as the yellow pigment biosynthesis polyketide synthase gene POX01430/alb1/wA. The results showed that expression of all three genes involved in starch degradation was downregulated in
the ΔPoxNsdD mutant compared to that in the ΔPoxKu70 strain during the whole induction period, except for expression of POX01356/Amy15A and POX09352/Amy13A at 4 h. Specifically, the POX01356/Amy15A, POX09352/Amy13A, and POX03890/amyr genes were significantly downregulated, by 35.9 to 82.8% (P < 0.05; Student’s t test), at 12, 24, and 48 h in the ΔPoxNsdD mutant compared to those in the ΔPoxKu70 parental strain.

Conversely, POX01356/Amy15A and POX09352/Amy13A were significantly upregulated, from 60.5% to 460.4%, at 4 h in the ΔPoxNsdD mutant (P < 0.01; Student’s t test) (Fig. 7A). In addition, transcription of the key gene POX01430/alb1/wA, involved in pigment biosynthesis, was increased 1.33- to 128.1-fold during the entire starch induction period (P < 0.05; Student’s t test) (Fig. 7A).

**PoxNsdD positively regulates genes involved in biomass degradation during both vegetative growth and conidiation but negatively regulates genes involved in conidiation only during vegetative growth in P. oxalicum.** RT-qPCR was continuously employed to measure the expression of the conidiation-regulated gene POX06534/brlA in the ΔPoxNsdD mutant 4, 12, 24, and 48 h after a shift from glucose to Avicel. The results showed that the transcription level of POX06534/brlA increased, by 209.6% to 1,519.4%, in the ΔPoxNsdD mutant during almost the whole induction period in comparison to that in the ΔPoxKu70 strain (P < 0.01; Student’s t test) (Fig. 7B). As shown in Fig. 7C, POX06534/brlA expression was barely noticeable in the ΔPoxKu70 strain under vegetative growth conditions, but its expression increased rapidly 12 h after induction of conidiation. Deletion of PoxNsdD resulted in a slight increase in POX06534/brlA expression during vegetative growth and in high levels 12, 24, and 36 h after induction of conidiation.

In addition, expression of the cbh1 gene POX05587/Cel7A-2 was also investigated during both vegetative growth and conidiation. POX05587/Cel7A-2 expression during...
vegetative growth was significantly higher than that during conidiation in the ΔPoxKu70 parental strain, and deletion of PoxNsdD decreased the accumulation of POX05587/Cel7A-2 mRNA throughout the whole growth cycle (Fig. 7C).

PoxNsdD binds the promoter regions of major genes involved in starch degradation, conidiation, and pigment biosynthesis and of TF-encoding genes in vitro. Analysis using the SMART website (http://smart.embl-heidelberg.de/) revealed that PoxNsdD contains a GATA-type zinc finger (ZnF_GATA) that specifically binds the DNA sequence NGATAR (N = A, T, C, or G; R = A or G) (29, 31). Screening of the major genes involved in starch degradation, conidiation, and pigment biosynthesis, including POX01356/Amy15A, POX09352/Amy13A, POX03890/amyr, POX06534/brlA, and POX01430/alb1/wA, revealed an average of 7.6 PoxNsdD-binding sites within 2 kb of their 5′ upstream regions (Fig. S2).

To confirm whether PoxNsdD indirectly or directly regulated expression of the tested genes described above, an in vitro electrophoretic mobility shift assay (EMSA) was performed. 6-Carboxyfluorescein (FAM)-tagged DNA fragments (about 1,000 bp) from the promoter regions of POX01356/Amy15A, POX09352/Amy13A, POX03890/amyr, POX06534/brlA, and POX01430/alb1/wA were amplified (Fig. S2), purified, and used as probes for EMSA, and the promoter region of POX05989, encoding β-tubulin, was used as the negative control. The putative DNA-binding domain PoxNsdD\textsubscript{335–494} was fused to thioredoxin (Trx), His, and S tags and recombinantly expressed and purified as described previously (30). As shown in Fig. 8, complexes of PoxNsdD\textsubscript{335–494} and the promoters of the target genes, POX01356/Amy15A, POX09352/Amy13A, POX03890/amyr, POX06534/brlA, and POX01430/alb1/wA, were observed, and the concentrations of complexes gradually increased with increasing amounts of fusion proteins (1.0 to 2.0 μg). The complexes were not observed with Trx-His-S or bovine serum albumin (BSA) alone, with only the promoters of the target genes, or with the promoter of POX05989 (Fig. 8). When protein-binding DNA fragments lacking the FAM label were used as competitive probes, the concentrations of the complexes gradually decreased with increasing amounts of the competitive probes (Fig. 8), suggesting that PoxNsdD bound specifically to the promoters of POX01356/Amy15A, POX09352/Amy13A, POX03890/amyr, POX06534/brlA, and POX01430/alb1/wA.
DISCUSSION

