Conservation and diversity of the regulators of cellulolytic enzyme genes in ascomycete fungi

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Abstract

In the last decade, various transcriptional activators of cellulolytic enzyme genes have been identified in ascomycete fungi. The regulatory system of cellulolytic enzymes is partially conserved, but also significantly diverse. For example, *Trichoderma reesei* has a system distinct from those of *Aspergillus* and *Neurospora crassa*: the former utilizes Xyr1 (the *Aspergillus* XlnR ortholog) as the major regulator of cellulolytic enzyme genes, while the latter use CLR-2/ClrB/ManR orthologs. XlnR/Xyr1 and CLR-2/ClrB/ManR are evolutionarily distant from each other.

Regulatory mechanisms that are controlled by CLR-2, ClrB, and ManR are also significantly different, although they are orthologous factors. Expression of *clr-2* requires the activation of another transcription factor, CLR-1, by cellobiose, while CLR-2 is constitutively active for transactivation. By contrast, ClrB activation requires cellobiose. While ClrB mainly regulates cellulolytic genes, ManR is essential to the activation of not only cellulolytic but also mannanolytic enzyme genes.

In this review, we summarize XlnR/Xyr1- and CLR-2/ClrB/ManR-dependent regulation in *N. crassa*, *A. nidulans*, *A. oryzae* and *T. reesei*, and emphasize the conservation and diversity of the regulatory systems for cellulolytic enzyme genes in these ascomycete fungi. In addition, we discuss on the role of McmA, an another transcription factor that plays an important role in recruiting ClrB to the promoters in *A. nidulans*.

Key words: cellulases, XlnR/Xyr1, CLR-2/ClrB/ManR, McmA, transcription factor

Introduction

A great number of glycoside hydrolase genes are present in the genome of a single filamentous fungus, with some possessing over 300, according to genome databases. Many of these genes are involved or predicted to be involved in the degradation of plant-derived polysaccharides, including starch, xylan, β-mannan, and cellulose. Most filamentous fungi, whether they are saprophytic or pathogenic, utilize plant-derived polysaccharides as a major source of carbon and energy. Filamentous fungi proliferate on the surface and/or inside of plants or plant residues, forming multicellular filaments. Such a lifestyle requires the ability to produce a wide variety of glycoside hydrolases to hydrolyze all the polysaccharides available on site.

Expression of polysaccharide-degrading genes is finely regulated, so that only a limited group of genes is activated, even in the presence of multiple polysaccharides. Such finely tuned regulation is mainly achieved through the induction and repression by transcriptional activators and repressors. Induction requires low molecular weight inducers, typically monosaccharides or disaccharides, which are specific to and derived from the polysaccharide to be degraded. By contrast, a wide variety of carbon sources act to repress the expression of polysaccharide-degrading genes. This negative regulatory system is called carbon catabolite repression (CCR), and regulates the expression of carbon source utilization genes in the presence of a better carbon source. Consequently, expression of genes for the utilization of a certain carbon source requires both the presence of an inducing molecule and the absence of repressing molecules. A typical case is the regulation of cellulolytic enzyme production. When cellulose or its degradation products, but not glucose, are solely present, expression of cellulolytic enzyme genes is induced by transcriptional activators. If other carbohydrates are present

at the same time, production of cellulolytic enzymes is significantly inhibited. Such regulation is reasonable considering that cellulose is the most recalcitrant polysaccharide owing to its crystalline structure. Filamentous fungi must have evolved a finely tuned regulatory mechanism to rank down the use of cellulose as a carbon source.

Uncovering molecular mechanisms underlying the hierarchy of polysaccharide utilization is critical to understanding the survival strategies of filamentous fungi in natural environments. This understanding also provides novel insights into the breeding of industrial fungi, with the aim of improving the production of biomass degrading enzymes. Due to the global interest in efficient biomass utilization as a strategy to cope with global warming, this field of research has been highly active in the last decade. Extensive research has been carried out with model filamentous fungi such as *Neurospora crassa* and *Aspergillus nidulans*, as well as industrial fungi such as *A. niger*, *A. oryzae*, and *Trichoderma reesei*. Here, we discuss the current knowledge on the regulation of cellulase production, with special focus on the main transcriptional activators involved.

