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Authors

Huberman, Lori B

Liu, Jason

Qin, Lina

et al.

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Review

Regulation of the lignocellulolytic response in filamentous fungi



Lori B. HUBERMAN, Jason LIU, Lina QIN, N. Louise GLASS*

The Energy Biosciences Institute and the Department of Plant and Microbial Biology, University of California, Berkeley, CA 94704, USA

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ABSTRACT

Lignocellulose is an abundant waste product of agricultural and processing industries that can be utilized as a renewable fuel substitute for petroleum-based fuels. Saprophytic filamentous fungi are an important source of plant cell wall degrading enzymes necessary to break down the complex carbohydrates found in plant cell walls into simple sugars. Zinc binuclear cluster transcription factors activate the transcription of plant cell wall degrading enzymes when fungal cells are exposed to plant biomass. Nutrient sensing pathways that prioritize the use of preferred carbon sources act upstream of these transcription factors to inhibit the energy intensive production of cell wall degrading enzymes when unnecessary, while downstream feedback from the fungal secretory system also acts to regulate the production of these enzymes. Understanding the regulation of the fungal lignocellulolytic response will be important as we strive to increase the efficiency of production of these enzymes.

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1. Introduction

Lignocellulose is an abundant waste product of many agricultural and processing industries that can be utilized for the production of renewable, carbon-neutral cellulosic fuel substitutes for petroleum based fuels. One of the major bottlenecks in the economic feasibility of cellulosic fuels is the cost-efficiency of converting complex carbohydrate polymers found in lignocellulose into soluble sugars for fermentation.

Market studies have shown that ~25 cents of every dollar of cellulosic bioethanol goes to the production of the enzymes that are used to degrade lignocellulose (Humbird et al., 2011). Saprophytic filamentous fungi are capable of degrading the carbohydrate polymers in the plant cell wall, including cellulose, hemicellulose, and pectin, through secretion of glycosyl hydrolases and other carbohydrate active enzymes (CAZymes). The current industrial standard of cellulase production comes from a mutagenized strain of the soft mold,

Abbreviations CAZymes, carbohydrate active enzymes; CCR, carbon catabolite repression; PKA, cAMP dependent protein kinase A; ER, endoplasmic reticulum; UPR, unfolded protein response; ERAD, endoplasmic reticulum-associated degradation.

* Corresponding author.

E-mail addresses: huberman@berkeley.edu (L. B. Huberman), jason.liu@berkeley.edu (J. Liu), bioqinln@berkeley.edu (L. Qin), lglas@berkeley.edu (N. L. Glass).

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Trichoderma reesei. Originally isolated from the Solomon Islands off of canvas tents in the 1950's, *T. reesei* was later realized to be a prolific source of cellulases in the 1970's through mutagenesis studies (Mandels and Reese, 1957; Mandels et al., 1971). Only in the last couple decades, however, has the mechanism of lignocellulolytic gene regulation been revealed.

While there are a number of pathways involved in CAZyme regulation, we focus on three broad cellular mechanisms: direct transcriptional regulation, upstream regulation by nutrient sensing pathways, and regulatory feedback from the secretory pathway (Fig 1). The expression of genes encoding CAZymes important for plant biomass deconstruction is directly regulated by transcription factors that activate transcription when cells are exposed to carbohydrates found in plant cell walls. General nutrient sensing pathways that prioritize the use of preferred carbon sources act upstream of the

direct activation of genes encoding CAZymes to inhibit the energy intensive production of plant biomass degrading enzymes. Feedback from downstream factors, such as the fungal secretory system, also acts to regulate the production/secretion of these enzymes.

2. Direct transcriptional regulation of genes encoding lignocellulolytic enzymes

Saprophytic filamentous fungi have evolved to degrade lignocellulose through the expression and secretion of plant cell wall degrading enzymes. In order to utilize the available nutrients in the most efficient manner, filamentous fungi only express these CAZymes when plant cell wall material is available in the absence of preferred carbon sources. The transcriptional induction of plant cell wall degrading enzymes is

