Fungal deconstruction of the plant cell requires a complex orchestration of a wide array of intracellular and extracellular enzymes. In *Neurospora crassa*, CLR-1, CLR-2, and XLR-1 have been identified as key transcription factors regulating plant cell wall degradation in response to soluble sugars. The XLR-1 regulon was defined using a constitutively active mutant allele, resulting in hemicellulase gene expression and secretion under noninducing conditions. To define genes directly regulated by CLR-1, CLR-2, and XLR-1, we performed chromatin immunoprecipitation and next-generation sequencing (ChIPseq) on epitope-tagged constructs of these three transcription factors. When *N. crassa* is exposed to plant cell wall material, CLR-1, CLR-2, and XLR-1 individually bind to the promoters of the most strongly induced genes in their respective regulons. These include promoters of genes encoding cellulases for CLR-1 and CLR-2 (CLR-1/CLR-2) and promoters of genes encoding hemicellulases for XLR-1. CLR-1 bound to its regulon under noninducing conditions; however, this binding alone did not translate into gene expression and enzyme secretion. Motif analysis of the bound genes revealed conserved DNA binding motifs, with the CLR-2 motif matching that of its closest paralog in *Saccharomyces cerevisiae*, Gal4p. Coimmunoprecipitation studies showed that CLR-1 and CLR-2 act in a homocomplex but not as a CLR-1/CLR-2 heterocomplex.

**IMPORTANCE** Understanding fungal regulation of complex plant cell wall deconstruction pathways in response to multiple environmental signals via interconnected transcriptional circuits provides insight into fungus/plant interactions and eukaryotic nutrient sensing. Coordinated optimization of these regulatory networks is likely required for optimal microbial enzyme production.
known to be involved in plant biomass deconstruction or utilization but also genes encoding hypothetical proteins, uncharacterized transporters, and transcription factors. DNA binding motifs for CLR-1, CLR-2, and XLR-1 were identified, and physical interactions of CLR-1 and CLR-2 were explored. This in-depth study illuminated the regulation and interactions of genes/proteins involved in plant biomass degradation and provided hypotheses that will help guide the optimization of pathways for increased enzyme production in filamentous fungi.

RESULTS

CLR-1 target gene regulon. We first tested how variants of CLR-1 (including epitope tags, promoter sequences, and genome localization) affected chromatin immunoprecipitation-sequencing (ChIPseq) results. One strain contained a C-terminal green fluorescent protein (GFP)-tagged clr-1 allele regulated by the promoter from the clock-controlled-gene-1 (pccg-1-clr-1-gfp) strain, which is constitutively active under these experimental conditions (18), and integrated into the his-3 locus in a Δclr-1 deletion strain. A second clr-1 strain carried the smaller V5 epitope at the C terminus integrated at the resident clr-1 locus, thus preserving the native clr-1 promoter (clr-1-V5). The pccg-1-clr-1-gfp strain had reduced endoglucanase activity, while the clr-1-V5 strain had wild-type (WT) enzyme activity and protein secretion (Fig. 1A).

The control Δclr-1 strain showed no enzyme activity or protein secretion. Constitutive expression of clr-1-gfp via the pccg-1 promoter under sucrose conditions yielded no detectable enzyme activity; under these conditions, ccg-1 drives expression of downstream genes at higher levels than the clr-1 native promoter, even under conditions of cellulose (Avicel) exposure. These data indicate that the presence of CLR-1 under noninducing conditions was insufficient for induction of a cellulolytic response (Fig. 1B).

To define target promoters bound by CLR-1, we performed ChIPseq on clr-1-gfp and clr-1-V5 strain cultures switched to Avicel for 4 h, a condition that strongly induces lignocellulolytic genes (6, 19). A strain carrying cytosolic GFP under the regulation of the pccg-1 promoter was used as a control for normalization (see Materials and Methods). Comparison of the CLR-1-GFP and CLR-1-V5 libraries showed that 93% of the top 500 CLR-1-V5 binding sites overlapped with at least one of the CLR-1-GFP libraries (see Fig. S1 in the supplemental material). CLR-1-V5 peaks without a corresponding peak in the CLR-1-GFP libraries were characterized by lower fold enrichment, were located within nonpromoter regions, or had high background levels obscuring the signal. These results indicate that there was not a bias between the GFP and V5 epitopes and that promoter differences of the tagged clr-1 genes did not play a significant role in ChIPseq results.

Under Avicel conditions, CLR-1 was significantly enriched at 203 promoter regions representing 293 genes due to the presence of binding sites located in the promoter regions of 90 divergently transcribed genes. CLR-1 gene targets included 16 predicted glycosyl hydrolases, including the major exoglucanases encoded by cbh-1, gh6-2, and gh6-3 (20) (see Dataset S1 in the supplemental material). CLR-1 also bound at locations upstream of 8 putative transporter genes, including clt-2 (21) and the cellobionic acid transporter gene cht-1 (22, 23) and 6 transcription factor genes, including clr-2, xlr-1, vib-1 (all implicated in cellulase or hemicellulase regulation) (6, 8, 24, 25), cpc-1 (regulation of amino acid metabolism) (26, 27), a homolog to tanmA (nitrogen metabolism) (28), the circadian rhythm modulator gene freq, and NCU03184, which contains a zinc finger domain.