Transcription factors involved in the regulation of cellulolytic enzyme genes

Many transcriptional activators are involved in the regulation of cellulolytic enzyme genes (Table 1). These are roughly classified into two groups based on whether or not they are essential for induction in the presence of cellulose. The essential transcriptional activators include: *N. crassa* CLR-1 and CLR-2, *A. nidulans* ClrB, *A. oryzae* ManR, *Penicillium oxalicum* ClrB, and *T. reesei* Xyr1 and Ace3 (Coradetti et al. 2012; Häkkinen et al. 2014; Li et al. 2015; Ogawa et al. 2013; Stricker et al. 2006), among which CLR-2, ClrB, and ManR are orthologs. The other group consists of the

transcriptional activators required for full expression but not essential to induction. For example, the Hap complex enables an elevated level of transcription of the endoglucanase gene (*eglA*) in *A. nidulans* (HapB/C/E), and the cellobiohydrolase gene (*cbh2*) in *T. reesei* (Hap2/3/5) (Endo et al. 2008, Tanaka et al. 2001; Zeilinger et al. 2001). In *A. nidulans*, PacC upregulates expression in response to alkaline pH (Kunitake, et al. 2016). In the other ascomycetes, PacC orthologs also modulate the expression of cellulolytic genes in response to ambient pH, but whether the regulation is positive or negative varies depending on the organism (Campos Antonieto et al. 2017; Häkkinen et al. 2015; He et al. 2014). In *A. aculeatus*, ClbR enhances the expression of cellulolytic enzyme genes in the presence of cellulose, but significant induction is still detectable in the ClbR deletion mutant (Kunitake et al. 2015; Kunitake et al. 2013). *A. nidulans* McmA is involved in induction of the major cellulolytic enzyme genes by interaction with ClrB, but McmA-independent induction by ClrB has also been observed (Li et al. 2016a; Yamakawa et al. 2013). Ace2, a transcriptional activator specific to *T. reesei*, partially participates in the expression of cellulolytic genes (Aro et al. 2001). It binds to a sequence similar to that of Xyr1 on the *cbh1* promoter, and modulates its expression on cellulose.

As shown in Table 1, most transcriptional activators involved in cellulolytic enzyme genes have a Zn2C6 ($Zn(II)_2Cys_6$) DNA-binding motif that is specific to fungi. Due to this characteristic motif, they are frequently called Zn2C6 transcription factors or fungal specific transcription factors, but they exhibit striking differences in their regulatory mechanisms. For example, the activity of *Saccharomyces cerevisiae* Gal4p in galactose utilization is regulated by the Gal3p-controlled interaction of Gal4p and Gal80p, in which Gal80p inhibits Gal4p-dependent transcriptional activation (Bhat and Iyer 2009).

Hap1p, which regulates genes required for respiration, forms a large complex with molecular chaperones Hsp90, 70, and 40. Once a heme binds to Hap1p, the complex structure changes to enable its DNA-binding ability (Lan et al. 2004). Put3p and Leu3p activities are inhibited by intramolecular masking of their activation domains in the absence of inducing molecules (Sellick and Reece 2003; Wang et al. 1999). While these factors are present in the nuclei under both inducing and non-inducing conditions, *Aspergillus* transcription factors NirA and AmyR, which are involved in nitrate assimilation and starch utilization, respectively, are regulated at the level of nuclear localization. They mainly exist in the cytoplasm under non-inducing conditions and accumulate in the nuclei under inducing conditions (Bernreiter et al. 2007; Makita et al. 2009; Murakoshi et al. 2012).

A number of Zn2C6 transcription factors specifically regulate the utilization of certain carbon and nitrogen sources, and some examples have been described in the previous paragraph. All the transcriptional activators that are so far known to be essential to the induction of cellulolytic enzyme genes are also Zn2C6 transcription factors. Thus, clarification of the regulatory mechanisms supported by these factors in cooperation with the non-essential factors is important for understanding the conservation and diversity of the cellulose utilization system in filamentous fungi. It should be noted that currently available information is mainly derived from not only model fungi *N. crassa* and *A. nidulans*, but also *A. oryzae*, *A. niger*, and *T. reesei*, which are used for specific industrial purposes.

