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Intracellular β-Glucosidases CEL1a and CEL1b Are Essential for Cellulase Induction on Lactose in *Trichoderma reesei*

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Lactose (1,4-*O*-β-D-galacto-pyranosyl-D-glucose) induces cellulolytic enzymes in *Trichoderma reesei* and is in fact one of the most important soluble carbon sources used to produce cellulases on an industrial level. The mechanism underlying the induction is, however, not fully understood. In this study, we investigated the cellular functions of the intracellular β-glucosidases CEL1a and CEL1b in the induction of cellulase genes by lactose in *T. reesei*. We demonstrated that while CEL1a and CEL1b were functionally equivalent in mediating the induction, the simultaneous absence of these intracellular β-glucosidases abolished *cbh1* gene expression on lactose. D-Galactose restored the efficient cellulase gene induction in the Δ*cel1a* strain independently of its reductive metabolism, but not in the Δ*cel1a* Δ*cel1b* strain. A further comparison of the transcriptional responses of the Δ*cel1a* Δ*cel1b* strain complemented with wild-type CEL1a or a catalytically inactive CEL1a version and the Δ*cel1a* strain constitutively expressing CEL1a or the *Kluyveromyces lactis* β-galactosidase LAC4 showed that both the CEL1a protein and its glycoside hydrolytic activity were indispensable for cellulase induction by lactose. We also present evidence that intracellular β-glucosidase-mediated lactose induction is further conveyed to XYR1 to ensure the efficiently induced expression of cellulase genes.

Cost-effective conversion of plant cell wall-derived polysaccharides holds the potential for production of an environmentally clean and renewable source of energy and platform chemicals (1). *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is well known for its high capacity to secrete large amounts of lignocellulosic enzymes that release fermentable sugars and has thus been developed into one of the most prolific industrial cellulase producers. High-yield production of the bulk of the plant cell wall-degrading machinery in *T. reesei* is, however, dependent on induction by insoluble substrates that include cellulose, hemicellulose, and mixtures of plant polymers. Considering the ease of manipulation and the complication of separating enzymes from insoluble plant cell wall materials, soluble inducing substrates are usually preferred or required (2). Among others, the disaccharide lactose (1,4-*O*-β-D-galacto-pyranosyl-D-glucose) is an important and economic soluble carbon source for cellulase production by *T. reesei*. However, the induced cellulase yields on lactose are usually lower than those on cellulose (3, 4). Understanding the differences in the inducing efficiency and the mode by which lactose triggers cellulase formation would be helpful for improving the performance of industrial strains.

In fungi, catabolism of lactose is thought to proceed either by extracellular hydrolysis and subsequent uptake of the resulting sugar monomers or by uptake of the disaccharide followed by intracellular hydrolysis (4). For *T. reesei*, it has been assumed that lactose metabolism relies on the first strategy, based on several findings, including the absence of apparent orthologs for lactose permease and intracellular β-galactosidase in the *T. reesei* genome (4, 5). The resulting D-galactose moiety from lactose can be converted either by the Leloir pathway before being channeled into the glycolytic pathway or by a second pathway, initiated by a xylose reductase-mediated reduction of galactose to galactitol (6). While deletion of the first gene of either pathway, *gal1* or *xyl1*, drastically reduces cellulase gene expression during growth on lactose (7), it has been shown that D-galactose alone is not an inducer and that overexpression of *bga1*, encoding the major extracellular β-galactosidase, almost abolishes the cellulase induction on lactose (5, 8). On the other hand, it was recently reported that three major facilitator superfamily (MFS) transporters behave as lactose permeases and are involved in cellulase induction (9–11). Our previous results also demonstrated that the absence of the intracellular β-glucosidase CEL1a delayed the induction of cellulase gene expression on lactose (12). Eleven predicted β-glucosidase-encoding genes exist in the genome of *Trichoderma reesei*, including those for the well-characterized major extracellular β-glucosidase BglI as well as the intracellular β-glucosidases CEL1a and CEL1b (12, 13). However, it remains unclear how these intracellular β-glucosidases affect the induction of cellulases by lactose.

In the present study, we extrapolate from the previous observation that CEL1a affects the induction of cellulase gene expression on lactose. We demonstrate that simultaneous deletions of *cel1a* and *cel1b* almost abolish the induction. We further present evidence that CEL1a-associated hydrolytic activity participates in a process beyond lactose catabolism to initiate efficient cellulase induction. We discuss a possible role of intracellular β-glucosidases in contributing to cellulase biosynthesis induced by lactose.

MATERIALS AND METHODS

Strains and cultivation conditions. *Trichoderma reesei* TU-6 (ATCC MYA-256) (14), a uridine-auxotrophic derivative of *T. reesei* QM9414

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TABLE 1 *Trichoderma reesei* strains used in this study

Strain name or genotype	Reference
TU-6	14
Δ*cel1a*	12
Δ*cel1b*	12
Δ*cel1a* Δ*cel1b*	12
ΔtriβG	12
Δ*cel1a* Δ*cel1b* CEL1b I174C	12
Δ*cel1a* Δ*xyl1*	This study
Δ*cel1a* Δ*bga1*	This study
Δ*cel1a* Δ*cel1b* CE*crt1*	This study
Δ*cel1a* Δ*cel1b* CE*xyr1*	This study
Δ*cel1a* CE*lac4*	This study
Δ*cel1a* CE*cel1a*	This study
Δ*cel1a* CE*cel1a* (E367A)	This study
Δ*cel1a* Δ*cel1b* NE*cel1a*	This study
Δ*cel1a* Δ*cel1b* NE*cel1a* (E367A)	This study

(ATCC 26921) with a mutant *pyr4* gene, was used as the parental strain. *Trichoderma reesei* strains used are listed in Table 1. All the strains were maintained on malt extract agar supplemented with 10 mM uridine when necessary. *Escherichia coli* DH5α was used for conventional gene cloning and vector construction. *Escherichia coli* Origami B(DE3) was used as a host for production of recombinant proteins.

For transcription and cellulase production analysis, *T. reesei* strains were pregrown in 1-liter Erlenmeyer flasks on a rotary shaker (200 rpm) at 30°C in 250 ml Mandels-Andreotti medium with glycerol (1% [vol/vol]) as the carbon source for 48 h. Mycelia were harvested by filtration and washed twice with a medium without a carbon source. Equal amounts of mycelia were then transferred to a fresh medium containing 1% (wt/vol) lactose or other carbon sources, as indicated, without peptone, and incubation was continued for the indicated times.

Plasmids. For expression of CEL1a and its mutant derivative in *Escherichia coli*, the coding sequence of *cel1a* or *cel1a* (E367A) was amplified from the cDNA of *T. reesei* or pNE*cel1a* (E367A) (see below) by use of primers harboring EcoRI and HindIII sites and ligated into pET32a(+) to obtain pET32a-*cel1a* or pET32a-*cel1a* (E367A).

For *bga1* and *xyl1* gene deletion in the Δ*cel1a* strain, a hygromycin resistance cassette containing the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) promoter from *T. reesei* and the hygromycin resistance gene from pRLMex30 (15) was amplified using primers *hph*-F and *hph*-R and then ligated into pMD19-T to obtain pMD*hph*. The 2-kb upstream and downstream flanking sequences of the *bga1* gene were digested by SalI/SpeI and XbaI/NotI, respectively, and ligated into the corresponding sites of pMD*hph* sequentially to obtain pMD*bga1hph*. In the same way, the 2-kb upstream region of the *xyl1* coding sequence was digested by BamHI/NotI, and the 2-kb downstream region was digested by SpeI/SalI, and the fragments were ligated into pMD*hph* to obtain pMD*xyl1hph*.