The binding profiles of CLR-1-GFP under sucrose versus cellulose (Avicel) conditions showed a large degree of overlap, with 68% of the Avicel-bound promoters also being bound under sucrose conditions (see Dataset S1 in the supplemental material). CLR-1 binding signals on sucrose were generally weaker than on Avicel, although many highly bound promoters on Avicel completely lacked signal on sucrose. Genes that exhibited Avicel-specific binding (see Dataset S1) included those encoding cellulases (cbh-1, gh6-2, gh5-1, gh61-4, gh2-2, gh11-2, gh55-1, and gh74-1), transporters (cht-1 and NCU11342), and xylose reductase (xyr-1). To validate our ChIPseq results, we conducted targeted ChiP-quantitative PCR (qPCR) experiments using four genes: gh6-3, cbb-1, gh6-2, and gh61-4. In keeping with our ChIPseq data, the ChiP-qPCR experiments showed that enrichment of CLR-1-GFP and CLR-1-V5 under Avicel conditions was more prominent at the promoters of gh6-3 and cbb-1 and less prominent at the promoters of gh6-2 and gh61-4 (see Fig. S2). ChiP-qPCR of CLR-1-GFP on sucrose also mirrored the ChIPseq data, with enrichment at the gh6-3 promoter but not at the cbb-1, gh6-2, or gh61-4 promoter, confirming their Avicel-specific binding patterns (see Fig. S2). These results also showed that CLR-1 was competent to bind the promoters of target genes, including...
Concordance of CLR-1 ChIPseq enrichment with differential expression of genes that require CLR-1 for induction. (A) Fold change in gene expression of the wild-type parental strain (FGSC 2489) versus the ∆clr-1 mutant on Avicel. Genes with significant binding in ChIP experiments are shown in red. (B) Venn diagram showing overlap of genes differentially expressed in the WT versus ∆clr-1 strain under noninducing conditions results in robust cellulolytic activity (9). These observations suggest fundamental differences between the regulatory mechanisms of CLR-1 and CLR-2. To better understand these differences, we performed ChIPseq on a strain containing N-terminally tagged mCherry-clr-2 that was regulated by the ccd-1 promoter and resided at the his-3 locus (mc-clr-2) (9). The mc-clr-2 strain grew normally on sucrose and showed robust growth on Avicel, with higher cellulase activity and protein secretion than the wild-type parental strain (Fig. 1A), which is consistent with previous observations (9).

The ChIPseq libraries from the mc-clr-2 strain grown on Avicel were normalized to a cytosolic mCherry ChIPseq library (see Materials and Methods). CLR-2 bound to 114 promoter sites upstream of 164 genes (see Dataset S1 in the supplemental material). As described above for CLR-1, we compared the MC-CLR-2 ChIPseq data set with the constitutively expressed clr-2 RNAseq

| NCU no. | Locus | Annotation or domain |
|---------|-------|----------------------|
| NCU00130 | ghi-1-1 | Intracellular β-glucosidase |
| NCU00206™ | clih-1 | Cellobiose dehydrogenase |
| NCU00326 | gh6-1 | Calcium homeostasis protein |
| NCU00762™ | gh5-1 | Glycosylhydrolyase family 5 |
| NCU00801™ | cdt-1 | Cellobextrin transporter |
| NCU00836™ | gh6-1-7 | Polysaccharide monoxygenase (AA9 family) |
| NCU01050™ | gh6-1-4 | Polysaccharide monoxygenase (AA9 family) |
| NCU01059 | gh47-3 | Glycosyl hydrolyase family 47 (alpha mannosidase) |
| NCU01944 | Hypothetical protein |
| NCU02240™ | gh6-1-6 | Polysaccharide monoxygenase (AA9 family) |
| NCU02485 | AMP-binding domain |
| NCU02915™ | RhoGAP domain |
| NCU02916™ | gh6-1-3 | Polysaccharide monoxygenase (AA9 family) |
| NCU05067™ | gh7-1 | Endoglucanase |
| NCU05574 | Acetyltransferase domain |
| NCU05846™ | Domain of unknown function DUF1479 |
| NCU05863 | ATPase (AAA) domain |
| NCU05864™ | Hypothetical protein |
| NCU05955™ | gh74-1 | Cel74a; xylolucanase |
| NCU06704 | gh6-3 | Ribosome-associated membrane protein RAMP4 |
| NCU07190™ | gh6-3 | Glycosylhydrolyase family 6 |
| NCU07339™ | Hypothetical protein |
| NCU07540™ | cbh-1 | Cellobiohydrolase |
| NCU07487 | gh6-3 | Periplasmic β-glucosidase |
| NCU07897™ | CET domain |
| NCU07898™ | gh61-13 | Polysaccharide monoxygenase (AA9 family) |
| NCU08042 | clr-2 | Transcription factor |
| NCU08115 | msh3 | DNA mismatch repair protein |
| NCU08412™ | Endo-β-1,4-mannanase |
| NCU08750 | Isoamyl alcohol oxidase |
| NCU08755 | gh3-3 | Secreted β-glucosidase |
| NCU08784 | Short-chain dehydrogenase domain |
| NCU09505 | Alpha/beta hydrolase domain |
| NCU09523™ | Hypothetical protein |
| NCU09524™ | Cellulose binding domain |
| NCU09680™ | gh6-2 | Glycosylhydrolyase family 6 |
| NCU09689 | Alpha/beta hydrolase domain |
| NCU09764 | gh61-14 | Polysaccharide monoxygenase (AA9 family) |
| NCU11342 | MFS hexose transporter |

* NCU numbers in bold represent promoter regions of genes bound by both CLR-1 and CLR-2.
* Promoter regions of genes (NCU numbers) bound by CLR-1 that may regulate 2 genes in opposite orientations.