XlnR/Xyr1-mediated regulation of cellulolytic enzyme genes

XlnR and its orthologs are essential to the expression of xylanolytic enzyme genes

(Klaubauf et al. 2014). A single exception is *Magnaporthe oryzae* Xlr1, which regulates pentose catabolism genes, but not xylanolytic enzyme genes (Battaglia et al. 2013). However, the degree of their involvement in cellulase regulation differs greatly depending on the fungus. In *T. reesei*, Xyr1 is essential for the expression of both xylanolytic and cellulolytic enzyme genes in the presence of xylan as well as cellulose (Stricker et al. 2006), while the involvement of XLR-1 in *N. crassa* and Xyr1 in *Fusarium graminearum* in the regulation of cellulolytic enzyme genes is not significant (Brunner et al. 2007; Sella et al. 2016; Sun et al. 2012). In *Aspergillus*, XlnR is essential for the expression of cellulolytic enzyme genes in the presence of xylan or D- xylose (Gielkens et al. 1999; Marui et al. 2002a; Noguchi et al. 2009; van Peij et al. 1998). In addition, in *A. oryzae*, XlnR is weakly involved in their expression in the presence of cellulose or cellobiose, as its overexpression causes elevated expression of the targets (Marui et al. 2002a). These observations suggest a minor role of the XlnR orthologs in the regulation of cellulolytic enzyme genes in most filamentous fungi, except for *T. reesei*. *T. reesei* might have employed specific tactics for biomass degradation.

There are a few reports on the molecular mechanisms of XlnR-dependent regulation. Hasper et al. conducted a molecular study of *A. niger* XlnR, and proposed that a putative coiled-coil region is involved in its nuclear import. The C-terminus downstream of the coiled-coil region has an inhibitory function, which is followed by the activation domain at the extreme C-terminus (Hasper et al. 2004). Noguchi et al. reported that *A. oryzae* XlnR is rapidly phosphorylated in the presence of D-xylose and dephosphorylated by its removal (Noguchi et al. 2011). This suggests that XlnR activity is regulated by reversible phosphorylation in response to the presence or absence of D-xylose at least in *A. oryzae*. Our recent studies revealed that alanine substitution of

two serine residues (S556 and S552) in *A. oryzae* XlnR abolishes the inducer-dependent phosphorylation and expression of the xylanolytic enzyme genes (unpublished data). The serine residues and the surrounding sequence are highly conserved among XlnR orthologs and paralogs of ascomycete fungi, including *N. crassa* and *T. reesei*, implying that inducer-dependent and reversible phosphorylation is a widespread mechanism to regulate the activity of XlnR orthologs and paralogs. In *A. oryzae*, XlnR weakly regulates cellulose- and cellobiose-induced expression (Marui et al. 2002a); however, cellobiose does not cause XlnR phosphorylation. This implies that induction by cellobiose involves a mechanism different from that by D-xylose.

In *T. reesei*, Xyr1 is essential to the induction of both xylanolytic and cellulolytic enzyme genes (Fig. 1) (Stricker et al. 2006). Another regulator, Ace3, is also essential to the expression of cellulolytic enzyme genes but only modulates that of xylanolytic enzyme genes (Häkkinen et al. 2014). The essential involvement of both Xyr1 and Ace3 for the expression of cellulolytic genes suggests that both the inducing molecules and an unidentified environmental signal are required for Xyr1/Ace3-dependent expression, which is not required for the expression of xylanolytic genes. Xyr1 and Ace3 cross-regulate each other, as deletion of one partially down-regulates the other (Häkkinen et al. 2014; Ma et al. 2016). Uncovering the precise regulatory mechanisms of Xyr1 and Ace3 is of great concern, because this partial down-regulation does not explain the absolute requirement of both factors for the expression of cellulolytic genes in the presence of cellulose. However, the Ace3-dependent system is not widely conserved among ascomycetes, because only a few limited species possess an Ace3 ortholog (Huberman et al. 2016). *Aspergillus*, *A. fumigatus*, and *A. clavatus* possess the ortholog, but *A. nidulans*, *A. oryzae* and *A. niger* do not. *N. crassa* also does not possess

an ortholog, while *P. oxalicum* does.