In order to complement the Δ*cel1a* Δ*cel1b* strain with CEL1a and CEL1a (E367A) under the control of the native *cel1a* promoter, the *cel1a* disruption vector pUC*cel1apyr4* (12) was digested with XbaI and SalI to remove the *pyr4* gene, followed by ligation with the hygromycin resistance cassette digested with the same enzymes to create pUC*cel1ahph*. This plasmid was digested with XbaI, dephosphorylated using shrimp alkaline phosphatase (TaKaRa), and further ligated with the coding region for CEL1a to obtain pNE*cel1a*. A mutant *cel1a* gene encoding a E367A substitution in wild-type (WT) CEL1a was created by oligonucleotide-mediated mutagenesis of the *cel1a* gene, using a two-step fusion PCR with pNE*cel1a* as the template. The resulting PCR product was ligated into pUC*cel1ahph* to obtain pNE*cel1a* (E367A).

To achieve the constitutive expression (CE) of *xyr1*, *cel1a*, *cel1a* (E367A), and *cel1b*, the coding sequences of these genes were amplified from the cDNA of *T. reesei* or the pNE*cel1a* (E367A) plasmid and were ligated into pFL3 (pIG1783 with the stop codon of *egfp* deleted), which contains a *gpd* promoter and a *trpC* terminator, to obtain pFL3*xyr1*, pFL3*cel1a*, pFL3*cel1a* (E367A), and pFL3*cel1b* (16). To achieve the constitutive expression of *crt1* and *lac4*, the coding sequences of *crt1* and *lac4* were placed under the control of a constitutive *pki* promoter. DNA fragments corresponding to the *pki* promoter and the *cbh2* terminator from *T. reesei* were amplified from pRLMex30, digested with HindIII/SphI and KpnI/EcoRI, respectively, and ligated into pUC19 to obtain pUC*pki-cbh2*. The coding sequences of *crt1* and *lac4* were further inserted into pUC*pki-cbh2* after being digested with SphI/XbaI to generate pCE*crt1* and pCE*lac4*, respectively. Oligonucleotides used in this study for plasmid construction, gene deletion, and probe preparation are listed in Table S1 in the supplemental material.

Production of recombinant CEL1a in *E. coli*. For purification of CEL1a and its mutant derivative, *E. coli* Origami B(DE3) cells transformed with pET32a-*cel1a* or pET32a-*cel1a* (E367A) were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 100 μM, and the incubation was continued at 20°C for 16 h. The proteins were purified from the supernatant with Ni-nitrilotriacetic acid-agarose (Qiagen), essentially as previously described (12).

Construction of *T. reesei* strains. For *xyl1* and *bga1* gene deletions in the Δ*cel1a* strain, plasmids pMD*xyl1hph* and pMD*bga1hph* were linearized by SalI and XbaI, respectively, before transformation (see Fig. S1 in the supplemental material). For constitutive expression of *xyr1* or *crt1*, the Δ*cel1a* Δ*cel1b* strain was transformed with plasmid pFL3*xyr1* or pCE*crt1* (together with pRLMex30) to obtain the Δ*cel1a* Δ*cel1b* CE*xyr1* and Δ*cel1a* Δ*cel1b* CE*crt1* strains. For constitutive expression of *cel1a*, *cel1a* (E367A), and *lac4* in the Δ*cel1a* strain, the Δ*cel1a* strain was transformed with plasmids pFL3*cel1a*, pFL3*cel1a* (E367A), and pCE*lac4* (together with pRLMex30) to obtain the Δ*cel1a* CE*cel1a*, Δ*cel1a* CE*cel1a* (E367A), and Δ*cel1a* CE*lac4* strains, respectively. For constitutive expression of *cel1a* or *cel1b* in the Δ*cel1a* Δ*cel1b* strain, the Δ*cel1a* Δ*cel1b* strain was transformed with plasmid pFL3*cel1a* or pFL3*cel1b* to obtain the Δ*cel1a* Δ*cel1b* CE*cel1a* and Δ*cel1a* Δ*cel1b* CE*cel1b* strains. In order to express CEL1a and CEL1a (E367A) under the control of the native *cel1a* promoter, the Δ*cel1a* Δ*cel1b* strain was transformed with plasmid pNE*cel1a* or pNE*cel1a* (E367A) linearized by HindIII to obtain the Δ*cel1a* Δ*cel1b* NE*cel1a* and Δ*cel1a* Δ*cel1b* NE*cel1a* (E367A) strains (see Fig. S2). Transformation was carried out essentially as described by Penttila et al. (17). Transformants were selected on minimal medium for resistance to 100 μg/ml of hygromycin. For ectopic complementation via constitutive expression, all the recombinant strains were verified for the presence of the entire expression cassette by PCR, and at least two transformants were analyzed for the relevant phenotypes.

Nucleic acid isolation and hybridization. Fungal mycelia were harvested by filtration, washed with tap water, and frozen in liquid nitrogen. Fungal genomic DNA was isolated according to the instructions of an EZNA fungal DNA miniprep kit (Omega Biotech). Total RNA was isolated by use of the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Southern and Northern hybridization analyses were performed with a digoxigenin-based nonradioactive system from Roche Applied Science, as described previously (18). Relative transcription levels were normalized to that of the 18S rRNA control by densitometry, using the software program ImageJ (http://rsb.info.nih.gov/ij).

Quantitative RT-PCR (qRT-PCR). Total RNA was further digested by use of a Turbo DNA-free kit (Ambion) to eliminate genomic DNA contamination. Reverse transcription (RT) was performed using a PrimeScript RT reagent kit (TaKaRa) according to the manufacturer's instructions. Quantitative PCRs were performed using SYBR green supermix (TaKaRa) on a Bio-Rad myIQ2 thermocycler (Bio-Rad). Reactions were performed in triplicate with a total volume of 20 μl, including 250 nM (each) forward and reverse primers and template cDNA. Data were analyzed using the relative quantitation/comparative threshold cycle ($\Delta\Delta C_T$)

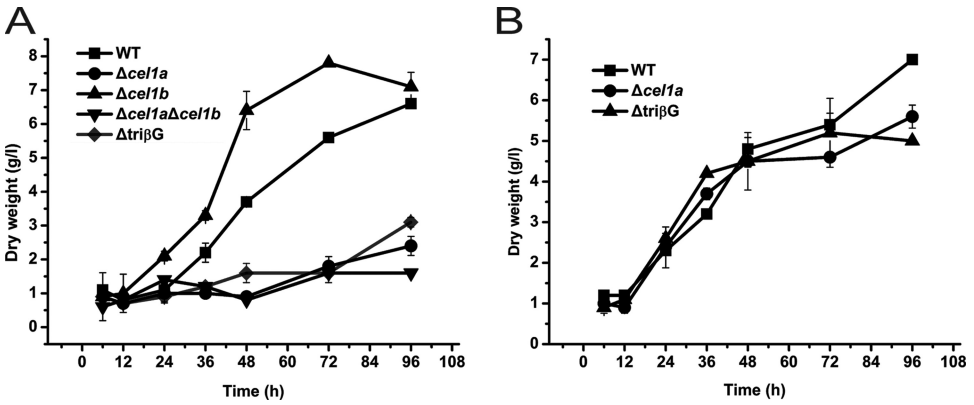


FIG 1 Disruption of *cel1a*, *cel1b*, and *bgl1* affects growth of *T. reesei* on lactose but not on galactose. Growth of WT and β-glucosidase deletion strains in shake flasks with 1% (wt/vol) lactose (A) or 1% (wt/vol) galactose (B) as the carbon source was analyzed by measuring dry weight. Data shown are the means for results from three independent experiments. Error bars show the standard deviations (SD) for these replicates.

method and were normalized to an endogenous control (actin), with expression on glycerol as the reference sample.