Previous work identified the key TF PoxNsdD in *P. oxalicum* HP7-1 by comparative transcriptomic profiling and genetic analyses (30). PoxNsdD shares 57 to 64% identity with NsdD in *A. nidulans* FGSC A4 (accession number XP_660756.1), *Aspergillus flavus* NRRL3357 (accession number XP_002376041.1), and *Aspergillus fumigatus* AF293 (accession number XP_754237.1). PoxNsdD contributes to the production of cellulase and xylanase under induction by Avicel. In the present study, the regulatory roles of PoxNsdD were further explored in detail, and the results showed that PoxNsdD not only repressed conidiation in *P. oxalicum*, like its homologue NsdD in *Aspergillus*, but also regulated plant biomass-degrading enzyme production, including amylase production.
and pigment biosynthesis. Importantly, PoxNsdD could directly regulate the expression of the major genes involved in these processes.

NsdD and its orthologues are identified as TFs with the conserved DNA-binding domain ZnF_GATA in many filamentous fungi (22–29). Hitherto in the reported literature and this work, NsdD and its orthologues primarily played six roles, including (i) repressing asexual development in *Aspergillus* spp. (25, 29), *F. fujikuroi* (27), *S. sclerotiorum* (28), and *P. oxalicum*; (ii) activating sexual development in *Aspergillus nidulans* (22), *S. sclerotiorum* (28), and *S. macrospora* (24); (iii) affecting the production of secondary metabolites, such as the dark mycelial pigment and gliotoxin in *Aspergillus* spp., under specific conditions (25, 29); synthesizing the polyketide synthase (PKS)-derived pigments in *F. fujikuroi* (27) and *P. oxalicum*; (iv) regulating light responses in *N. crassa* (23) and *B. cinerea* (26); (v) regulating fungal virulence in the necrotrophic plant pathogen *B. cinerea* (26); and (vi) regulating the production of plant biomass-degrading enzymes and the expression of these enzyme genes in *P. oxalicum*, as identified previously (30) and in this study.

Conidiation is well known to be strictly controlled by a central regulatory pathway of three TFs, BrlA, AbA, and WetA, acting in concert with other genes, such as FLB, as an integrative part of the fungal life cycle (32). NsdD plays a negative regulatory role in conidiation via repressing brlA expression in conidia and growing hyphae in *Aspergillus*. In contrast, in developing cells, the removal of NsdD with the addition of VosA activates brlA expression, leading to further activation of conidiation (25, 29). Similarly, in the present study, both RT-qPCR and RNA sequencing revealed that POX06354/brlA expression was also derepressed in the ΔPoxNsdD mutant compared to that in the ΔPoxKu70 parental strain. Furthermore, by screening of the PoxNsdD regulon, ~40% (17/41 DEGs)
of DEGs were predicted to be involved in conidiogenesis in *P. oxalicum*, including 16 markedly upregulated genes and 1 downregulated gene (POX03389). Of these 16 genes, three key TF genes (POX06534/brlA, POX07025/abaA, and POX07099/flbD) are known to activate fungal conidiation (32), six (POX01390/aygA, POX01391/abrA, POX01430/alb1/wA, POX01431/abrB/yA, POX03412/arpA, and POX03413/arpB) are involved in pigment biosynthesis (33, 34), three (POX01651/rodA-like gene, POX01764/rodA, and POX06595/rodB) encode hydrophobins (35), two (POX03398/axl2 and POX05090/tmpA) encode transmembrane proteins (36), one (POX00872/nimX) controls cell division (37), and one encodes the velvet protein VosA, which represses fungal conidiation (32). The remaining DEG, POX03389, is an ortholog of phiA that is involved in phialide development in *A. nidulans*. However, how expression of these genes is regulated by PoxNsdD in *P. oxalicum* needs further study.
This study revealed that PoxNsdD regulates the production of plant biomass-degrading enzymes in filamentous fungi. Exactly how PoxNsdD simultaneously regulates such disparate processes is of significant interest. Throughout the life cycle of *P. oxalicum* grown on Avicel, the *cbh1* gene POX05587/Cel7A-2 is expressed at high levels in order to provide nutrients for growth and/or substrates for other cellulases by degrading Avicel. In contrast, the key activator gene *brlA*, involved in asexual reproduction, is expressed at high levels only during conidiation. Accordingly, PoxNsdD...
activates the expression of genes encoding plant biomass-degrading enzymes, such as CBH1, and represses the expression of genes involved in fungal asexual development, such as brlA. Lee et al. (29) reported that NsdD encodes two distinct peptides, NsdDα and NsdDβ, in Aspergillus. Western blotting confirmed that NsdDα specifically accumulates in hyphae, whereas NsdDβ is expressed constitutively throughout the life cycle. Whether PoxNsdD similarly encodes two peptides which separately regulate the expression of genes involved in plant biomass degradation and asexual reproduction in P. oxalicum merits further study.