CLR-2/ClrB/ManR-mediated regulation of cellulolytic enzyme genes

In contrast to *T. reesei*, the XlnR orthologs do not play a major role in the induction of cellulolytic enzyme genes in other ascomycetes. CLR-2 in *N. crassa*, ClrB in *A. nidulans*, and ManR in *A. oryzae* are the transcription factors essential for cellulase induction in the presence of cellulose and cellobiose (Fig. 1) (Coradetti et al. 2012, Coradetti et al. 2013; Ogawa et al. 2012; Ogawa et al. 2013). These factors are orthologous to each other, but their regulatory mechanisms are strikingly different. Induction of cellulolytic enzyme genes in *N. crassa* appears to involve serial steps: recognition of cellobiose by the transceptors CDT-1 and CDT-2 (Znameroski et al. 2014), expression of *clr-1* and activation of its product CLR-1, *clr-2* expression mediated by CLR-1, and finally activation of cellulolytic enzyme genes by CLR-2 (Coradetti et al. 2012; Coradetti et al. 2013). As CLR-2 is active even in the absence of the inducers, induction of *clr-2* directly correlates with the expression of the cellulolytic enzyme genes.

In an experiment combining RNA-seq and ChIP-seq, Craig et al. identified 39 possible targets of CLR-1 in response to an inducer (Avicel), including eighteen enzyme genes predicted to be involved in plant biomass degradation, two transporter genes, and *clr-2* (Craig et al. 2015). Considering the experimentally observed essential roles of both CLR-1 and CLR-2 for the production of cellulolytic enzymes, direct up-regulation by CLR-1 of plant biomass-degrading genes might be an initiation step, boosting cellulolytic enzyme production by CLR-2.

In *A. nidulans*, induction of cellulolytic enzyme genes requires inducing molecules,

even if *clrB* is constitutively expressed, indicating that ClrB is not constitutively active like CLR-2 (Coradetti et al. 2013). In addition, *clrB* expression is not tightly regulated by the presence of cellulose because *clrB* expression increases only 2.5-fold in the presence of cellulose as compared to that in the presence of no carbon source (Coradetti et al. 2013). These results suggest that ClrB activity is directly regulated by inducers. The slight up-regulation might serve to enhance the expression of cellulolytic enzyme genes after the inducing molecules have been sensed. Although the precise mechanisms underlying the activation of ClrB by cellobiose or cellulose are yet to be clarified, a putative cellobiose transceptor in *A. nidulans* CltB might be involved in signal transduction (Dos Reis et al. 2016). *A. nidulans* possesses the *clr-1* ortholog *clrA*, but the effect of its deletion is minor as compared to that of the deletion of *clr-1* in *N. crassa*, causing only a 2- to 4-fold decrease in cellulase gene expression (Coradetti et al. 2012). Thus, CLR-1 and ClrA obviously participate differently in cellulase gene regulation in *N. crassa* and *A. nidulans*. While CLR-1 is essential to the expression of cellulolytic enzyme genes, ClrA only modulates their expression levels. Interestingly, *T. reesei* does not possess a CLR-1 ortholog despite its wide conservation as a transcription factor among ascomycete fungi (Coradetti et al. 2012; Tani et al. 2014), again suggesting a distinct evolutionary path in *T. reesei*.