Enzyme and protein assays. Cellobiohydrolase and β-glucosidase activities were determined by measuring the amount of *p*-nitrophenol, using *p*-nitrophenyl-D-cellobioside (pNPC; Sigma) and *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma), respectively, as substrates. β-Galactosidase activity was measured with *o*-nitrophenyl-D-galactopyranoside (ONPG; Sigma) as the substrate. The assays were carried out in 200-μl reaction mixtures including 50 μl of culture supernatant or cell extract and 50 μl of the respective substrate (5 mM; pNPC, pNPG, or ONPG) in 50 mM sodium acetate buffer (pH 5.0). The reaction mixtures were incubated at 45°C for 30 min for pNPC and pNPG and at 30°C for 30 min for ONPG. Cellular extracts used for assay of intracellular β-glucosidase activity were prepared as follows. *T. reesei* strains were grown on Mandels-Andreotti medium with lactose or glycerol as the carbon source. The mycelia were harvested and washed twice with 0.9% NaCl. Lysates were prepared by grinding the mycelia into a fine powder under liquid nitrogen, and the powder was then suspended in 50 mM sodium phosphate buffer (pH 7.0) with protease inhibitors. Cell debris was removed by centrifugation at 14,000 × *g* for 10 min. One unit of activity corresponds to transformation of 1 μmol of substrate per min under the test conditions. The activity of CEL1a toward lactose was measured as follows. The reaction mixture (200 μl), containing 50 μl of a 4% lactose solution, 50 μl purified CEL1a protein (50 μg), and 100 μl 50 mM sodium phosphate buffer (pH 7.0), was incubated at 30°C for 30 min. The reaction was stopped by heating for 10 min in a boiling water bath. The products were analyzed by high-performance liquid chromatography (HPLC) as previously described (12).

Total secreted and intracellular proteins were determined using the method of the Bradford protein assay, with bovine serum albumin (BSA) as the standard. SDS-PAGE and Western blotting were performed according to standard protocols (19). CBH1 was immunoblotted using a polyclonal antibody raised against amino acids 426 to 446 of the protein, as previously described (12). Relative induced CBH1 production was analyzed semiquantitatively by densitometry, using the software program ImageJ (http://rsb.info.nih.gov/ij).

Biomass determination. Mycelial dry weight was recorded by withdrawing 5-ml aliquots from the culture, centrifuging them at 10,000 × *g* for 10 min, and drying them to a constant weight at 80°C.

Statistical analyses. Student's *t* tests were performed on all sets of data. All the differences mentioned fulfill the criterion of having a *P* value of <0.05.

RESULTS

The absence of intracellular β-glucosidases CEL1a and CEL1b compromises cellulase induction by lactose. Our previous result showed that deletion of the major intracellular β-glucosidase-encoding gene, *cel1a*, delayed the induced expression of cellulase genes on lactose (12). To investigate the effects of the absence of other β-glucosidases on cellulase induction by lactose, a strain deleted for *cel1b*, encoding another highly expressed intracellular β-glucosidase upon lactose incubation, a double (Δ*cel1a* Δ*cel1b*) mutant strain, and a triple (ΔtriβG) mutant strain with simultaneous deletions in *cel1a*, *cel1b*, and *bgl1* were used to analyze growth and cellulase gene expression. While the absence of CEL1b facilitated the growth on lactose compared with that of the WT, deletion of *cel1a* led to a severe growth defect (Fig. 1A). Additional deletions of *cel1b* and *bgl1* did not build upon the compromised growth in the absence of CEL1a. In contrast, the Δ*cel1a* and ΔtriβG strains grew as well as the WT on galactose (Fig. 1B). Analysis of the induced production of CBH1 on lactose demonstrated that compared with the case of the Δ*cel1a* strain, the absence of CEL1b hardly affected the efficient induction and resulted only in slightly less production of CBH1 and a lower exoglucanase activity than those of the parental strain at the later stage of induction (Fig. 2A and B). Simultaneous absence of CEL1a and CEL1b, however, abolished the detection of secreted CBH1 and extracellular pNPC hydrolytic activity. A triple mutant carrying a deletion of *bgl1* displayed the same noninduction phenotype as that of the Δ*cel1a* Δ*cel1b* strain. Further examination of the endogenous mRNA by qRT-PCR revealed that the defective cellulase gene induction observed in the deletion strains occurred at the transcriptional level, with the Δ*cel1a* Δ*cel1b* and ΔtriβG mutants showing no significantly induced transcription of *cbh1* (Fig. 2C). Taken together, these results indicate that intracellular β-glucosidases CEL1a and CEL1b are critical for the efficient induction of cellulase gene expression on lactose.

CEL1a and CEL1b are functionally equivalent in cellulase induction by lactose. Given the very different phenotypes displayed by the single and double deletion mutants, we tried to probe the independent roles of CEL1a and CEL1b in the induction on lactose. Analysis of mRNA levels of *cel1a* and *cel1b* in the WT, Δ*cel1a*, and Δ*cel1b* strains revealed that while the absence of *cel1b* resulted in a slightly higher transcription level of *cel1a*, deletion of *cel1a* led to a significant delay in the transcription of *cel1b* on lactose (Fig. 3A). This delayed induction of *cel1b* was largely corrected by the addition of a small amount of galactose, which also restored the