Studies on the regulatory mechanism for amylase genes in fungi are minimal. In P. oxalicum, the major amylase gene, Amy15A, is positively regulated by the G protein PGA3 (38), the casein kinase CK2 (39), the heterochromatin protein Hep1 (40), and the TFs LaeA (16), PrtT for activating the expression of genes encoding extracellular proteinases (41) and AmyR (13). However, it is unknown whether these regulators, except for AmyR, directly or indirectly regulate amylase genes. In the present study, under induction by soluble starch, PoxNsdD positively regulated the expression of the α-amylase gene POX09352/Amy13A, the glucoamylase gene POX01356/Amy15A, and their activator gene, POX03890/amyr, via direct binding to their promoters.

When cultivated in media containing different carbon sources, such as glucose, Avicel, or soluble corn starch, ΔPoxNsdD cultures became yellow, suggesting that PoxNsdD repressed the expression of genes involved in pigment biosynthesis, which might be similar to the effect of Csm1 of F. fujikuroi (27). Mining of the whole genome of P. oxalicum HP7-1 by using antibiotics and secondary metabolite analysis shell (antiSMASH) (42) revealed 93 putative BGCs for secondary metabolites (J. X. Feng et al., unpublished data). Approximately one-third of BGCs were found in the PoxNsdD regulon, indicating that the ΔPoxNsdD mutant might biosynthesize a larger number of emericellin, leucinostatin, roquefortine C/meleagrin, beaucorvatin, cytochalasin, malbrancheamide, and viridicatumtoxin compounds. Remarkably, PKSs contribute to pigment biosynthesis in filamentous fungi, such as that of the pigments melanin, mitorubrin, and mitorubricin acid, as well as monascorubrin, citrinin, and ankaflavin, in Talaromyces (formerly Penicillium) marneffei (43). The transcriptional levels of two genes in cluster 14, POX01430/alb1/wA and POX01431/abrB/yA, increased 365- and 95.9-fold in the ΔPoxNsdD mutants; these genes encode the putative polyketide synthase Alb1/wA and the laccase AbrB/yA, which likely contribute to the colorful phenotype of the ΔPoxNsdD mutant. POX01430/alb1/wA produced the yellow heptaketide napht抱pyrone YWA1 intermediate asexual spore pigment in A. nidulans (44). In addition, P. oxalicum was predicted to biosynthesize citrinin as the secondary metabolite encoded by BGC cluster 17. Deletion of PoxNsdD significantly affected the expression of POX01612 (log2 fold change = −1.25), POX01614 (log2 fold change = −1.39), and POX01615 (log2 fold change = 5.97) under induction by Avicel.

Bioinformatics analysis revealed that POX01612 contains an HPP motif and may be a transporter protein, while POX01615 is a mannosyltransferase 1 and POX01614 is a hypothetical protein. Changes in the expression of these genes might enhance citrinin biosynthesis, thereby affecting culture color. In general, natural pigments secreted by fungi are complex mixtures. For example, the red pigment of T. marneffei contains more than 16 chemical compounds, including amino acid conjugates of monascorubrin and rubropunctatin (45). Therefore, the regulation of genes involved in pigment biosynthesis by PoxNsdD merits further study in P. oxalicum.