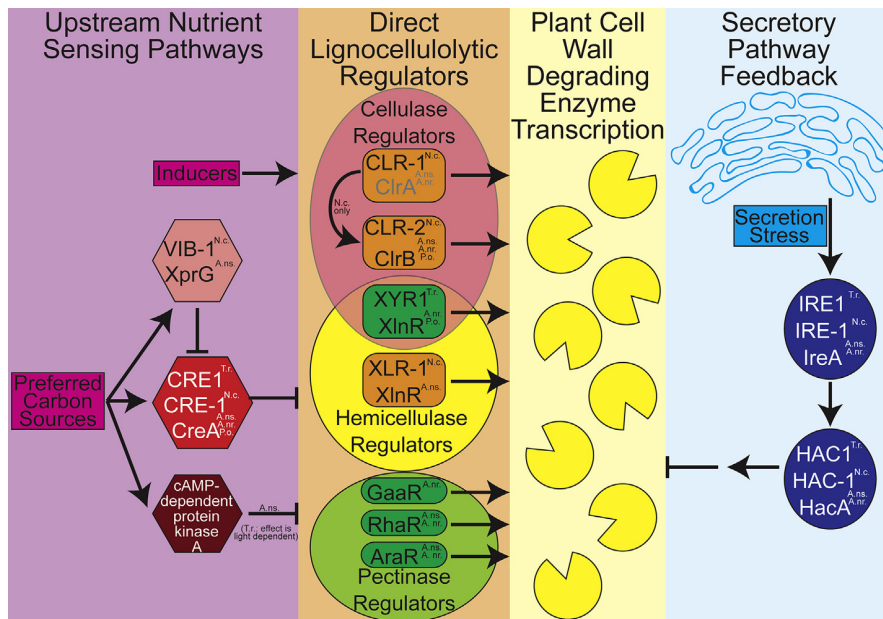


Fig. 1 – Filamentous fungi control transcription of lignocellulolytic CAZymes through direct regulators, upstream nutrient sensing pathways, and downstream secretory pathway feedback. *Upstream nutrient sensing pathways:* Cells prioritize the utilization of preferred carbon sources over plant biomass through the transcription factor CRE1/CRE-1/CreA, which is regulated in part by VIB-1/XprG. When activated, CRE1/CRE-1/CreA represses transcription of both direct lignocellulolytic regulators and plant cell wall degrading enzymes. The cAMP-dependent protein kinase A (PKA) also plays a role in nutrient sensing, although its effect is species dependent. Inducers derived from the carbohydrates in plant cell walls activate lignocellulolytic regulators. *Direct lignocellulolytic regulators:* In *T. reesei*, *A. niger*, and *P. oxalicum*, XYR1/XlnR activates transcription of both cellulase and hemicellulase genes, while in *N. crassa* and *A. nidulans*, XLR-1/XlnR only regulates transcription of hemicellulase genes. Cellulase gene transcription in *N. crassa*, *A. nidulans*, and *A. niger* is regulated by CLR-1/ClrA and CLR-2/ClrB. In *N. crassa*, CLR-1 activates transcription of some genes necessary for cellulose utilization as well as the main transcriptional activator of cellulase transcription, CLR-2. The CLR-2 homolog, ClrB is involved in cellulase transcription in *A. nidulans*, *A. niger*, and *P. oxalicum*. ClrA appears to play a small role in the regulation of cellulase transcription in *A. nidulans* and *A. niger*, although it does not control ClrB transcription. Pectin utilization is regulated by several transcription factors, which each regulate the utilization of individual pectin components: GaaR regulates galacturonic acid utilization, RhaR regulates rhamnose utilization, and AraR regulates arabinose utilization. *Secretory pathway feedback:* Secretion stress activates IRE1/IRE-1/IreA, which cleaves a non-canonical intron from the transcription factor *hac1/hac-1/hacA* allowing for its activation. HAC1/HAC-1/HacA activates the unfolded protein response, which may cause downregulation of the transcription of plant cell wall degrading enzymes. (A.nr. = *Aspergillus niger*, A.ns. = *Aspergillus nidulans*, N.c. = *Neurospora crassa*, P.o. = *Penicillium oxalicum*, T.r. = *Trichoderma reesei*).

tightly controlled by transcription factors that are specifically activated by the presence of plant cell wall material.

Primary transcription factors

In fungi, the zinc binuclear cluster class of transcription factors is often involved in the regulation of nutrient sensing pathways. The most prominently studied member of this class is the transcriptional regulator of the galactose utilization pathway in *Saccharomyces cerevisiae*, GAL4 (MacPherson et al., 2006). Like the *S. cerevisiae* galactose utilization pathway, primary transcription of genes encoding the major lignocellulolytic enzymes in many species of filamentous fungi is promoted by a zinc binuclear cluster transcription factor. In the industrially important species, *T. reesei* and *Aspergillus niger*, the major transcriptional regulator of cellulose and hemicellulose degrading enzymes is XYR1/XlnR (Rauscher et al., 2006; van Peij et al., 1998a; van Peij et al., 1998b). However, in *Neurospora crassa* and *Aspergillus nidulans* primary transcription of genes encoding cellulases and hemicellulases has been decoupled. In *N. crassa*, the transcription factor CLR-2 drives expression of genes required for cellulose degradation, and the orthologous protein, ClrB, modulates the expression of cellulolytic enzymes in *A. nidulans* (Coradetti et al., 2012, 2013). Hemicellulose degradation is regulated by XLR-1 and XlnR, the *N. crassa* and *A. nidulans* orthologues of XYR1,

respectively (Klaubauf et al., 2014; Sun et al., 2012; Tamayo et al., 2008). In species such as *Penicillium oxalicum*, cellulase and hemicellulase gene regulation appears only partially decoupled, with roles for both ClrB and XlnR in cellulase regulation (Li et al., 2015) (Fig 1 and Table 1).