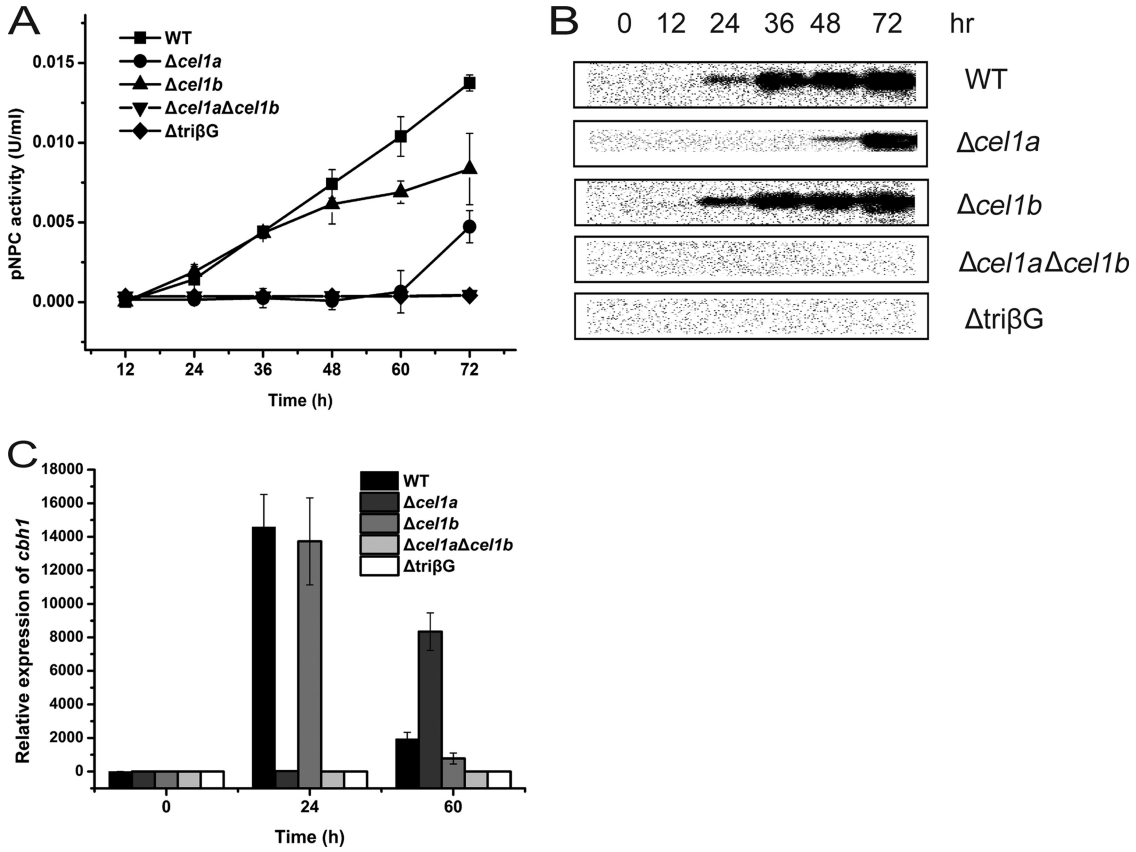


FIG 2 Absence of intracellular β-glucosidases compromises cellulase induction by lactose. (A) Exoglucanase activity analysis of the culture supernatants of WT and mutant strains deleted for β-glucosidase genes grown on 1% (wt/vol) lactose. The differences between the WT and Δ*cel1b* strains were found to be statistically relevant, by Student's *t* test, at the later stages of induction ($P = 0.025$ for 60 h and $P = 0.029$ for 72 h). (B) Western blot analysis of CBH1 secreted into the culture supernatants of WT and mutant strains grown on 1% (wt/vol) lactose. Equal amounts of culture supernatant relative to biomass were loaded for all strains. (C) Quantitative RT-PCR analysis of gene expression of *cbh1* in WT and mutant strains after induction by 1% (wt/vol) lactose for different periods, as indicated. Values in this figure are the means for three biological replicates. Error bars show the SD for these replicates.

efficient cellulase induction in the Δ*cel1a* strain (Fig. 3B) (see below). To further tease apart the differences in the effects of CEL1a and CEL1b, two recombinant strains were constructed to constitutively express either CEL1a or CEL1b in the Δ*cel1a* Δ*cel1b* strain. Analysis of the induced expression of *cbh1* in the respective recombinant strains revealed that expression of either CLE1a or CEL1b alone was fully capable of mediating the induced expression of *cbh1* on lactose (Fig. 3C and D). These results indicate that CEL1a and CEL1b are functionally equivalent in mediating the induction of cellulase and that CEL1b plays an important role in induced expression of cellulase genes in the absence of CEL1a.

Addition of galactose restores cellulase induction in the Δ*cel1a* strain but not in the Δ*cel1a* Δ*cel1b* strain. To ask whether the growth and cellulase induction defects on lactose displayed in the absence of CEL1a and CEL1b are due to the inefficient hydrolysis of lactose, we determined the extracellular β-galactosidase activity of the mutant strains. Analysis of the ONPG hydrolytic activity of the supernatant from the culture grown on lactose revealed that the extracellular β-galactosidase activity was drastically reduced in the Δ*cel1a*, Δ*cel1a* Δ*cel1b*, and ΔtriβG strains compared with the WT and Δ*cel1b* strains (Fig. 4A). These results correlated well with the observed growth phenotype of the mutant strains on lactose. We wondered whether the inefficient hydrolysis of lactose, and thus release of the D-galactose moiety, accounts for the cellulase induction defect in the β-glucosidase mutants. We therefore tested the effect of addition of different amounts of galactose to the lactose medium on the induced production of CBH1. Addition of galactose at a molar ratio of 1 to 5 relative to lactose restored the growth of the Δ*cel1a* strain as well as the Δ*cel1a* Δ*cel1b* and ΔtriβG mutants on lactose (Fig. 4B). A significant increase in the extracellular β-galactosidase activity was also detected for the Δ*cel1a*, Δ*cel1a* Δ*cel1b*, and ΔtriβG strains under such conditions compared with those induced only by lactose (Fig. 4C). However, an efficient induction of *cbh1* expression, to a level even higher than that of the WT, was observed only for the Δ*cel1a* strain, not for the double- and triple-deletion mutant strains (Fig. 4D and E). Galactose alone at such a concentration had no induction effect (see Fig. S3 in the supplemental material). Moreover, reintroduction of a CEL1b mutant (CEL1b I174C) with a higher pNPG hydrolytic activity than that of wild-type CEL1b into the Δ*cel1a* Δ*cel1b* strain (14) recovered the ability of the double mutant to induce *cbh1* expression upon incubation with lactose and galactose (Fig. 4F). The restored induction was not the result of accelerated growth in the presence of galactose. Indeed, addition of glycerol had no effect on the induction defect in the Δ*cel1a* and Δ*cel1a* Δ*cel1b* strains, despite their improved