Although ClrB in *A. nidulans* and ManR in *A. oryaze* are close orthologs, with over 60% amino acid sequence identity, their physiological roles are slightly different. ManR is essential for the expression of cellulolytic and mannanolytic enzyme genes (Ogawa et al. 2012; Ogawa et al. 2013). However, in the regulation of mannanolytic enzyme genes in *A. nidulans*, the major role is played not by ClrB but by its paralog AN6832 (Li et al., 12th European Conference on Fungal Genetics, Seville, 2014). Orthologs of this paralog

are not present in *Sordariomycetes* such as *N. crassa*, *F. oxysporum*, and *T. reesei* (Tani et al. 2014). In *Eurotiomycetes*, *Penicillium* and only limited species of *Aspergillus*, which are phylogenetically closely related to *A. nidulans*, possess the paralog. Such phylogenetic distribution suggests that the ClrB paralog emerged after the separation of *Sordariomycetes* and *Eurotiomycetes*, and then several *Aspergillus* spp. including *A. oryzae* and *A. niger* subsequently lost the gene.

Involvement of McmA in cellulolytic enzyme gene regulation

In *A. nidulans*, ClrB and McmA are involved in the induction of cellulolytic enzyme genes (Fig. 1) (Li et al. 2016a; Yamakawa et al. 2013). McmA is a member of SRF-MADS box proteins, which are conserved between a wide variety of eukaryotes. A MADS box protein is sometimes called a combinatorial transcription factor that functions through the interaction with a variety of co-functioning transcription factors (Messenguy and Dubois 2003). McmA recruits ClrB to the $CCGN_2CCN_6GG$ sequence (Cellulose Responsive Element; CeRE), in which McmA recognizes CCN_6GG, and ClrB the preceding CCG (Li et al. 2016a). Mutation of McmA causes a decrease in the expression of major cellulolytic enzyme genes, namely *eglA* and *eglB* encoding endoglucanases, *cbhA* and *cbhD* encoding cellobiohydrolases, *bglI* and AN10124 encoding β–glucosidases, and AN3860 encoding putative polysaccharide monooxygenase. However, the degree of decrease caused by the mutation varies depending on the genes. This is due to the presence of the McmA-independent ClrB binding site $CGGN_8CCG$, which leads to McmA-independent expression (Li et al. 2016a).

As a combinatorial transcription factor, McmA is also involved in the regulation of

some hemicellulolytic enzyme genes such as *manB* and *xlnC*, a protease gene *prtA*, and genes encoding regulators of asexual and sexual development (Li et al. 2016a; Li, et al. 2016b). A number of genes other than these are also under the control of McmA, based on a preliminary survey by RNA sequencing, although most of them are uncharacterized. It is unknown whether McmA responds to a certain environmental stimulus, and if so, what the stimulus is. It is reported that the phosphorylation status of serine-162 of a human SRF-MADS protein may constitute a novel switch that directs target gene expression into proliferation or differentiation programs (Iyer et al. 2006), and that the phosphorylation status of *S. cerevisiae* Mcm1p changes in the presence of salt stress (Kuo et al. 1997). These suggest that McmA functions to modulate ClrB-dependent expression, in response to unknown stimuli that change its phosphorylation status.

Future perspectives

Most transcriptional activators that are important for the regulation of cellulolytic enzyme genes have been identified to date, revealing incomplete conservation of the regulatory systems among ascomycete fungi. The *T. reesei* system is obviously distinct. *N. crassa*, *A. nidulans*, and *A. oryzae* share the essential factor CLR-2/ClrB/ManR, but their regulatory systems are not the same. It is apparent that each regulatory system is quite complex. In the recent past decade, genome-wide transcriptional analysis predicted sets of genes under the regulation of transcriptional activators. However, it is obviously difficult to identify the genes directly regulated by a certain factor by such transcriptional studies, because differential expression can be as a result of primary as well as secondary effects of deletion/overexpression. Comprehensive DNA binding analysis accompanied by transcriptional analysis may be useful for better prediction of

directly regulated genes (Craig et al. 2015; Kojima et al. 2016). At the same time, precise research on the regulatory systems at the protein level is important for complete understanding of the regulation of cellulolytic enzyme genes, especially considering the remarkable diversity of the regulatory systems. Evolutionary and ecological considerations of the observed diversity are also required.