CLR-2 target gene regulon. Constitutive expression of clr-1 in media lacking a cellulolytic inducer did not result in cellulase activity (Fig. 1B). In contrast, constitutive expression of ∆clr-2 under noninducing conditions results in robust cellulolytic activity (9). These observations suggest fundamental differences between the regulatory mechanisms of CLR-1 and CLR-2. To better understand these differences, we performed ChIPseq on a strain containing N-terminally tagged mCherry-clr-2 that was regulated by the ccd-1 promoter and resided at the his-3 locus (mc-clr-2) (9). The mc-clr-2 strain grew normally on sucrose and showed robust growth on Avicel, with higher cellulase activity and protein secretion than the wild-type parental strain (Fig. 1A), which is consistent with previous observations (9).
By combining ChIPseq and RNAseq data (9) obtained from strains carrying the constitutively expressed clr-2 allele, we identified 54 genes that were bound by CLR-2 and were dependent on CLR-2.

| TABLE 2 Genes upregulated and differentially expressed in wild-type strain versus a clr-2 constitutive expression strain and whose promoter region was bound by CLR-2 |
|---------------------------------------------------------------|
| NCU no. | Locus | Annotation or domain |
|--------|-------|----------------------|
| NCU0206<sup>+</sup> | cdh-1 | Cellobiose dehydrogenase |
| NCU00762<sup>+</sup> | gh5-1 | Glycosyl hydrolase family 5 |
| NCU00801<sup>+</sup> | cdt-1 | Celldextrin transporter |
| NCU00836<sup>+</sup> | gh61-7 | Polysaccharide monoxygenase (AA9 family) |
| NCU00870 | | SET domain |
| NCU01049<sup>+</sup> | | Fascidin domain |
| NCU01500<sup>+</sup> | gh61-4 | Polysaccharide monoxygenase (AA9 family) |
| NCU01076 | | Hypothetical protein |
| NCU01900<sup>+</sup> | gh43-2 | Xylosidase/arabinosidase |
| NCU01209 | apr-3 | Endothiasepin |
| NCU02138 | | Hypothetical protein |
| NCU02240<sup>+</sup> | gh61-1 | Celldextrin transporter |
| NCU02855 | gh11-1 | Endo-1,4-β-xylanase |
| NCU02915<sup>+</sup> | | RhoGAP domain |
| NCU02916<sup>+</sup> | gh61-3 | Polysaccharide monoxygenase (AA9 family) |
| NCU03180<sup>+</sup> | | Hypothetical protein |
| NCU03181<sup>+</sup> | | Acetylxylan esterase |
| NCU03328<sup>+</sup> | gh61-6 | Polysaccharide monoxygenase (AA9 family) |
| NCU03329<sup>+</sup> | Domain of unknown function (DUF3632) |
| NCU04850 | gh55-1 | Exo-β-1,3-glucanase |
| NCU04854 | gh7-2 | Endoglucanase |
| NCU04870<sup>+</sup> | cel-1 | Acetyl xylan esterase |
| NCU05057<sup>+</sup> | gh7-1 | Endoglucanase |
| NCU05121 | gh45-1 | Glycosyl hydrolase family 45 |
| NCU05846<sup>+</sup> | | DUF1479 |
| NCU05864<sup>+</sup> | | Hypothetical protein |
| NCU05924 | gh10-1 | Endo-1,4-β-xylanase |
| NCU05955<sup>+</sup> | gh74-1 | Cel74a; xyloglucanase |
| NCU05956<sup>+</sup> | gh2-2 | β-Galactosidase |
| NCU06277 | | Microtubule-associated protein domain |
| NCU07143 | | 6-Phosphogluconolactonase |
| NCU07190<sup>+</sup> | gh6-3 | Exoglucanase 3 |
| NCU07225<sup>+</sup> | gh11-2 | Endo-1,4-β-xylanase |
| NCU07326 | gh32 | Glycosyl hydrolase family 32 |
| NCU07339<sup>+</sup> | | Hypothetical protein |
| NCU07340<sup>+</sup> | chh-1 | Cellobiohydrolase |
| NCU07760 | gh61-2 | Polysaccharide monoxygenase (AA9 family) |
| NCU07787 | ccg-14 | Clock-controlled protein; cerato-platanin domain |
| NCU07897<sup>+</sup> | | HET domain |
| NCU07898<sup>+</sup> | gh61-13 | Polysaccharide monoxygenase (AA9 family) |
| NCU08114<sup>+</sup> | cdt-2 | Celldextrin transporter |
| NCU08397 | | Oligopeptide transporter domain |
| NCU08398 | | Aldose 1-epimerase |
| NCU08409 | trp-3 | Triptophan synthetase |
| NCU08412<sup>+</sup> | | Endo-1,4-mannanase |
| NCU08760 | gh61-5 | Polysaccharide monoxygenase (AA9 family) |
| NCU09416 | | Cellulose-binding GDSL lipase/acylhydrolase |
| NCU09523<sup>+</sup> | | Hypothetical protein |
| NCU09524<sup>+</sup> | cedt-1 | Celldextrin binding domain |
| NCU09582 | ce4-1 | Chitin deacetylase |
| NCU09680<sup>+</sup> | gh6-2 | Exoglucanase |
| NCU09764 | gh61-14 | Polysaccharide monoxygenase (AA9 family) |
| NCU09775 | gh54-1 | α-N-Arabinofuranosidase |

<sup>+</sup> NCU numbers in bold indicate promoter regions of genes bound by both CLR-1 and CLR-2.