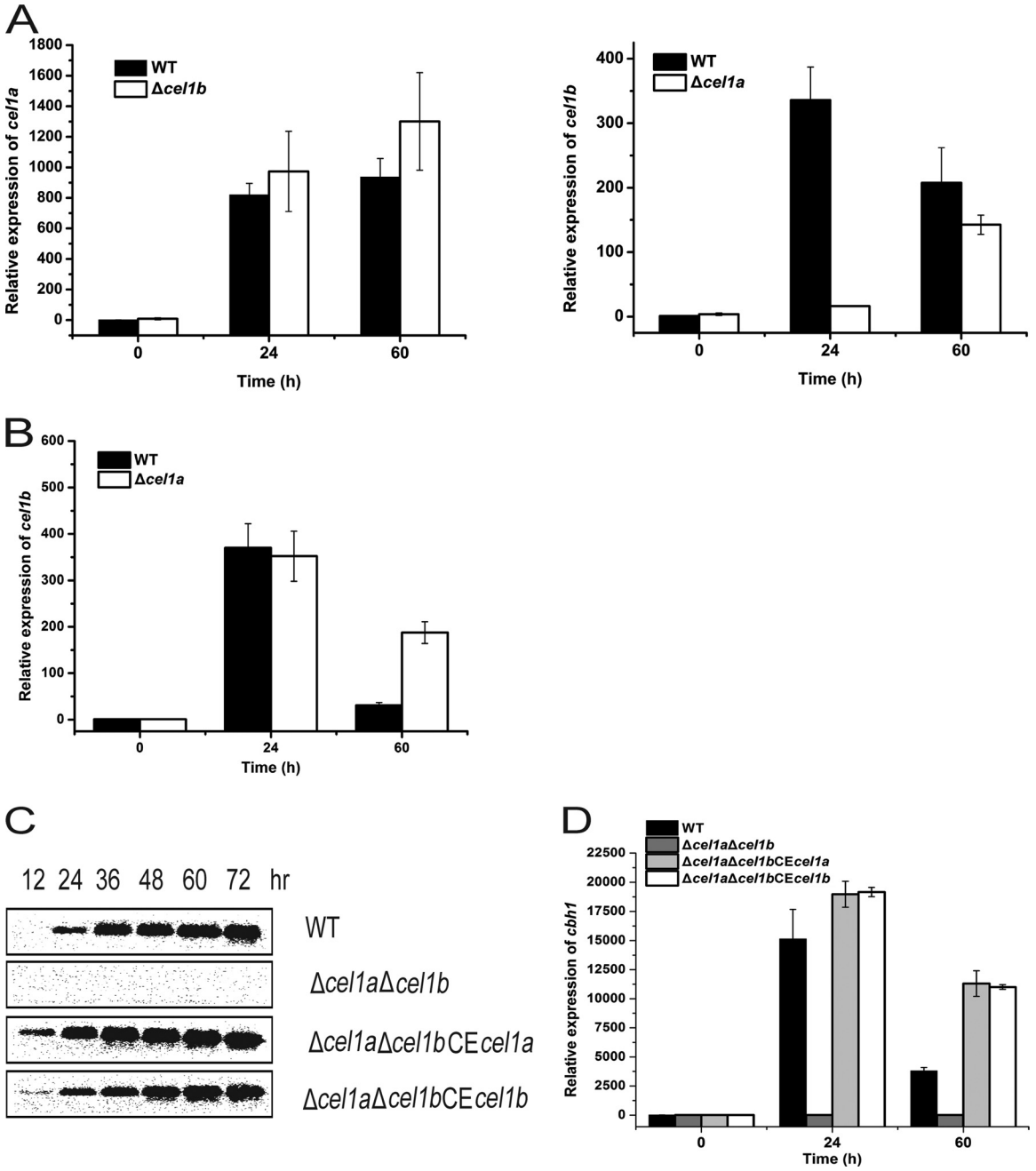


FIG 3 Either CEL1a or CEL1b is capable of inducing cellulase gene expression. (A) Transcription of *cel1a* (left) or *cel1b* (right) was analyzed by quantitative RT-PCR analysis of the WT, Δ*cel1a*, and Δ*cel1b* strains cultivated on 1% (wt/vol) lactose. Statistical analyses indicated significant differences in the transcription of *cel1a* between the WT and Δ*cel1b* strains ($P = 0.043$ for 24 h and $P = 0.026$ for 60 h) as well as in the transcription of *cel1b* between the WT and Δ*cel1a* strains ($P = 0.0041$ for 24 h). (B) Transcription of *cel1b* was analyzed by quantitative RT-PCR analysis of the WT and Δ*cel1a* strains cultivated on 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose. (C) Western blot analysis of CBH1 secreted into the culture supernatants of the WT, Δ*cel1a* Δ*cel1b*, Δ*cel1a* Δ*cel1b* CE*cel1a*, and Δ*cel1a* Δ*cel1b* CE*cel1b* strains grown on 1% (wt/vol) lactose. Equal amounts of culture supernatant relative to biomass were loaded for all strains. (D) Transcription of *cbh1* was analyzed by quantitative RT-PCR analysis of the WT, Δ*cel1a* Δ*cel1b*, Δ*cel1a* Δ*cel1b* CE*cel1a*, and Δ*cel1a* Δ*cel1b* CE*cel1b* strains during growth on 1% (wt/vol) lactose. Values in this figure are the means of results from at least three biological replicates. Error bars show the SD for these replicates. For the Δ*cel1a* Δ*cel1b* CE*cel1a* and Δ*cel1a* Δ*cel1b* CE*cel1b* strains, at least three independent transformants were determined to possess similar phenotypes, and results for a representative transformant of each are shown.

growth compared with that on lactose (see Fig. S4). These data suggest that inefficient extracellular hydrolysis of lactose may not account for the induction defect in the absence of CEL1a and CEL1b.

Neither extracellular hydrolysis of lactose nor metabolism of galactose is involved in the restored induction in the Δ*cel1a* strain. To further exclude the possibility that the restored cellulase induction upon addition of galactose in the absence of CEL1a is a

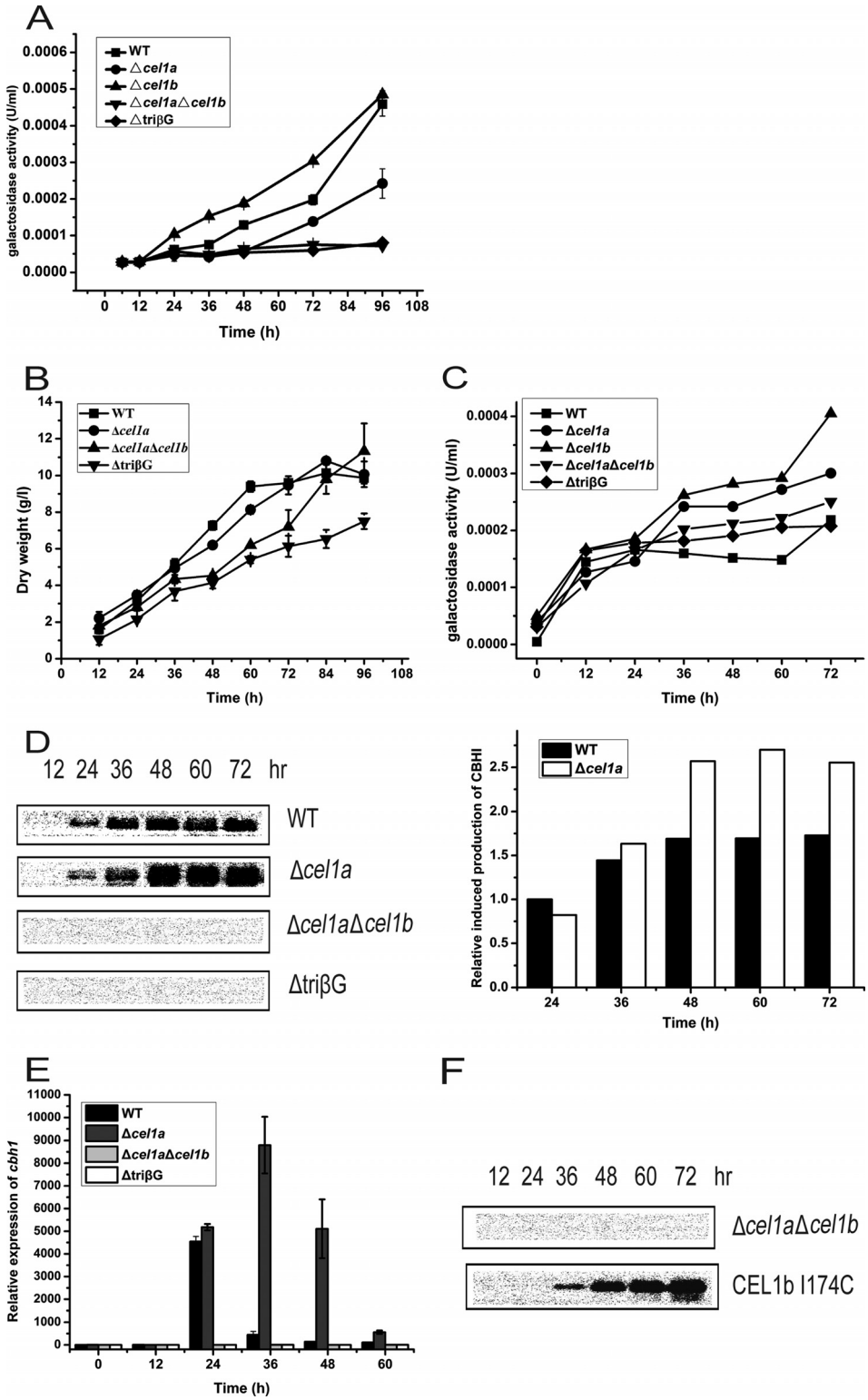


FIG 4 Galactose restores cellulase induction in the Δ*cel1a* strain but not in the Δ*cel1a* Δ*cel1b* strain. (A) Analysis of extracellular β-galactosidase activity in the culture supernatants of WT and mutant strains grown on 1% (wt/vol) lactose. (B) Growth of WT and β-glucosidase deletion strains in shake flasks with 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose as the carbon source. (C) Analysis of extracellular β-galactosidase activity in the culture supernatants of WT and mutant strains grown on 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose. Data points are the means of results from triplicates. SD are <10%. Statistical analyses indicated significant differences in the galactosidase activities of the Δ*cel1a*, Δ*cel1a* Δ*cel1b*, and ΔtriβG strains between conditions with lactose and lactose plus galactose at all the relevant time points ($P < 0.001$). (D) (Left) Western blot analysis of CBH1 in the culture supernatants of the WT, Δ*cel1a*, Δ*cel1a* Δ*cel1b*, and ΔtriβG strains after induction with 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose. (Right) CBH1 expression was quantitated by scanning densitometry of the developed membranes. Equal amounts of culture supernatant relative to biomass were loaded for all strains. (E) Gene expression of *cbh1* as analyzed by quantitative RT-PCR after induction with 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose for different periods. The differences in transcription of *cbh1* between the WT and Δ*cel1a* strains were found to be statistically relevant by Student's *t* test ($P = 0.015$ at 24 h and $P < 0.001$ at 36 h, 48 h, and 60 h). (F) Western blot analysis of CBH1 in the culture supernatants of the Δ*cel1a* Δ*cel1b* strain and the Δ*cel1a* Δ*cel1b* strain complemented with CEL1b I174C after induction with 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose. Equal amounts of culture supernatant relative to biomass were loaded for all strains. All values are the means of results from three biological replicates. Error bars show the SD for these replicates.