There are a number of questions to be answered. How is the cellobiose signal transduced to CLR-1 in *N. crassa* and ClrB/ManR in *Aspergillus*? What kind of molecular mechanisms are involved in their activation? Is the McmA-involved regulatory system conserved among ascomycete fungi, and how is McmA activity regulated? Repressing mechanisms of cellulolytic enzyme genes, so called carbon catabolite repression (CCR), also need to be studied for an in-depth understanding of the regulatory mechanisms involved. CreA-dependent CCR is well-known (Dowzer and Kelly 1991), and the possible CreB/CreC deubiquitinating complex had been thought to be involved in regulation of CreA activity (Lockington and Kelly 2002). Recent studies have indicated that CreC is essential for CreA function and stability (Ries et al. 2016), but CreB does not directly target CreA (Alam et al. 2016a; Alam, et al. 2016b). Furthermore, a malfunction of CreA is not sufficient for the complete derepression of cellulolytic enzyme genes, at least in *A. nidulans* in our experience.

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Table 1 List of transcriptional activators involved in the regulation of cellulolytic enzyme genes

Organism	Activator	Motif	binding consensus	Reference
A. nidulans	**ClrB***	Zn2C6	$CGGN_8CCG$ $CCGN_2YCN_6GG$**	(Coradetti, et al. 2012, Li, et al. 2016a)
	ClrA	Zn2C6	N/A***	(Coradetti, et al. 2012)
	HapB/C/E	histone-fold	CCAAT	(Endo, et al. 2008, Tanaka, et al. 2001)
	PacC	C2H2	GCCARG	(Kunitake, et al. 2016))
	McmA	MADS	YCN_6GG	(Li, et al. 2016a, Yamakawa, et al. 2013)
A. niger	XlnR	Zn2C6	GGCTARA	(Gielkens, et al. 1999, van Peij, et al. 1998)
A. oryzae	**ManR**	Zn2C6	CAGAAT****	(Ogawa, et al. 2012:Ogawa, 2013 #514)
	XlnR	Zn2C6	GGCTARA*****	(Marui, et al. 2002a, Marui, et al. 2002b)
A. aculeatus	ClbR	Zn2C6	N/A	(Kunitake, et al. 2015, Kunitake, et al. 2013)
N. crassa	**CLR-2**	Zn2C6	$CGGN_{11}CCG$	(Coradetti, et al. 2012, Coradetti, et al. 2013, Craig, et al. 2015)
	CLR-1	Zn2C6	$CGGN_5CGGNCCG$	(Coradetti, et al. 2012, Coradetti, et al. 2013, Craig, et al. 2015)
T. reesei	**Xyr1**	Zn2C6	GGCWWWW	(Furukawa, et al. 2009, Stricker, et al. 2006)

	Ace2	Zn2C6	GGCTAATAA	(Aro, et al. 2001)
	Ace3	Zn2C6	N/A	(Häkkinen, et al. 2014)
	Hap2/3/5	histone-fold	CCAAT	(Zeilinger, et al. 2001)
P. oxalicum	**ClrB**	Zn2C6	N/A	(Li, et al. 2015)
	ClrB-2	Zn2C6	N/A	(Li, et al. 2015)
	ClrC	b-Zip	N/A	(Lei, et al. 2016)

* Transcriptional activators essential to the induction of cellulolytic enzyme genes on cellulose are shown in bold.