Pectin is a far more heterogeneous component of lignocellulosic biomass than either cellulose or hemicellulose, and this may account for the lack of a single transcription factor regulating pectin utilization (Mohnen, 2008). Several conserved zinc binuclear cluster transcription factors required for the regulation of genes encoding enzymes important for the transport and utilization of the component saccharides have been identified. In *Botrytis cinerea* and *A. niger*, the utilization of galacturonic acid, the most abundant pectin component, is regulated by GaaR (Alazi et al., 2016; Zhang et al., 2016), while in *A. niger* and *A. nidulans*, the utilization of rhamnose and arabinose, two minority pectin components, is regulated by RhaR and AraR, respectively (Battaglia et al., 2011a, 2011b; Gruben et al., 2014; Pardo and Orejas, 2014) (Fig 1 and Table 1). The identification of additional transcription factors may be necessary to fully elucidate the regulation of pectin utilization.

In *T. reesei*, over-expression of XYR1 increases the transcription of downstream targets, but does not activate the full hemicellulolytic response (Mach-Aigner et al., 2008). Thus, XYR1/XlnR/XLR-1 requires activation/de-repression

Table 1 – Orthologs of the transcription factors discussed in this review.

Regulated by	<i>N. crassa</i> OR74A	<i>A. nidulans</i> FGSC A4	<i>A. niger</i> CBS 513.88	<i>T. reesei</i> QM6a	<i>P. oxalicum</i> 114-2	Role
Cellulose	<i>ace1</i>	<i>sltA</i>	An16g02040	<i>ace1</i>	<i>aceA</i>	Negative regulator of cellulase transcription
	NCU09333	AN2919		Trire2_75418	PDE_01988	
	N/A	N/A	N/A	<i>ace2</i>	N/A	Positive regulator of cellulase transcription
	N/A	N/A	N/A	Trire2_78445		
				<i>ace3</i>	PDE_09536	
				Trire2_77513		
Cellulose and hemicellulose	<i>clr-1</i>	<i>clrA</i>	<i>clrA</i>	N/A	PDE_04046	
	NCU07705	AN5808	An05g00020			
	<i>clr-2</i>	<i>clrB</i>	<i>clrB</i>	Trire2_26163	<i>clrB</i>	
	NCU08042	AN3369	An12g01870		PDE_05999	
	NCU04058	AN4324	An04g00480	Trire2_65315	<i>clrC</i>	
					PDE_09023	
Cellulose and hemicellulose			<i>xlnR^a</i>	<i>xyr1^a</i>	<i>xlnR^a</i>	Positive regulator of cellulase and hemicellulase transcription
			An15g05810	Trire2_122208	PDE_07674	
Hemicellulose	xlr-1^b	xlnR^b				Positive regulator of hemicellulase transcription
Pectin	NCU06971	AN7610				
	NCU09033	<i>rhaR</i>	<i>rhaR</i>	Trire2_79871	PDE_00828	Positive regulator of rhamnose utilization
		AN5673	An13g00910			
	NCU04295	AN10548	<i>gaaR</i>	Trire2_67339	PDE_06088	Positive regulator of galacturonic acid utilization
Preferred carbon source	N/A	<i>araR</i>	An04g00780	N/A	PDE_04461	Positive regulator of arabinose utilization
		AN0388	An04g08600			
	<i>cre-1</i>	<i>creA</i>	<i>creA</i>	<i>cre1</i>	<i>creA</i>	Negative regulator of lignocellulase transcription
	NCU08807	AN6195	An02g03830	Trire2_120117	PDE_03168	
ER stress	<i>hac-1</i>	<i>hacA</i>	<i>hacA</i>	<i>hac1</i>	PDE_01436	Positive regulator of unfolded protein response
	NCU01856	AN9397	An01g00160	Trire2_46902		

Phenotypic evidence exists for genes in bold.

a Orthologs regulated by cellulose and hemicellulose.

b Orthologs regulated by hemicellulose.

prior to the induction of (hemi)cellulase gene transcription. Activation/de-repression can occur through a single, conserved amino acid substitution in XYR1/XLR-1 that is sufficient for constitutive expression of the major cellulases and hemicellulases in *T. reesei* and for constitutive expression of hemicellulase genes in *N. crassa*. The conserved amino acid lies inside a putative domain of unknown function found in Gal4-like zinc binuclear cluster transcription factors (Craig et al., 2015; Derntl et al., 2013). How this region regulates function of XYR1/XLR-1 is not clear, nor is the mechanism of activation/de-repression of XYR1/XLR-1 understood.