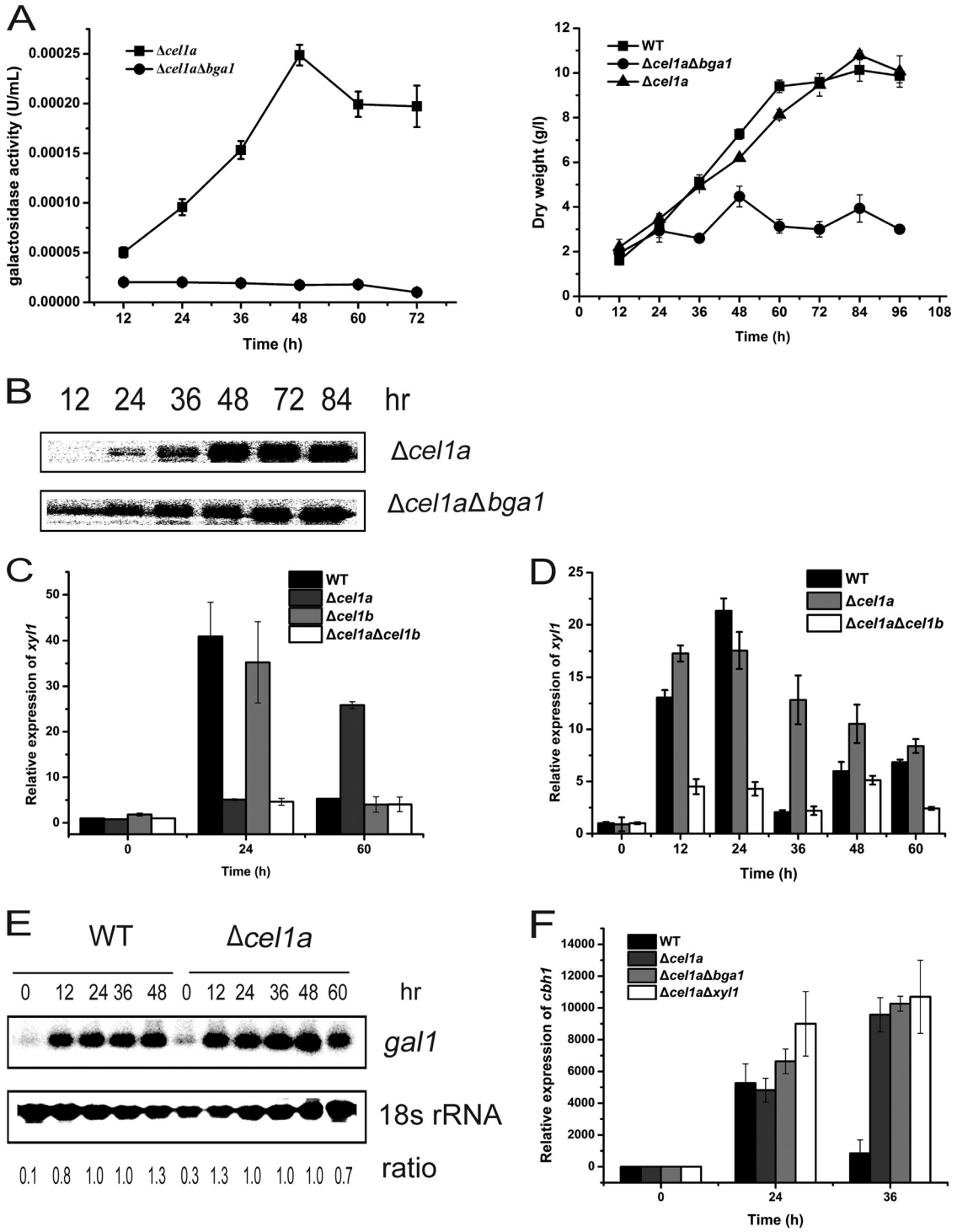


FIG 5 Roles of *bga1* and *xyl1* in the restored *cbh1* induction in the Δ*cel1a* strain. (A) Extracellular β-galactosidase activities (left) and growth curves (right) for the Δ*cel1a* and Δ*cel1a* Δ*bga1* strains grown on 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose. (B) Western blot analysis of extracellularly secreted CBH1 of the Δ*cel1a* and Δ*cel1a* Δ*bga1* strains grown on 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose. Equal amounts of culture supernatant relative to biomass were loaded for all strains. (C and D) Quantitative RT-PCR analyses of *xyl1* gene expression of wild-type and mutant strains induced by solely 1% (wt/vol) lactose (C) or 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose (D). (E) Northern blot analysis of *gal1* mRNA and 18S rRNA of the WT and Δ*cel1a* strains cultured on 1% (wt/vol) lactose. The values below the panels indicate the ratios of the intensities of the *gal1* signals measured by densitometry to that of the 18S rRNA control. (F) Quantitative RT-PCR analysis of *cbh1* gene expression in the WT, Δ*cel1a*, Δ*cel1a* Δ*bga1*, and Δ*cel1a* Δ*xyl1* strains induced by 1% (wt/vol) lactose plus 0.1% (wt/vol) galactose for different periods, as indicated. All values are the means of results from three biological replicates. Error bars show the SD for these replicates.