The gene regulatory network is integrative in cells but is complex and multidegree owing to specific ecological niches, requiring regulation of the signaling pathway for vegetative growth, secretion of extracellular enzymes, and the conidiation pathway. This results in cogovernance by various regulators, such as velvet proteins, G-proteins, and RAS proteins, that have global regulatory roles (32). Similarly, PoxNsdD coregulates the production of enzymes involved in biomass degradation, conidiation, and pigmentation in P. oxalicum.

In conclusion, the PoxNsdD gene of P. oxalicum was characterized as an ortholog of the NsdD gene encoding a GATA-type zinc finger TF. The present study not only provides evidence consistent with the general roles of NsdD orthologs in regulating...
asexual development and the production of secondary metabolites but also confirms that PoxNsdD plays a key role in regulating the production of enzymes that digest plant biomass into simple sugars, such as glucose, to be used as nutrients by host cells. These observations provide novel insights into the molecular mechanisms of transcriptional regulation of genes encoding plant biomass-degrading enzymes and of the elaborate cross-regulation between the signaling pathway for vegetative growth and the conidiation pathway in filamentous fungi.

**MATERIALS AND METHODS**

**Fungal strains and culture conditions.** The *P. oxalicum* wild-type strain HP-7 (China General Microbiological Culture Collection Center [CGMCC] no. 10781) was isolated from subtropical forest soil in the Guangxi Zhuang Autonomous Region, China (46). The ΔPoxKu70 (CGMCC no. 3.15650) and ΔPoxNsdD (CGMCC no. 12967) mutants were constructed by knocking out the PoxKu70 gene in HP-7 and the PoxNsdD gene in the ΔPoxKu70 strain, respectively (14, 30). All *P. oxalicum* strains were maintained on potato dextrose agar (PDA) plates at 4°C. Fungal spores were harvested 6 days after inoculation onto PDA plates at 28°C and then resuspended in 0.1% Tween 80 for subsequent reproduction.

For enzymatic activity and RT-qPCR assays, 1 × 10⁸ spores were precultured at 28°C for 24 h in liquid medium consisting of 100 ml of modified minimal medium (MMM) containing 4 g/liter (NH₄)₂SO₄, 4 g/liter KH₂PO₄, 0.6 g/liter CaCl₂, 0.6 g/liter MgSO₄·7H₂O, 0.005 g/liter FeSO₄·7H₂O, 0.0016 g/liter MnSO₄, 0.0017 g/liter ZnCl₂, 0.002 g/liter CoCl₂, and 1 ml of Tween 80 supplemented with 1% (wt/vol) glucose. Pregrown hyphae were transferred to MMM containing 1% (wt/vol) soluble corn starch for 2 to 4 days (for enzymatic activity assay) or 4, 12, 24, and 48 h (for RT-qPCR) at 28°C.

For phenotypic investigation, a certain number of spores were inoculated onto solid medium plates containing MMM plus agar and 1% (wt/vol) glucose, Avicel, or soluble corn starch at 28°C for 2 to 4 days, and PDA plates were used as the control. In addition, *P. oxalicum* strains were inoculated into liquid medium under similar conditions to investigate pigment biosynthesis.

For RNA sequencing of *P. oxalicum* strains under noninduced conditions, 1 ml of spore suspension at a concentration of 1 × 10⁸ cells per ml was used to inoculate 100 ml of MMM containing 1% (wt/vol) Avicel as the sole carbon source. Inoculated cultures were shaken at 180 rpm and 28°C for 72 h.

**Extraction of total DNA and RNA.** Extraction of total DNA and RNA from *P. oxalicum* strains was performed according to the method described by Zhao et al. (14). Briefly, a lysate reagent (40 mM Tris-HCl, 10 mM EDTA, 20 mM sodium acetate, and 1% sodium dodecy sulfate, pH 8.0) was added to grind mycelial powder at a ratio of 1:100 (vol:wt), incubated at room temperature for 10 min, and then centrifuged at 11,300 × g for 10 min to collect total DNA. Total RNA was extracted using a TRIzol RNA kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The concentrations of extracted total DNA and RNA were determined by measuring the A₂₆₀ and by electrophoresis on a 1% agarose gel.