Aberrant expression of *N. crassa* CLR-2 through a constitutive promoter drives a nearly complete cellulase transcriptional response, even during growth on a normally repressive carbon source, indicating that CLR-2 does not require post-translational activation (Coradetti et al., 2013). This is not the case for other CLR-2 orthologs, however, since constitutive expression of *A. nidulans* and *P. oxalicum* ClrB does not result in constitutive activation of cellulolytic genes (Coradetti et al., 2013; Li et al., 2015). In *N. crassa*, regulation of *clr-2* transcription is governed by a second zinc binuclear cluster transcription factor, CLR-1 (Coradetti et al., 2012). The direct transcriptional targets of CLR-1 also include genes necessary for the breakdown of cellulose and import of soluble products (Craig et al., 2015). Together, CLR-1 and CLR-2 transcriptionally activate genes that make up the full cellulolytic response. However, unlike *clr-2*, constitutive expression of *clr-1* does not cause expression of target genes, although CLR-1 binds cis-regulatory elements even under non-inducing conditions (Coradetti et al., 2012; Craig et al., 2015). These data indicate that CLR-1 also requires activation/de-repression. Elucidation of the molecular mechanism by which CLR-1 and XYR1/XlnR/XLR-1 are activated/de-repressed will be an important step in understanding transcriptional regulation of lignocellulolytic genes.

Although XYR1/XlnR/XLR-1 regulate different target genes in different fungi, the binding motifs of XYR1/XlnR in *T. reesei* and *A. niger* show conservation with those of *N. crassa* XLR-1 (Craig et al., 2015; Furukawa et al., 2009; van Peij et al., 1998a). These three species have a conserved 5'-GGCTWW-3' consensus sequence in the 5' cis regulatory elements of the target genes (Craig et al., 2015; van Peij et al., 1998a; Zeilinger et al., 1998). The promiscuity of regulons amongst homologous transcription factors is further reflected as *N. crassa* CLR-2 has a consensus sequence nearly identical with its closest yeast paralogue, Gal4, but without a clear function in the *N. crassa* galactose utilization pathway (Bram and Kornberg, 1985; Craig et al., 2015; Giniger et al., 1985). The evolution of CLR-2/ClrB and XYR1/XlnR/XLR-1 as the major transcription factors of CAZymes is poorly understood and may represent a functional shift in environmental niches from an ancestral lifestyle.

Accessory transcription factors

Two accessory transcription factors in *T. reesei*, ACE1 and ACE2, were originally identified from a yeast one-hybrid screen that promoted activation of a selectable marker tethered to the promoter of the cellulase gene, *cbh1* (Saloheimo et al., 2000). While both ACE1 and ACE2 promote transcription

of genes driven by the *cbh1* promoter in *S. cerevisiae*, the two transcription factors appear to have opposing roles in the regulation of cellulase transcription *in vivo*. *T. reesei* cells lacking *ace2* had no change in the expression of cellulases on sophorose (a gratuitous inducer of cellulases in *T. reesei* (Mandels et al., 1962; Sternberg and Mandels, 1979)), but decreased cellulase expression after growth on cellulose (Aro et al., 2001). Conversely, cells lacking *ace1* showed an increase in the transcription of the major cellulases, implying a negative regulatory role for ACE1 (Aro et al., 2003). A third *T. reesei* accessory transcription factor, ACE3, which plays a positive role in CAZyme regulation, was identified by mining transcriptome data for transcription factors that appear to be co-regulated with genes involved with lignocellulose utilization. Several other transcription factors, including the *T. reesei* *clr-2* homolog, also appear to be co-regulated with genes encoding CAZymes or sugar transporters and could be targets for future investigation (Hakkinen et al., 2014).

Studies on the promoter region of the *T. reesei* cellulase gene *cbh2* identified an 11 base pair region necessary for transcriptional activation, termed the *cbh2* activating element, or CAE. Characterization of this region showed a multi-transcription factor binding arrangement that led to the identification of a potential role for the HAP2/3/5 complex in cellulase regulation, as well as possible binding sites for XYR1 and ACE2 (Zeilinger et al., 1998, 2001). In *S. cerevisiae*, the HAP complex functions in glucose-repressible transcriptional activation (Guarente et al., 1984). A study investigating nucleosome positioning at the *cbh2* regulatory region also implicated the shifting of nucleosome complexes in the promotion of *cbh2* transcription (Zeilinger et al., 2003).