<sup>+</sup> NCU numbers in bold and in italics indicate promoter regions of genes bound by both CLR-2 and XLR-1.

<sup>+</sup> NCU numbers indicate promoter regions that may regulate 2 genes in opposite orientations.
Coimmunoprecipitation experiments were performed with CLR-1 and CLR-2. (A) Coimmunoprecipitation experiments were performed on a strain bearing CLR-1-GFP and CLR-1-V5. Data are from the same gel, differentially blotted with either α-GFP or α-V5 antibodies. Intervening control and blank lanes were removed. CLR-1-V5 is 81 kDa, and CLR-1-GFP is 105 kDa. Molecular mass markers (135 kDa, 95 kDa, and 72 kDa) are shown in the left lane. (B) Coimmunoprecipitation experiments were performed with a strain bearing MC-CLR-2 and CLR-2-V5. Data are from the same gel, differentially blotted with either α-V5 or α-mCherry (mCh) antibodies. Intervening control and blank lanes were moved. CLR-2-V5 is 93 kDa in size, while MC-CLR-2 is 117 kDa. Molecular mass markers (135 kDa and 95 kDa) are shown in the right lane. (C) Lack of detection of coimmunoprecipitation of CLR-1-C/CLR-2 heterocomplexes in a strain bearing CLR-1-V5 and MC-CLR-2. Data are from the same gel, differentially blotted with either α-V5 or α-mCherry antibodies. Molecular mass markers (135 kDa, 95 kDa, and 72 kDa) are shown in the left lane. The intervening lanes were removed. (D) Cartoon of CLR-2 binding as a homodimer to its predicted DNA binding motif.

CLR-2 for expression (Fig. 3B and Table 2). These included genes encoding 31 enzymes predicted to act on plant-derived polysaccharides, two cellodextrin transporter genes (cdt-1 and cdt-2 [21]), a predicted oligopeptide transporter gene (NCU08397), seven genes encoding proteins with biochemical domains, four genes encoding enzymes with uncharacterized roles in plant cell wall deconstruction, and nine genes that either encoded hypothetical proteins or contained a conserved domain of unknown biochemical function (DUF or HET).

CLR-1 and CLR-2 function as homodimers. Over half of the genes that both were dependent upon functional CLR-1 for expression and had promoters that were bound by CLR-1 were also regulated and bound by CLR-2 (Tables 1 and 2). Five of these genes encoded lytic polysaccharide monooxygenases (LPMOs; AA9 family) involved in the oxidative cleavage of cellulose (32–34). In addition, the promoter of a cellodextrin dehydrogenase gene, cdh-1, which encodes an enzyme involved in pH-dependent electron transfer to LPMOs (33, 35), was also bound by both CLR-1 and CLR-2.

An inspection of the promoter regions of these 21 dually regulated genes showed that CLR-1 and CLR-2 bound in close proximity to each other. Dimerization of Zn2C6 transcription factors can occur via hydrophobic repeats that form a coiled-coil interaction region adjacent to the Zn2C6 domain (36). Analysis of CLR-1 and CLR-2 revealed a high probability of the presence of a coiled-coil structure in CLR-2 and, to a lesser extent, in CLR-1 (Fig. 4D; see also Fig. S3 in the supplemental material) (37). To test the hypothesis that CLR-1 and CLR-2 function either as homocomplexes or heterocomplexes, we first constructed strains that simultaneously expressed clr-1-gfp and clr-1-V5 (see Text S1). As shown in Fig. 4A, CLR-1-V5 coimmunoprecipitated with CLR-1-GFP from Avicel-exposed mycelia, indicating that CLR-1 forms a homocomplex. To assess whether CLR-2 forms a homocomplex, we constructed a strain that carried a clr-2 allele tagged with a V5 epitope at the clr-2 locus and which showed WT endoglucanase levels (Fig. 1). Using a strain bearing both mc-clr-2 and clr-2-V5 strain constructs, MC-CLR-2 and CLR-2-V5 were coimmunoprecipitated, indicating that CLR-2 also forms a homocomplex (Fig. 4B). To test whether CLR-1 and CLR-2 function in a heterocomplex, a strain was constructed that expressed clr-1-gfp and also expressed mc-clr-2 (see Text S1). However, although both CLR-1-GFP and MC-CLR-2 could be individually immunoprecipitated from the clr-1-gfp; mc-clr-2 strain (Fig. 4C), coimmunoprecipitation of CLR-1-GFP with MC-CLR-2 was not detected, suggesting that CLR-1 and CLR-2 do not form a heterocomplex. To investigate this further, we sought to determine if the ability of constitutively expressed mc and clr-2 genes to induce cellulase expression under sucrose conditions was dependent on the presence of CLR-1. To do this, we crossed the mc-clr-2 strain with a clr-1 deletion strain (Δclr-1). The resulting pcg-1-mc-clr-2; Δclr-1 strain was still capable of secreting cellulases even under sucrose conditions (Fig. 1B), supporting the notion that a CLR-1/CLR-2 heterocomplex is not a requirement for activation of cellulase gene transcription.