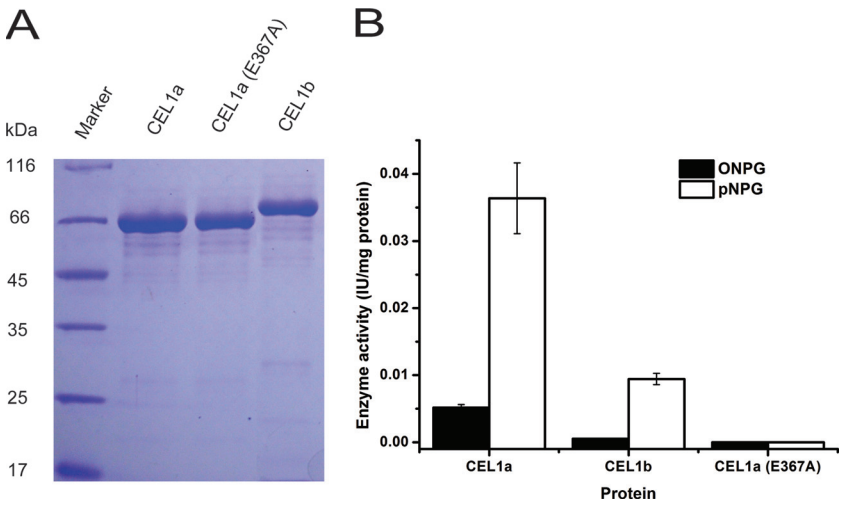
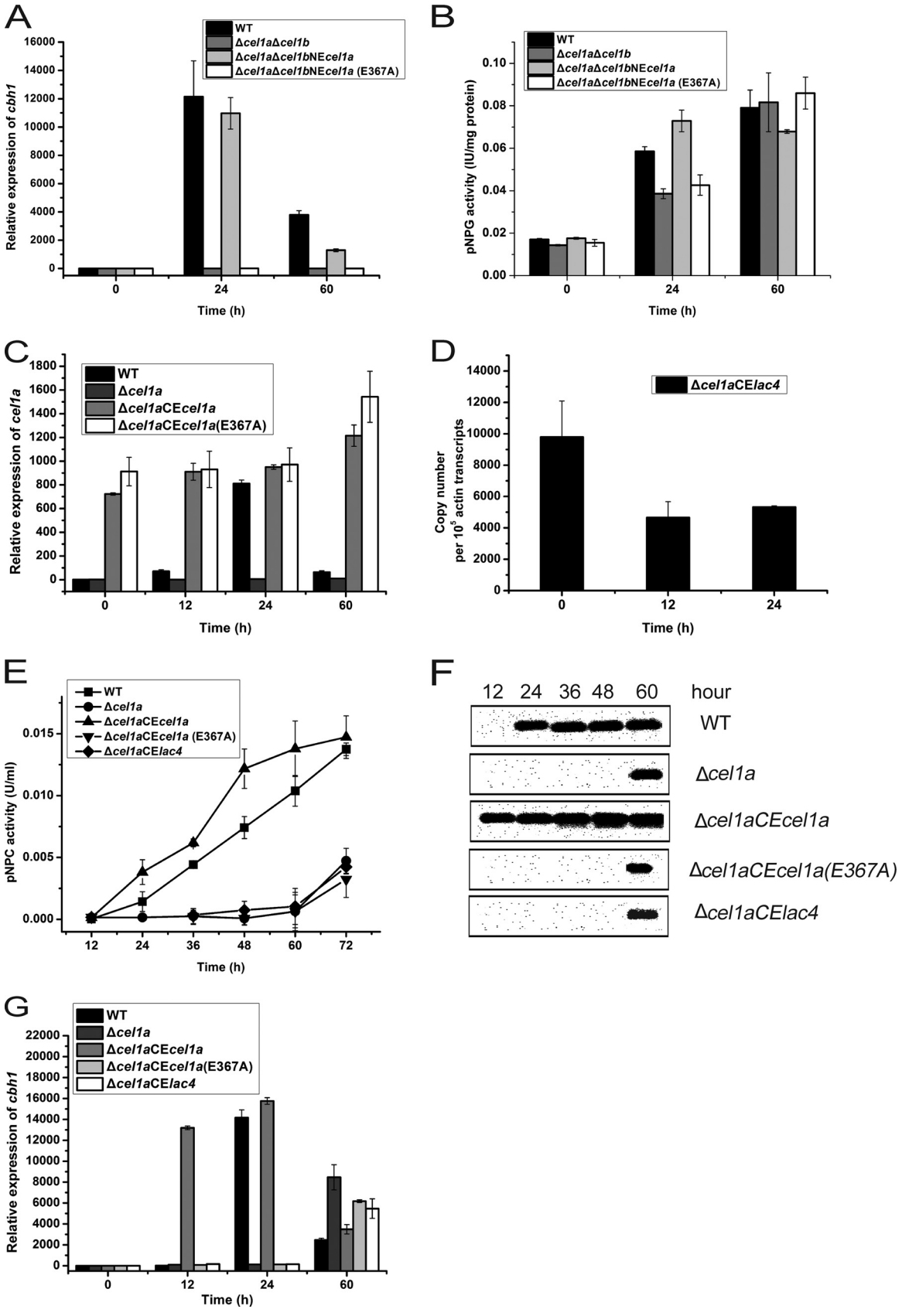


FIG 6 Enzymatic characterization of recombinant CEL1a, CEL1b, and CEL1a (E367A). (A) SDS-PAGE analysis of purified CEL1a, CEL1b, and CEL1a (E367A) produced in *E. coli*. (B) Recombinant CEL1a and CEL1b exhibit β-glucosidase activity and low levels of β-galactosidase activity, while CEL1a (E367A) completely lacks both activities. ONPG and pNPG were used as the substrates. Values are the means of results from three replicates. Error bars show the SD for these replicates.

result of enhanced extracellular lactose hydrolysis, we deleted *bga1* from the Δ*cel1a* strain and analyzed its response to lactose induction. While the absence of BGA1 drastically reduced the extracellular β-galactosidase activity and cell growth in the presence of galactose and lactose, its deletion did not interfere with the restored efficient induction observed in the Δ*cel1a* strain (Fig. 5A, B, and F). Metabolism of galactose by either the reduction pathway or the Leloir pathway has been shown to be involved in the induced expression of cellulase genes on lactose (6, 7). In the absence of CEL1a, induction of *xyl1*, encoding a D-xylose reductase necessary for β-galactosidase and cellulase induction, was found to be delayed on lactose but was restored in the presence of lactose and galactose (Fig. 5C and D). In contrast, transcription of *gal1*, encoding galactose kinase, was not affected in the absence of CEL1a (Fig. 5E). We further tested the effect of deletion of *xyl1* on the restored induction. As shown in Fig. 5F, in contrast with the results reported for the WT (7), the absence of XYL1 did not compromise the galactose-restored transcription of *cbh1* in the Δ*cel1a* strain. These data implicate that unlike the case in the WT, galactose metabolism may not be involved in the restored cellulase induction upon addition of galactose in the absence of CEL1a.

Both CEL1a and its associated hydrolytic activity are responsible for the efficient induction of cellulase genes. Although no apparent intracellular β-galactosidase-encoding gene exists in the *T. reesei* genome and negligible intracellular compared with extracellular β-galactosidase activity was detected during culture on lactose (5), we still wondered whether CEL1a and CEL1b are capable of hydrolyzing lactose. We heterologously expressed and purified CEL1a and CEL1b from *Escherichia coli* and tested their lactose hydrolytic activity. The purified CEL1a protein exhibited apparent hydrolytic activity toward ONPG and lactose, though the hydrolysis was much less efficient than that toward pNPG (Fig. 6A and B; see Fig. S5 and Table S2 in the supplemental material). As expected, targeted change of the catalytic residue Glu367 to Ala (E367A) in CEL1a resulted in the complete loss of both pNPG- and ONPG-targeted hydrolytic activities (Fig. 6A and B). To gain insight into the possibility that CEL1a-associated glycoside hydrolytic activity is involved in the induction process, we reintroduced the WT or catalytic mutant *cel1a* gene back into the endogenous *cel1a* locus so that the expression of the gene was under the control of its own promoter in the Δ*cel1a* Δ*cel1b* strain. Compared with the reintroduced WT CEL1a protein, which fully recapitulated the cellulase induction by lactose, CEL1a (E367A) was incapable of induction (Fig. 7A). Further analysis of the intracellular β-glucosidase activity demonstrated that a corresponding increase in the activity at the early stage of lactose induction (24 h) was observed only in cells expressing WT CEL1a, not for the CEL1a (E367A)-complemented strain (Fig. 7B). To further test whether CEL1a-associated hydrolytic activity toward lactose is fully responsible for cellulase induction, we constitutively expressed WT CEL1a, CEL1a (E367A), or the β-galactosidase LAC4 from *Kluyveromyces lactis* in the Δ*cel1a* strain. Transcription of the reintroduced *cel1a*, *cel1a* (E367A), and *lac4* genes occurred efficiently even before induction on lactose (Fig. 7C and D). Analysis of the induced expression of *cbh1* revealed that while constitutive expression of WT CEL1a resulted in relatively more induced expression of *cbh1* than that with the WT strain, cells expressing CEL1a (E367A) or LAC4 displayed the same kinetics of *cbh1* induction as that in the Δ*cel1a* strain on lactose (Fig. 7E to G). Altogether, these data suggest that either a specific kinetics of lactose hydrolysis achieved only by CEL1a and CEL1b or intracellular processing of lactose beyond sole hydrolysis mediated by these β-glucosidases may account for their important roles in cellulase induction by lactose.