There is some evidence for a role for accessory transcription factors in other fungal species. The *A. niger* *clr-1* and *clr-2* homologs, *clrA* and *clrB*, have been implicated in lignocellulase gene regulation (Raulo et al., 2016), and a basic leucine zipper transcription factor, ClrC, modulates cellulase expression in *P. oxalicum* (Lei et al., 2016). Homologs of *ace1* are present in the genomes of *N. crassa*, *A. nidulans*, *A. niger*, and *P. oxalicum*, although these species lack clear homologs of *ace2* and only *P. oxalicum* has a close *ace3* homolog. However, a role for homologs of *ace1/ace2/ace3* in regulating CAZyme gene transcription has not been established in these other species. Similarly, while clear homologs of *P. oxalicum* ClrC exist in *A. nidulans* and *A. niger*, these homologs have not been implicated in the lignocellulolytic response. In *N. crassa*, chromatin immunoprecipitation-high throughput sequencing (ChIP-seq) of sequences bound by CLR-1, CLR-2, and XLR-1 identified several uncharacterized transcription factors, potentially involved in modulating the enzyme response to plant cell wall carbohydrates (Craig et al., 2015).

3. Upstream nutrient sensing pathways regulate lignocellulase transcription

While direct transcriptional induction of the genes involved in plant cell wall degradation is the first step of enzyme production, there are many competing signals that control whether these enzymes will be produced. One of these mechanisms,

known as carbon catabolite repression (CCR), involves the downregulation of genes necessary for consumption of a less preferred carbon source when a more preferred carbon source is present.

CreA/CRE1/CRE-1-mediated carbon catabolite repression

In filamentous fungi, CCR is regulated by the zinc binuclear cluster transcription factor, CreA/CRE1/CRE-1. CreA was first identified in *A. nidulans* (Arst et al., 1990; Arst and Bailey, 1977; Bailey and Arst, 1975) and later homologs were identified in many other filamentous fungi, including *N. crassa* and *T. reesei* (de la Serna et al., 1999; Ilmen et al., 1996). In *T. reesei*, a *cre1* truncation mutant was identified after selection for CCR mutants in an effort to generate cellulase hyper-secreting strains (Ilmen et al., 1996). The regulation of CreA/CRE1/CRE-1 appears to be at least partially dependent on intracellular localization. Under repressing conditions, CreA/CRE1/CRE-1 is found in the nucleus where it can regulate the expression of target genes, and when under de-repressing conditions, CreA/CRE1/CRE-1 exits the nucleus (Brown et al., 2013; Cupertino et al., 2015; Lichius et al., 2014). However, the genetic regulation of CreA/CRE1/CRE-1 appears to be fairly divergent amongst the filamentous fungi. In *A. nidulans*, the AMP-activated kinase, SnfA, is necessary for nuclear export of CreA (Brown et al., 2013), but in *T. reesei*, while phosphorylation of CRE1 is necessary for de-repression of target genes, this phosphorylation is not dependent on the SnfA homolog (Cziferszky et al., 2002, 2003).

Although CCR has primarily been thought of as glucose-related repression, in organisms capable of consuming a wide variety of carbon sources of varying complexity, like saprophytic filamentous fungi, CCR has expanded to include a range of preferred carbon sources. During the utilization of plant cell wall carbohydrates, CCR can be activated not only by exogenously added sugars, but also by breakdown products resulting from plant cell wall degradation. For example, in *A. nidulans*, xylose is an inducer of xylanase genes, but this response to xylose can be severely curtailed by the concurrent presence of glucose in the media (Orejas et al., 1999, 2001; Piñaga et al., 1994). Cells containing mutations in CreA have substantial transcription of xylanase genes even in the presence of glucose. These mutations in CreA also vastly increase the amount of xylanases produced in the presence of xylose as a sole carbon source. Presumably, xylose acts not only as an inducer of xylanase gene expression through the transcriptional activator XlnR, but also as a repressor of xylanase gene expression through the transcriptional repressor CreA (Orejas et al., 1999, 2001; Piñaga et al., 1994; Tamayo et al., 2008). In *N. crassa*, deletion of *cre-1* causes an increase in cellulase production in strains grown on crystalline cellulose, presumably due to repression caused by the glucose, cellobiose, and other cellulose breakdown molecules released by the secreted cellulases (Sun and Glass, 2011).