Construction of a xlr-1 mutant that expresses hemicellulases under noninducing conditions. It was recently shown that a point mutation in T. reesei xyr1 (Fig. 5A) rendered Xyrl constitutively active (38) and that overexpression of wild-type xyr1 was sufficient for activity under noninducing conditions (39). We therefore assessed whether constitutive expression of a strain with the wild-type xlr-1 gene (pcg-1-xlr-1-gfp strain) or a strain carrying the homologous T. reesei mutation (A828V) in xlr-1 (pcg-1-xlr-1A828V strain) resulted in constitutive hemicellulase expression in N. crassa. Neither the wild-type strain nor the strain with constitutively expressed xlr-1 secreted active xylanases under no-carbon conditions. However, a strain bearing the xlr-1A828V mutation secreted active xylanases when switched to no-carbon media (Fig. 5B) and secreted significantly more active xylanase than either the WT or xlr-1-gfp-tagged strain when switched to xylan (Fig. 5C).

RNAseq analyses of the xlr-1A828V mutant, the Δxlr-1 mutant, and the WT strain revealed the presence of both xlr-1-dependent and xlr-1-independent xylan-induced genes. As shown in Fig. 6A, the pattern of induction and expression of the dominant hemicellulase genes in the xlr-1A828V mutant under no-carbon conditions was remarkably similar to that of a WT strain exposed to xylan (see Dataset S3 in the supplemental material). A cluster of 50 xylan-inducible genes were responsive to the xlr-1A828V mutant and the WT strain under xylan conditions (Fig. 6B; cluster 1) and were dominated by xylanases and xylose-utilization genes. XLR-1-independent, xylan-induced genes in a second cluster (100 genes) were dominated by pectinases (Fig. 6B; cluster 2). These genes were induced in strains switched to pectin media (7), suggesting that this large cluster of genes is induced by pectin contamination of the xylan substrate and not by xylan per se.

XLR-1 target gene regulon. To identify direct targets of XLR-1, we used a pcg-1-xlr-1-gfp; Δxlr-1 strain, which showed endoxylanase activity and secreted protein levels comparable to those seen with the WT strain (Fig. 5C). Under xylan conditions, XLR-1-GFP bound to 63 sites, corresponding to promoters for 84 genes (see Dataset S1 in the supplemental material), including...
activity of the strain bearing the constitutively activating mutation in the alanine-to-valine mutation and alignment to the corresponding region of a parental wild type, a strain carrying pTAAA (Fig. 5A). 

Deletion mutants of xlr-1 showed enrichment for the motif GGN (Fig. 5B), and the gene for a xylose transporter [NCU06138] and two transcription factors (CLR-1, CLR-2, and XLR-1) formed direct-target regulons for CLR-1, CLR-2, and XLR-1. Within this network, the CLR-1, CLR-2, and XLR-1 regulons are clearly distinct but overlap in many of the issues associated with translating raw ChIPseq data into meaningful assignment of biological function. Network analyses showed that CLR-1, CLR-2, and XLR-1 bind the promoters of major hemicellulase genes and also of genes encoding xylosidase and xylan degradation, including the CDT-2 transporter (44). Two of these targets, cdtd and gh11-2, were also bound by CLR-1 (see Dataset S1 in the supplemental material), suggesting a direct role for all three transcription factors in their regulation. By combining genome-wide expression studies using RNAseq and direct binding studies using ChIPseq to identify the direct-target regulons for CLR-1, CLR-2, and XLR-1, we resolved many of the issues associated with translating raw ChIPseq data into meaningful assignment of biological function. Network analyses showed that CLR-1, CLR-2, and XLR-1 bind the promoters of genes encoding cellulases and hemicellulases and also of genes encoding a wide array of other transcription factors and transporters. These genes included genes encoding cellulases, xylanases, and other enzymes specific for other polymers (pectin, for example), while some genes with broader functionality (such as those encoding general oligosaccharide transporters) must be additively regulated. This gene set was dominated by genes encoding secrete enzymes required for deconstruction of xylan and by genes encoding enzymes involved in xylooligosaccharide or xylose utilization and a xylose/glucose transporter. In addition, other uncharacterized sugar transporters (NCU04537 and NCU05350) were in this gene set (Table 3).

Network analysis of lignocellulose deconstruction. The plant cell wall is a multivariate structure that requires the orchestrated and concerted action of enzymes involved in cellulose, hemicellulose, pectin, and lignin activity for deconstruction. Four target genes were directly bound by both CLR-2 and XLR-1 and were also differentially expressed in the clr-2 and xlr-1 mutant strains versus the WT strain. These genes included gh3-2 and gh5-3 (encoding xylosidase/arabinosidase), cel-1 (acetyl xylan esterase), gh11-2 and cdtd (cellodextrin transporter), which are predicted to be involved in xylan degradation, including the CDT-2 transporter (44). Two of these targets, cdtd and gh11-2, were also bound by CLR-1 (see Dataset S1 in the supplemental material), suggesting a direct role for all three transcription factors in their regulation. By combining genome-wide expression studies using RNAseq and direct binding studies using ChIPseq to identify the direct-target regulons for CLR-1, CLR-2, and XLR-1, we resolved many of the issues associated with translating raw ChIPseq data into meaningful assignment of biological function. Network analyses showed that CLR-1, CLR-2, and XLR-1 bind the promoters of genes encoding cellulases and hemicellulases and also of genes encoding a wide array of other transcription factors and transporters. These genes included genes encoding cellulases, xylanases, and other enzymes specific for other polymers (pectin, for example), while some genes with broader functionality (such as those encoding general oligosaccharide transporters) must be additively regulated. This gene set was dominated by genes encoding secrete enzymes required for deconstruction of xylan and by genes encoding enzymes involved in xylooligosaccharide or xylose utilization and a xylose/glucose transporter. In addition, other uncharacterized sugar transporters (NCU04537 and NCU05350) were in this gene set (Table 3).