**Light microscopy.** Colonies were photographed using a Canon EOS 6D digital camera (Canon, Beijing, China). For light or fluorescence microscopy, harvested hyphae were transferred into fresh MMM on a microscope slide and covered with a coverslip. Slides were analyzed using an Olympus DP480 microscope (Olympus Corporation, Tokyo, Japan). Photomicrographs were taken and analyzed using cellSens Dimension digital imaging software (Olympus).

**Enzymatic activity assay.** The activities of raw cassava starch-degrading enzymes and soluble starch-degrading enzymes were tested by measuring the reducing sugars released during hydrolysis. The 500-µl reaction mixtures consisted of 450 µl of 1% raw cassava flour or soluble potato starch in 0.1 M citrate-phosphate buffer (pH 4.5) and 50 µl of diluted enzyme solution. Reaction mixtures were incubated at 65°C for 30 min and then transferred to boiling water for 10 min to stop the reaction. Inactivated enzymes were used as a control. The concentration of reducing sugars released was determined using the 3,5-dinitrosalicylic acid method (47). One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of reducing sugars per min from the reaction substrates. Triplicate independent experiments were performed for each sample.

**RNA sequencing.** Total RNA sequencing of *P. oxalicum* strains was performed as described by Zhao et al. (14). Briefly, a DNA library with an average length of 100 bp was constructed for each sample and evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an ABI StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The constructed cDNA libraries were sequenced by use of an Illumina HiSeq 4000 system, and generated reads were mapped onto the genome of the wild-type strain HP-7 for functional annotation using BWA v0.7.10-r789 (http://sourceforge.net/projects/bio-bwa/files/) and Bowtie2 v2.1.0 (48). Expression levels (fragments per kilobase of exon per million mapped reads [FPKM]) were analyzed using RAEM v1.2.12 software (49) and the NOISeq tool (http://www.bioconductor.org/packages/release/bioc/html/NOISeq.html). Genes with log₂ ratios (\(\Delta_{\text{PoxNsdD}}\) _FPKM/\(\Delta_{\text{PoxKu70}}\) _FPKM) of ≥1 and probabilities of ≥0.8 were defined as significantly differentially expressed. Three biological replicates were analyzed for each sample.