Studies on the transcriptional response to CCR have elucidated that the mechanism of CreA/CRE1/CRE-1 repression works through regulation of both the genes necessary for degradation and consumption of plant cell wall material as well as the transcription factors which control their expression (Fig 1 and Table 1). In *A. nidulans*, CreA regulates xylanase genes as well as expression of their transcriptional regulator, xlnR

(Orejas et al., 1999, 2001; Tamayo et al., 2008). In *N. crassa* and *P. oxalicum*, regulation of cellulolytic genes as well as the transcription factor *clr-2/clrB* is thought to be under CCR control (Coradetti et al., 2012; Li et al., 2015; Sun and Glass, 2011; Xiong et al., 2014b). Like in *N. crassa* and *A. nidulans*, in *T. reesei*, CRE1 regulates genes necessary for utilization of plant biomass as well as the transcription factors which regulate their expression. Interestingly, expression of the primary regulator of cellulase genes, *xyr1*, appears to be repressed by CRE1 when glucose is available, while also requiring CRE1 for full expression under some inducing conditions (Antonieto et al., 2014; Portnoy et al., 2011). As transcriptomics and proteomics become an increasingly commonplace tool in biological investigations, additional mechanisms of CreA/CRE1/CRE-1-mediated CCR as well as the specific response of cells to soluble carbohydrates released from plant biomass will likely be revealed.

Other factors involved in carbon catabolite repression

Although CreA/CRE1/CRE-1 is an important player in CCR, other proteins also regulate CCR and the expression of genes encoding plant cell wall degrading enzymes (Fig 1). The cAMP-dependent protein kinase A (PKA) is a highly conserved protein kinase involved in the sensing of sugar and the regulation of metabolism, which often plays an opposing role to the AMP-kinase, SnfA/SNF1 (Barrett et al., 2012; Santangelo, 2006; Thompson-Jaeger et al., 1991). In *T. reesei*, a homolog of a PKA catalytic subunit, *pkac1*, regulates cellulase gene expression, most likely through the regulation of upstream transcription factors, such as *xyr1*, and may also mediate interactions between cellulase production and the circadian clock (Schuster et al., 2012). In *A. nidulans*, a PKA catalytic subunit, PkaA affects cellulase production and CCR. When grown on crystalline cellulose, cells lacking *pkaA* express glycosyl hydrolases, as well as their upstream regulators *xlnR* and *clrB*, at higher levels earlier in the cellulose response. Cells lacking PkaA are partially glucose-blind, allowing cellulase production under conditions where CCR is active in wild type cells (de Assis et al., 2015).

Another actor in the glucose response, which may be regulated by PKA, is a transcription factor involved in the regulation of extracellular proteases in response to carbon and nitrogen starvation, *vib-1/xprG* (de Assis et al., 2015; Dementhon et al., 2006; Katz et al., 1996, 2006). In *N. crassa*, VIB-1 is necessary for full cellulase and hemicellulase induction and appears to be an upstream regulator of genes involved in CCR, including *cre-1* and the *N. crassa* homologs of two genes with potential roles in the ubiquitination pathway, *creB* and *creD* (Boase and Kelly, 2004; Hynes and Kelly, 1977; Kelly and Hynes, 1977; Lockington and Kelly, 2001; Xiong et al., 2014b). VIB-1 also regulates the expression of *col-26*, the *N. crassa* homolog of *T. reesei* *bglR*, a zinc binuclear cluster transcription factor that positively regulates glycosyl hydrolase expression (Nitta et al., 2012; Xiong et al., 2014b).

From the above studies, it is clear that there are unidentified genes/proteins involved in CCR, and the mechanism of action of many identified genes, such as *col-26/bglR*, require additional elucidation. Although there are several highly conserved nutrient-sensing pathways that have been implicated in the lignocellulosic response, their role in the regulation of plant cell wall degrading enzyme transcription is still unclear. For

example, the TOR complex is involved in nutrient sensing in eukaryotes and has been shown to be highly phosphorylated under cellulolytic conditions in *N. crassa* (Wullschleger et al., 2006; Xiong et al., 2014a). Putative ubiquitination and deubiquitination enzymes, such as CreB/CRE-2 and CreD, have been implicated in CCR and glycosyl hydrolase production potentially through ubiquitination of CreA itself, however, additional protein targets and the precise mechanism of action of the genes involved has yet to be identified (Boase and Kelly, 2004; Colabardini et al., 2012; Denton and Kelly, 2011; Lockington and Kelly, 2001, 2002; Ries et al., 2016; Xiong et al., 2014b). The elucidation of the role of these and other highly conserved actors like the AMP-activated kinase, SnfA/SNF1, in the repression of plant cell wall degrading enzymes could help illuminate the regulation of transcription under cellulolytic conditions as well as nutrient-sensing pathways in eukaryotic species.

4. Post-translational regulation of lignocellulase enzymes

Once plant cell wall degrading enzymes have been transcribed and translated, their regulation continues as they are trafficked through the secretory pathway. During the lignocellulolytic response, nascent glycosyl hydrolases are sent to the endoplasmic reticulum (ER) to be sorted and processed for secretion. This targeting to the ER results in increased protein flux through the secretory pathway, which can be accompanied by significant ER stress due to an increased demand for protein folding, disulfide bond formation, glycosylation, and sorting. Alleviating this stress is required to achieve the level of enzyme secretion typically seen in fungi involved in plant cell wall degradation and is accomplished through the activation of several regulatory feedback mechanisms (Benz et al., 2014; Tanaka et al., 2015). The involvement of these feedback mechanisms in the lignocellulosic response is a fairly new area of study, but several pathways have been implicated in linking secretion to lignocellulase enzyme production: the unfolded protein response (UPR), ER-associated degradation (ERAD), and transcriptional repression under secretion stress.