Discussion

The regulatory network coordinating plant cell wall hydrolysis and utilization reflects the complex and variable nature of plant cell wall polysaccharides. Some dedicated enzymes (such as cellulases and hemicellulases) need to be regulated independently of enzymes specific for other polymers (pectin, for example), while some genes with broader functionality (such as those encoding general oligosaccharide transporters) must be additively regulated. In response to multiple signals. Concurrently with the restructuring of the transcriptional landscape, the metabolic stress of shift-
ing global protein expression to largely secreted proteins requires fine-tuning of many aspects of intracellular carbon metabolism, secretion, and even cellular morphology. In N. crassa, CLR-1, CLR-2, and XLR-1 form the nexus of this complex regulatory lignocellulosic deconstruction network.

This report presents genome-wide analyses of the three major transcription factors required for deconstruction of the major components of the plant cell wall. A total of 39 and 54 genes in a core set are directly bound and regulated by CLR-1 and CLR-2, respectively, under cellulosic conditions, and 23 genes are bound and regulated by XLR-1 under xylan conditions. The CLR-1, CLR-2, and XLR-1 regulons were distinct but overlapped in some of the most highly and differentially expressed genes. A prime example is the gene encoding cellodextrin transporter 2, \textit{cdt-2}, which is bound and differentially expressed and contains the XLR-1 and CLR-2 motifs and a partial CLR-1 motif (CGGNC CG). Regulation by all three transcription factors is consistent with recent findings indicating that \textit{cdt-2} encodes a generalized oligosaccharide transporter capable of transporting both cellodextrins and xylodextrins (44, 45). CLR-1, CLR-2, and XLR-1 also each bind to genes encoding additional transcription factors, including ones that have a role in regulating nutrient sensing under cellulolytic conditions in \textit{N. crassa}, such as \textit{vib-1} (24), \textit{cpc-1} (\textit{cross-pathway-control-1}) (25), \textit{sah-2} (29), and \textit{hac-1}, which regulates the unfolded protein response and was recently shown to be required for cellulose utilization (30, 31). These transcription factors could act as drivers for second-tier gene expression, allowing more-nuanced regulation in response to different carbon sources.

Previously, \textit{clr-1} was identified as a target of the white-collar complex (WCC) composed of WC-1 and WC-2 (46), which is the major blue light and clock regulator in \textit{N. crassa} (47, 48). Light affects expression of cellulases in both \textit{N. crassa} and \textit{T. reesei} (25, 49). During light and circadian regulation, WCC activates the major circadian regulator, \textit{frq}, which functions as a negative regulatory element in the circadian negative-feedback loop (48, 50). We found that CLR-1 bound the promoter region of \textit{frq} under sucrose conditions.

\textbf{FIG 6} Identification of XLR-1 regulon, direct targets, and XLR-1 binding site. (A) RNAseq analyses of the most highly expressed hemicellulase genes in the \textit{xlr-1A828V} strain relative to the WT strain and a \textit{Δxlr-1} mutant shifted to sucrose (sucr), no-carbon (nc), or xylan medium conditions. (B) Hierarchical clustering of gene expression of the strains shown shifted to sucrose, no-carbon, or xylan conditions. Genes within cluster 1 are dependent upon XLR-1 for expression. (C) Venn diagram depicting overlap of genes that show differential expression (DE), genes that have similar expression patterns through hierarchical clustering (HC) in the WT strain versus the \textit{xlr-1A828V} strain under no-carbon conditions (Cuffdiff; \textit{Padj} = <0.05; 4-fold), and genes that showed significant binding of XLR-1 in their promoter regions (XLR-1 ChIP). (D) Consensus binding sequence for XLR-1 based on promoter regions bound by XLR-1 in the ChIPseq data.
TABLE 3  Genes upregulated and differentially expressed in the wild-type strains versus a xlr-1A828V constitutive expression strain and whose promoter regions was bound by XLR-1

| NCU no. | Locus | Annotation or domain |
|---------|-------|----------------------|
| NCU00292 | ceo-3 | Carboxy esterase |
| NCU00709 | gh3-8 | β-Xylosidase |
| NCU00891 | Xdh | Xyitol dehydrogenase |
| NCU01900 | gh43-2 | Xylosidase/arabinosidase |
| NCU02343 | gh51-1 | Alpha-1, arabinofuranosidase |
| NCU03322 | NCU04401 | Fructose-bisphosphate aldolase |
| NCU04537 | Monosaccharide transporter |
| NCU04870 | cel-1 | Acetyl xylan esterase |
| NCU05159 | ece-2 | Acetyl xylan esterase |
| NCU05350 | NCU06138 | Major facilitator transporter |
| NCU06143 | xy-31 | Xylose transporter |
| NCU06143 | gh115-1 | Putative glucuronidase |
| NCU06490 | NCU07510 | Hypothetical protein |
| NCU07225 | NCU07510 | Hypothetical protein |
| NCU08114 | cdt-2 | Celloextrin/xylodextrin transporter |
| NCU08189 | gh10-2 | Endo-1,4-β-xylanase |
| NCU08384 | NCU09652 | Xylose reductase |
| NCU09652 | gh43-5 | β-Xylosidase |
| NCU099705 | GAL10-like; UDP-glucose-4-epimerase |
| NCU110110 | 3-Hydroxyisobutyrate dehydrogenase |
| NCU11353 | NCU02343 | α-Xylose kinase |

* NCU numbers in bold indicate promoter regions bound by CLR-2 and XLR-1.

and Avicel conditions. The conditions under which our experiments were performed for ChIPseq and RNAseq analyses reduced or eliminated the light and clock inputs. However, the binding of the clr-1 promoter by the WCC and binding of the promoter of frq by CLR-1 suggest interplay among light, clock regulation, and plant cell wall deconstruction in filamentous fungi, which deserves further investigation.