**Quantitative reverse transcription-PCR.** The RT-qPCR assay was performed based on a previously described method (14). First-stand cDNA was synthesized using total RNAs from *P. oxalicum* strains as templates, using a PrimeScript RT reagent kit (TaKaRa Bio Inc., Dalian, China). Each RT-qPCR mixture contained 0.8 µl of 10 µM primers (Table 1), 0.2 µl of first-strand cDNA as the template, and 10 µl of SYBR Premix Ex Taq II (TaKaRa Bio Inc.). All reactions were run for 40 cycles of 3 s at 95°C and 30 s at 60°C. The fluorescence signal was measured at the end of each 80°C extension step. The relative expression of...
TABLE 1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POX06534/brlA-F</td>
<td>CCAGTGCCGCTTTCGTCAG</td>
</tr>
<tr>
<td>POX06534/brlA-R</td>
<td>GGAAGGGAAGTCGGGTTT</td>
</tr>
<tr>
<td>POX05587/Cel7A-2-F</td>
<td>GTACCTCCGATCTGATGAGG</td>
</tr>
<tr>
<td>POX05587/Cel7A-2-R</td>
<td>CCAAGGTGAGGAGCCTGTTG</td>
</tr>
<tr>
<td>POX01356/Amy13A-F</td>
<td>GCAACGGCCCTATCTGTCG</td>
</tr>
<tr>
<td>POX01356/Amy13A-R</td>
<td>GGGAGGTGCTGGTAATGG</td>
</tr>
<tr>
<td>POX01430/abl1/wA-F</td>
<td>TGGATGGTCCGCTTTGTAAG</td>
</tr>
<tr>
<td>POX01430/abl1/wA-R</td>
<td>GTAAAGGCGACGGGGTAG</td>
</tr>
<tr>
<td>POX03890/amyR-F</td>
<td>ACCCGTCAGGAACCCAC</td>
</tr>
<tr>
<td>POX03890/amyR-R</td>
<td>CATCCGATGCCGGTACG</td>
</tr>
<tr>
<td>POX09352/Amy13A-F</td>
<td>CAGGTCTTCAAGCCATCAG</td>
</tr>
<tr>
<td>POX09352/Amy13A-R</td>
<td>CCAAGCCAAACAGTCCCT</td>
</tr>
<tr>
<td>POX09352-Amy13-F</td>
<td>TCGGACCAACCCATAAAG</td>
</tr>
<tr>
<td>POX09352-Amy13-R</td>
<td>GAAACGCTTCTGATCCACACA</td>
</tr>
<tr>
<td>POX01356-Amy15-F</td>
<td>ATGAAGGATCTCAAGTACG</td>
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<tr>
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<td>FAM-AGTGATGGAGCTGTAGGAAAGA</td>
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<tr>
<td>POX03890-AmyR-R</td>
<td>FAM-CGGTCAGGAAGGAGAAGAGT</td>
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<td>POX06534/brlA-F</td>
<td>GGAACCTAAACCCGGCCCT</td>
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<tr>
<td>POX06534/brlA-R</td>
<td>FAM-CATGTCGCTGATTCTCCAAT</td>
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<td>POX01430/abl1/wA-F</td>
<td>AAAGGGTGTCCTGTCCAC</td>
</tr>
<tr>
<td>POX01430/abl1/wA-R</td>
<td>FAM-AAGGAGGAATGCTGTTG</td>
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<tr>
<td>POX5989/tubulin-F</td>
<td>ACCCTCATTGCCGGTCCTG</td>
</tr>
<tr>
<td>POX5989/tubulin-R</td>
<td>FAM-AACAGCTTATGAGTGAGGAGAACA</td>
</tr>
</tbody>
</table>

Recombinant expression of the sequence encoding the DNA-binding domain (PoxNsd) and electrophoretic mobility shift assays. Recombinant expression of the sequence encoding the putative DNA-binding domain (PoxNsd) in Escherichia coli, purification of the translated product, and EMSAs were performed as previously described (30). The DNA sequence encoding PoxNsd was flanked by Sall and Xhol restriction enzyme sites, was inserted into the expression vector pET-32a, which was digested with the same restriction enzymes. Cells harboring the resultant plasmid were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and cultured at 37°C for 20 h. The fusion protein containing PoxNsd alone with Trx, His, and S tags was purified using Ni-nitrilotriacetic acid (Ni-NTA) resin. Trx-His-S purified from E. coli cells harboring the empty vector pET-32a and bovine serum albumin (BSA) alone were used as negative controls. Meanwhile, ~1,000-bp fragments upstream of the ATG start codons of target genes, labeled with 6-carboxyfluorescein (FAM) at the 3’ terminus, were used as probes for EMSA, and a 500-bp DNA fragment from the promoter region of POX05989, encoding β-tubulin, was used as a negative control. The same DNA fragments without the FAM label were used as competitive probes following amplification with the corresponding primers (Table 1).

For in vitro binding experiments, ~40 ng of probe was mixed with various amounts (0 to 2.0 μg) of purified proteins in binding buffer (0.1 mg/ml BSA, 20 mM Tris-HCl, pH 8.0, 5% glycerol, 50 mM KCl, 1 mM dithiothreitol [DTT], and 0.5 μg sheared salmon sperm DNA) at 30°C for 30 min. For competitive binding experiments, a known amount of binding protein was mixed with various amounts of probes under the same conditions as those described above. Protein-DNA complexes were separated by agarose gel electrophoresis and visualized with a Bio-Rad ChemiDoc MP imaging system at an excitation wavelength of 489 to 506 nm.

Statistical analysis. The Student t test (two-tailed) was used for statistical analysis in Microsoft Excel (Office 2016, Microsoft, Redmond, WA, USA).

Accession number(s). All transcriptomic data are available from the Sequence Read Archive database under accession number SRA493765. The DNA sequence of PoxNsd is available from the GenBank database under accession number KY368171.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01039-18.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.
SUPPLEMENTAL FILE 2, XLS file, 0.2 MB.
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