Unfolded protein response

The UPR is an evolutionarily conserved phenomenon activated when unfolded proteins accumulate in the ER. This ER stress response was initially observed in mammalian cells expressing a mutant form of a virus that was unable to properly fold, and later found to act in the secretory pathway in fungal cells (Kozutsumi et al., 1988; Normington et al., 1989; Rose et al., 1989). Although there are some major differences between the metazoan and fungal UPR, the basic mechanism is highly conserved amongst fungi (Hollien, 2013) and has been most well characterized in *S. cerevisiae*. In *S. cerevisiae*, accumulation of unfolded proteins in the ER activates Ire1, an ER-membrane spanning protein with endonuclease function. When activated, Ire1 cleaves a non-canonical intron from the mRNA of the basic leucine-zipper transcription factor, HAC1, releasing it from transcriptional inhibition (Sidrauski and Walter, 1997). Efficiently translated Hac1 activates the transcription of a broad set of genes that allow for

enhanced ER folding capacity and more efficient protein trafficking (Kaufman, 1999; Mori et al., 1996). A similar role for IRE1 and HAC1 orthologs in the UPR has been reported for a number of filamentous fungal species (Arvas et al., 2006; Guillemette et al., 2007; Montenegro-Montero et al., 2015; Mulder et al., 2004; Saloheimo et al., 2003; Sims et al., 2005).

The UPR may also play a role in balancing the need for increased protein secretion with ER processing capacity during the lignocellulolytic response (Fig 1 and Table 1). In *N. crassa*, expression of *ire-1*, *hac-1*, and other proteins associated with the UPR is upregulated in response to crystalline cellulose (Benz et al., 2014). Deletion of either *ire-1* or *hac-1* results in a reduction in cellulase secretion, although cellulase transcription is unaffected (Fan et al., 2015; Montenegro-Montero et al., 2015). In a *T. reesei* strain selected for increased cellulase secretion, the UPR is activated earlier in the lignocellulolytic response than in wild type cells, although it is not clear how this affects glycosyl hydrolase production (Wang et al., 2014). Further investigation into how the UPR affects cellulase quality control and flux through the secretory pathway during cellulase secretion will be necessary to fully understand its role during the lignocellulolytic response.

Other ER stress responses

Two other protein quality control mechanisms that may function during the lignocellulolytic response and may be under the control of the UPR are ERAD and transcriptional repression under secretion stress (Carvalho et al., 2012; Fan et al., 2015; Travers et al., 2000). Proteins that are not correctly folded even after an extended stay in the ER are targeted for proteasomal degradation via the ERAD pathway (Lippincott-Schwartz et al., 1988; Sommer and Jentsch, 1993). Although the role of ERAD during the lignocellulolytic response has not been investigated in detail, it may play a role, since mutations that may inhibit accurate folding of CBH1 in *T. reesei* cause the upregulation of genes involved in ERAD as well as co-localization of the mutant CBH1 with the proteasome (Kautto et al., 2013). Transcriptional repression under secretion stress is a poorly understood phenomenon where filamentous fungi downregulate transcription of lignocellulose degrading enzymes when exposed to chemical stresses that target the secretory system or during expression of constitutively active HacA/HAC1/HAC-1 (Al-Sheikh et al., 2004; Carvalho et al., 2012; Pakula et al., 2003). Although there have been several studies which have identified promoter regions that may be necessary for this phenomenon, the transcription factors and other genes involved in mediating this process are still unknown (Pakula et al., 2003; Zhou et al., 2015). Future investigation will be necessary to determine the genetic actors involved in this feedback mechanism as well as a concrete role for transcriptional repression under secretion stress during the lignocellulolytic response.

5. Conclusions and future directions

Understanding the regulation of the lignocellulosic response by filamentous fungi will be necessary as we strive to make lignocellulosic biofuels a viable component of our renewable

energy repertoire. When filamentous fungi sense the presence of plant cell wall carbohydrates, cells activate transcription of enzymes necessary for complex carbohydrate degradation primarily via zinc binuclear cluster transcription factors. These CAZymes are also under the transcriptional control of signaling pathways that are involved in nutrient sensing or in sensing the energy state of the cell. Regulation of enzyme production continues post-translationally through phenomena including, but likely not limited to, the UPR, ERAD, and transcriptional repression under secretion stress.