Promoter regions of genes directly bound by CLR-1, CLR-2, and XLR-1 included genes encoding a number of hypothetical proteins or proteins that have predicted functional domains but that do not have a characterized connection to plant biomass deconstruction or utilization. In particular, constitutive expression of CLR-2 and XLR-1A828V resulted in secretion of cellulase and hemicellulases, respectively, under noninducing conditions. The CLR-1/CLR-2 and XLR-1 regulons, as determined on the basis of RNAseq data, are large (212 and 243 genes, respectively); approximately 50% of the genes in each of these regulons encode hypothetical proteins or proteins with a generalized biochemical function, thus making establishing priorities for biochemical characterization difficult. Identifying the genes that are directly regulated by CLR-1, CLR-2, and XLR-1 considerably reduced this list of genes. For example, two hypothetical proteins (encoded by NCU06490 and NCU07510) and two uncharacterized transporters (encoded by NCU05350 and NCU04537) are bound by XLR-1, suggesting that these proteins play a role in xylan degradation/ utilization and sugar transport, respectively. Similarly, 20 genes in the CLR-2 direct regulon (Table 2) encode proteins that do not have an obvious role in deconstruction of plant biomass.

Although CLR-1 bound to two-thirds of its regulon under sucrose conditions, the promoters of many cellulase genes were not bound; unlike the results seen with clr-2, constitutive expression of clr-1 did not result in significant cellulase activity. These data indicate that CLR-1 requires an activating step, presumably via cellulose sensing. In addition, carbon catabolite repression (CCR) functions to repress expression of cellulolytic genes under noninducing conditions (51). It is possible that CLR-1 (and its targets) is also subject to regulation by CCR, which may affect its activity and ability to bind target genes, with binding to some targets more strongly affected than binding to others.

By leveraging the ChIPseq data, we identified DNA binding motifs for CLR-1, CLR-2, and XLR-1. In a recent study, the binding sites of over 1,000 transcription factors were determined in vitro by protein binding microarrays (PBM) (52); XLR-1 and CLR-1 were included in that analysis. The PBM analyses identified only the conserved “A” residue as significant; however, the overall sequence, including nucleotides below the confidence threshold, matches the XLR-1 motif identified here. For the CLR-1 motif, the PBM analyses identified the CGG triplet common to all Zn_{2}C_{6} transcription factors (52). The CLR-2 binding site was not identified via PBM analyses (52), but here we show that the clr-2 DNA binding motif is identical to that of S. cerevisiae Gal4p, which is the closest paralog to CLR-2 in the N. crassa genome, consistent with the finding that proteins with conserved DNA binding domains bind highly similar DNA sequences (52). CLR-2 does not target galactose utilization genes, highlighting the conserved nature of these transcription factors even as their target genes have diverged over time. In addition, the regulation of CLR-2 is clearly different from the posttranslational regulation of Gal4p (53). By directly assaying their DNA binding locations in vivo on model plant cell wall substrates and tying that binding to functional induction of target genes, we have reaffirmed several known components of that network and highlighted new points of coordination among...
polymer saccharification, sugar transport, carbon metabolism, nutrient sensing, and cellular physiology. To engineer the next generation of hypersecreting industrial strains, all of these aspects will need to be explored and manipulated.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type strain (FGSC 2489) and gene deletion mutants were obtained from the Fungal Genetics Stock Center (FGSC) (54, 55). A detailed list of the constructed his-3-pccg-1-mc-clr-2; Δclr-2::Hyg; sad-1::Hyg; rid-1 A, the his-3-pccg-1-xr-1::Hyg; Δxlr-1::Hyg a, the clr-1-V5::Hyg a, the clr-2-V5::Hyg a, his-3-pccg-1-xr-1-gfp; Δxlr-1::Hyg a, and the his-3-pccg-1-clr-1-gfp; Δclr-1::Hyg a strains is provided in Text S1 in the supplemental material. All strains were propagated on 2% sucrose Vogel’s minimal medium (VMM) slants, grown in the dark at 30°C for 2 days, and transferred to conditions of constant light at 25°C for all downstream experiments.

Enzyme and secreted protein assays. CMCase and xylanase activity assays were carried out according to the protocols of the manufacturer (Megazyme) (S-ACMCL and S-AXBL), with slight modifications. Reaction mixtures were miniaturized to 200 μl in 100 mM sodium acetate (pH 5.0), and a lower substrate concentration (0.3%) was used with 5 to 20 μl of culture supernatants. After incubation at 50°C for 30 min, uncleaved polymers were precipitated with 1 ml of ethanol and relative enzyme activities measured by absorbance of the supernatant at 590 nm. Total protein was determined by Bradford assays (BioRad).