** Cooperative binding with McmA.

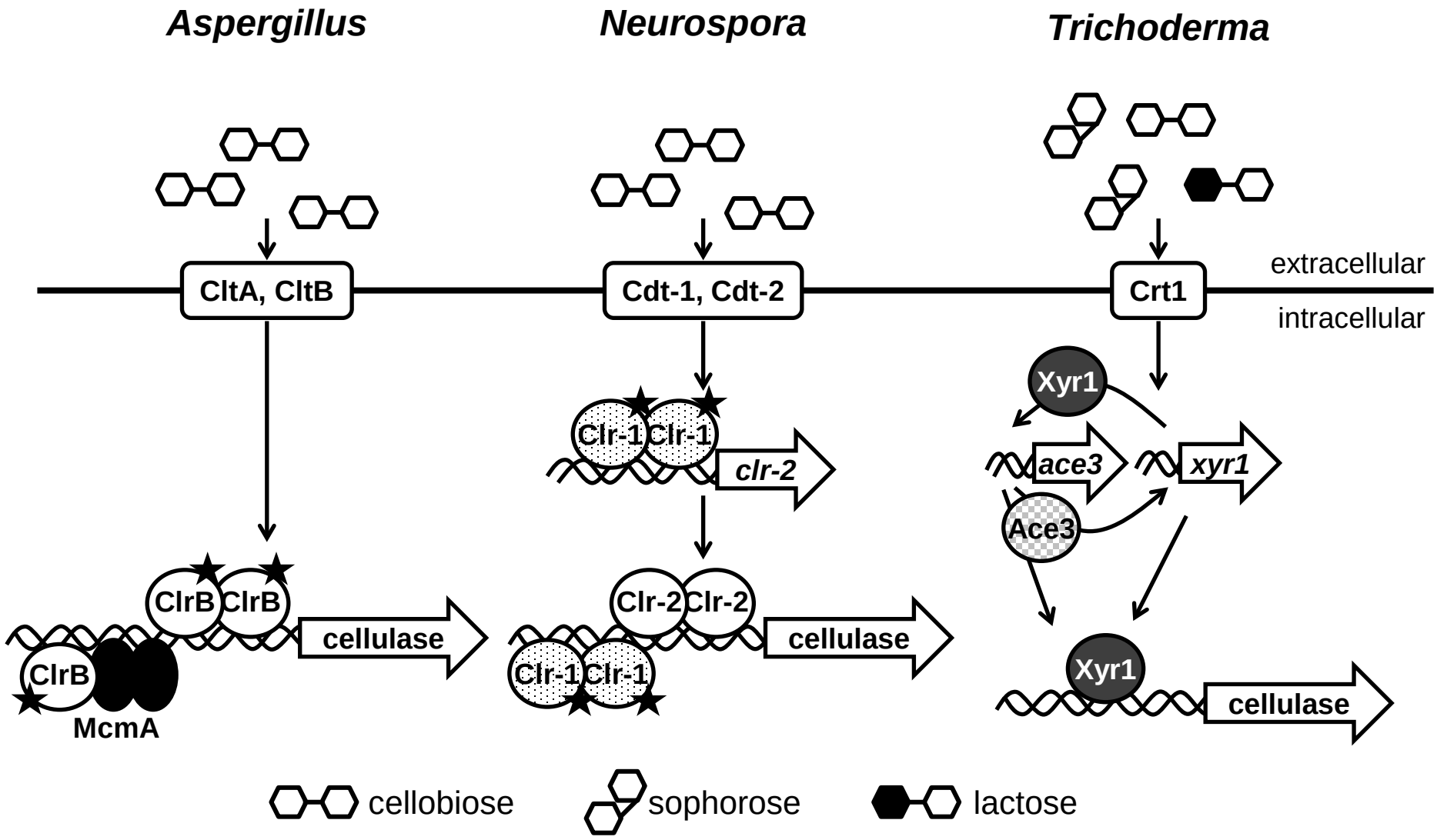
*** N/A: not available

**** Based on *in silico* analysis.

***** This XlnR binding consensus may need to be revised, as our extensive examination revealed that *A. oyrzae* XlnR binds to CGGNTAAW as a monomer and TTAGSCTAA as a dimer (unpublished).

Figure legend

Fig. 1 Schematic drawing of the transcriptional activation of cellulolytic enzyme genes in model fungi. Transcription factors activated via cellulose/cellobiose sensing are indicated with stars.


Aspergillus
Neurospora
Trichoderma
CltA, CltB
Cdt-1, Cdt-2
Crt1
extracellular
intracellular
Xyr1
Clr-1 Clr-1
clr-2
ace3
xyr1
Ace3
ClrB ClrB
cellulase
ClrB
McmA
Clr-2 Clr-2
cellulase
Clr-1 Clr-1
Xyr1
cellulase
cellobiose
sophorose
lactose