Although the identity of many of the primary transcription factors that activate CAZymes is known, constitutive expression of these transcription factors is not sufficient for a full lignocellulolytic response, indicating that post-translational activation or de-repression of these transcription factors is necessary (Coradetti et al., 2012; Derntl et al., 2013; Mach-Aigner et al., 2008; Tamayo et al., 2008). It is obvious that insoluble substrates, like cellulose itself, are competent to induce the cellulolytic or hemicellulolytic response, but it is likely that soluble sugars constitute the intracellular activation signal. The β -1,4-glucose dimer, cellobiose, and a transglycosylated form of this sugar, sophorose, are sufficient to elicit a cellulolytic response in *N. crassa* cells lacking the three major β -glucosidases and *T. reesei*, respectively (Mandels et al., 1962; Sternberg and Mandels, 1979; Znameroski et al., 2012). However, these molecules do not appear to be universal inducers, suggesting that each species may respond to a slightly different signal (Gielkens et al., 1999; Suzuki et al., 2010; Znameroski et al., 2012). Importantly, we do not know whether the inducer molecule directly activates primary transcription factors or whether additional, unidentified genes are involved.

Transcriptional activation or de-repression by nutrient sensing pathways is also necessary for full induction of the lignocellulolytic response. The effect of CCR is the best studied of these nutrient sensing pathways in filamentous fungi, but while the transcriptional effects of its most prominent player, CreA/CRE1/CRE-1, have been fairly well studied, there are clearly other proteins, such as VIB-1/XprG and COL-26/BglR, whose mechanisms of action require further study (de Assis et al., 2015; Nitta et al., 2012; Xiong et al., 2014b). Additionally, there are other highly conserved pathways that sense the nutritional state of the cell, such as PKA and TOR complex activation, that have been implicated in the lignocellulosic response, but whose role in plant cell wall degrading enzyme production needs further clarification (de Assis et al., 2015; Xiong et al., 2014a).

While it is well established that the lignocellulolytic response is under tight transcriptional control, the volume of proteins sent through the secretory pathway when filamentous fungi degrade plant cell wall material appears to induce significant stress on the secretory system. This may be why activation of the UPR and ERAD is associated with CAZyme secretion (Fan et al., 2015; Tanaka et al., 2015). Filamentous fungi also appear to respond transcriptionally to secretion stress. Future studies into the mechanism of how cells detect ER stress and the signal transduction mechanisms involved in transcriptional feedback from transcriptional repression under secretion stress will contribute to our understanding of the role that the secretory pathway plays in regulation of the

lignocellulolytic response. Recent work has also implicated other conserved mechanisms that affect secretion of plant cell wall CAZymes, including components of the clathrin complex and the sterol regulatory element binding protein (SREBP) pathway (Pei et al., 2015; Reilly et al., 2015). Characterization of these pathways and others that affect the secretion of plant cell wall CAZymes may help us to better understand how filamentous fungi have evolved to become the dominant plant biomass degrading organisms in nature.

Elucidation of nutrient sensing, carbon utilization, and secretion stress pathways can be harnessed in filamentous fungi to increase the efficiency of lignocellulosic breakdown for biofuels and specialty products to begin the long road necessary to replace fossil fuels as our go-to source of hydrocarbons.

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Lori B. Huberman is a postdoctoral fellow at the Energy Biosciences Institute at UC Berkeley in the laboratory of Dr. N. Louise Glass, where she works on nutrient sensing in *N. crassa*. She did her graduate work on mating in *S. cerevisiae* in Dr. Andrew Murray's laboratory at Harvard University, and received undergraduate degrees in Biology and Music from the Massachusetts Institute of Technology.

Jason Liu is a graduate student in Dr. N. Louise Glass's lab in the Energy Biosciences Institute at UC Berkeley where he studies cellulase secretion in *N. crassa*. He received his undergraduate degree from Northwestern University.

Lina Qin is a postdoctoral scholar in Dr. N. Louise Glass's lab at the Energy Biosciences Institute, UC Berkeley. She earned her B.S degree in biochemistry from Shanxi University in China and a PhD degree in biochemistry and molecular biology from Institute of Microbiology, Chinese Academy of Sciences. She is involved in a project using systems biology approaches

to study the mechanism of plant cell wall deconstruction by lignocellulolytic filamentous fungi.

N. Louise Glass earned her graduate degree in plant pathology at the University of California, Davis. She was a post-doctoral associate at the University of Wisconsin–Madison before starting an assistant professorship at the University of British Columbia (UBC) in the Department of Botany and The

Biotechnology Laboratory. In 1999, she joined the faculty at University of California, Berkeley (UCB), and ascended to the rank of professor in the Plant and Microbial Biology Department. In addition to her UCB faculty appointment, Glass was recently named Director for the Environmental Genomics & Systems Biology (EGSB) Division, Lawrence Berkeley National Laboratory.