Chromatin immunoprecipitation. Flasks containing 100 ml VMM–2% (wt/vol) sucrose were inoculated with 106 conidia and incubated 16 h at 25°C under conditions of constant light at 220 rpm. The experimental conditions for the ChiPseq data are provided in Table S1 in the supplemental material. The culture was filtered, rinsed with Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen), and transferred to 100 ml fresh VMM containing 1% cellulose (Avicel PH-101; Sigma-Aldrich), hem celulose (Beechwood xylan; Sigma-Aldrich), or sucrose as the sole carbon source for 4 h (3 biological replicates each for the clr-1-gfp, mc-clr-2, and xlr-1-gfp strains on Avicel, 3 biological replicates for the xlr-1-gfp strain on xylan, and 1 biological replicate for the clr-1-V5 strain on Avicel). In addition, N. crassa clr-1-gfp, mc-clr-2, pccg-1-gfp, and pccg-1-mCherry strains were grown for 16 h on sucrose and switched to Avicel for 24 h prior to fixation. Cells were fixed in 1% formaldehyde. After 15 min, the reaction was quenched by a 5-min incubation in 125 mM glycine. Cells were harvested by filtration, flash frozen in liquid nitrogen, and stored at −80°C. Chromatin immunoprecipitation was carried out using versions of previously published protocols (46) (briefly described in Text S1). ChiPseq files are available at the NCBI GEO database (accession no. GSE68517).

ChiPseq peak calling and motif analyses. Enriched peaks were identified with MACS (v1.4.2) (56) (see Dataset S1 in the supplemental material). Peaks that overlapped by 50% across replicates were identified with Bedtools (v2.16.2) (57). Peaks were manually curated to remove false positives. Surrounding genes were extracted with a custom Perl script. This list was manually curated to remove genes with no detectable transcription. A total of 300 bp of sequence data from either side of each summit were analyzed for enriched motifs with the MEME-Chip suite (v4.9.1): a compilation of MEME, DREME, CentriMo, and TomTom (58) (http://meme.nbcr.net/meme/).

Differential-expression analysis. RNAseq libraries included WT (FGSC 2489) and Δxlr-1 strains grown for 16 h in VMM and switched to xylan (Beechwood xylan; Sigma-Aldrich) for 4 h (3 biological replicates) and the xlr-1::Hyg a strain grown for 16 h in VMM and transferred to either sucrose or media containing no carbon source for 4 h (3 biological replicates). RNA libraries were generated following the Illumina protocols and sequenced on the Illumina HiSeq 2000 platforms. The expression sequence files are available at the NCBI GEO database (accession no. GSE68517). Mapping and analyses were as previously described (9) (see Text S1 in the supplemental material).

Hierarchical clustering analysis was performed with the Cluster 3.0/TreeView software suite (http://bonsai.hgc.jp/~mdehoon/software/clustertree/software.htm). Fragments per kilobase per million (FPKMs) were normalized with the average linkage method with Pearson’s centered correlation as the similarity metric. Network analysis was performed using cytoscape 3.1.1 (59).

Coimmunoprecipitation experiments. Strains were grown for 16 h on VMM and subsequently switched to Avicel for 4 h. One gram of mycelia was ground and suspended in 2 ml DPBS buffer with protease inhibitors (0.1 M phenylmethylsulfonyl fluoride [PMSF], Complete EDTA-free protease inhibitors). The suspension was processed with a Dounce homogenizer 10 times and cross-linked with 3 mM dithiobis succinimidyl propionate (DSP) for 2 h. The reaction was quenched with 1 M Tris (pH 7.5) to reach a final concentration of 25 mM Tris for 15 min at room temperature. Final concentrations of 1% NP-40 and 0.5% deoxycholate were added to disrupt nuclear membranes. Immunoprecipitation was carried out as described above for ChiP (mouse anti-GFP [Roche; 11811446001], rabbit anti-m-Cherry [BioVision; 5993-100], and rabbit anti-V5 [Abcam; ab9116]). Western blot analyses were performed as previously described (see Text S1 in the supplemental material).

ChiP-qPCR analysis. A CFX Connect real-time PCR machine (Bio-Rad) and DyNaMo HS Sybr green master mix (Thermo Scientific) were used for qPCR experiments. All primers (see Text S1 in the supplemental material) were assessed for amplification efficiency using a serial dilution of genomic DNA (data not shown). Negative controls (NC-1, NC-2, NC-3, and NC-4) were composed of regions devoid of nearby genes, and primers for the promoter region of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (NCU01528) were designed for use as the nontarget control. qPCR was carried out with chromatin-immunoprecipitated DNA, and fold enrichment was determined by comparing the antibody immunoprecipitated fraction to a no-antibody precipitated control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi:10.1128/mBio.01425-15/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.

Dataset S1, XLSX file, 0.2 MB.

Dataset S2, XLSX file, 0.1 MB.

Dataset S3, XLSX file, 1.3 MB.

Figure S1, PDF file, 0.2 MB.

Figure S2, PDF file, 0.4 MB.

Figure S3, PDF file, 0.1 MB.

Figure S4, PDF file, 1 MB.

Table S1, PDF file, 0.1 MB.

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J.P.C. performed the ChiP work, motif analysis, Co-IP work, and RNAseq analysis and drafted the manuscript. S.T.C. performed the xlr-1 mutation analysis, RNAseq library construction, and enzyme assays and aided in drafting the manuscript. T.L.S. created vector pTS12 and edited the manuscript. N.L.G. was involved in the study’s conception and design and in preparation and editing of the manuscript.

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