Regulation of expression of *xyr1* and *crt1* essential for lactose induction by CEL1a and CEL1b. XYR1 has been established as the key regulator of the cellulolytic response to Avicel or lactose in *T. reesei* (20, 21). Our recent results, together with others, also identified an MFS sugar transporter, CRT1 (Tr_3405), that is essential for lactose- or Avicel-induced cellulase gene expression (9). We therefore tested whether the absence of CEL1a and CEL1b affected the expression of the respective genes in response to lactose. Compared with that of the WT, transcription of both *xyr1* and *crt1* was drastically reduced in the Δ*cel1a* and Δ*cel1a* Δ*cel1b* strains during culture on lactose (Fig. 8A). In contrast to the case on lactose,


A
WT
Δcel1aΔcel1b
Δcel1aΔcel1bNEcel1a
Δcel1aΔcel1bNEcel1a (E367A)
Relative expression of cbh1
Time (h)
B
pNPG activity (IU/mg protein)
Time (h)
C
WT
Δcel1a
Δcel1aCEcel1a
Δcel1aCEcel1a(E367A)
Relative expression of cel1a
Time (h)
D
Δcel1aCElac4
Copy number per 10^5 actin transcripts
Time (h)
E
WT
Δcel1a
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Δcel1aCEcel1a (E367A)
Δcel1aCElac4
pNPC activity (U/ml)
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12 24 36 48 60 hour
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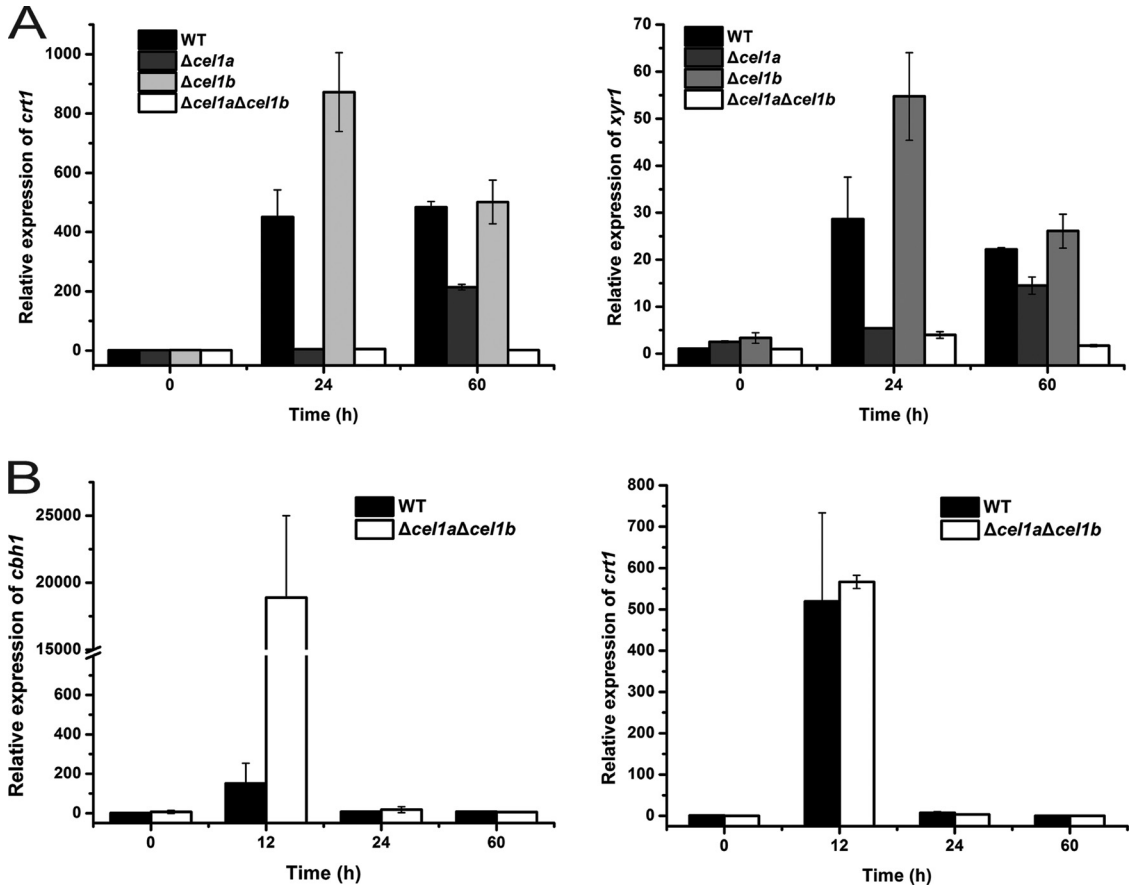

FIG 8 Transcriptional analysis of *xyr1*, *crt1*, and *cbh1* in response to lactose or cellobiose. (A) Transcription of *xyr1* and *crt1* in WT and β-glucosidase deletion strains cultured on 1% (wt/vol) lactose. Statistical analyses indicated significant differences in the transcription of *xyr1* or *crt1* between the WT and Δ*cel1a* strains ($P = 0.01$ at 24 h and $P = 0.002$ at 60 h for *xyr1*; $P = 0.001$ at 24 h and $P < 0.001$ at 60 h for *crt1*) as well as between the WT and Δ*cel1a* Δ*cel1b* strains ($P = 0.008$ at 24 h and $P < 0.001$ at 60 h for *xyr1*; $P = 0.001$ at 24 h and $P < 0.001$ at 60 h for *crt1*). (B) Transcription of *cbh1* and *crt1* in WT and β-glucosidase deletion strains cultured on 0.25% (wt/vol) cellobiose. Values are the means of results from three biological replicates. Error bars show the SD for these replicates.

induced transcription of *crt1* was not affected, and the *cbh1* transcript level was even significantly increased, in the Δ*cel1a* Δ*cel1b* strain on cellobiose (Fig. 8B), suggesting that the regulatory effect on the cellulolytic response by CEL1a and CEL1b is specific for lactose. To test whether the induction defect observed in the absence of CEL1a and CEL1b was solely due to the compromised expression of *crt1* or *xyr1*, we expressed *crt1* or *xyr1* under the control of the *pki* or *gpd* promoter in the Δ*cel1a* Δ*cel1b* strain and analyzed its response to lactose. No induced expression of *cbh1* was detected in the recombinant Δ*cel1a* Δ*cel1b* strain even under lactose induction, though the expression of *crt1* and *xyr1* in the recombinant strain occurred early on lactose and reached a level comparable to that of the WT (see Fig. S6 in the supplemental material), suggesting that, unlike the case for the WT strain with constitutive expression of Xyr1 (22), expression of *crt1* or *xyr1* is not sufficient to activate cellulolytic transcription, even upon induction, in the absence of CEL1a and CEL1b.

DISCUSSION

Although rarely present in its natural habitat, lactose is able to support the growth of cellulolytic *Trichoderma reesei* and to simultaneously induce its secretion of cellulases. Lactose has thus been one of the most important carbon sources for (hemi)cellulolytic enzyme production from *T. reesei* on a technical scale, despite the less-well-understood mechanism. In this study, we demonstrated that the absence of the intracellular β-glucosidases CEL1a and

FIG 7 Expression of catalytically inactive CEL1a (E367A) or *K. lactis* LAC4 is unable to restore cellulase induction. Transcription levels of *cbh1* (A) and intracellular β-glucosidase activities (B) in the WT, Δ*cel1a* Δ*cel1b*, Δ*cel1a* Δ*cel1b* NE*cel1a*, and Δ*cel1a* Δ*cel1b* NE*cel1a* (E367A) strains after induction with lactose (1% [wt/vol]) are shown. (C) Transcript levels of *cel1a* in *T. reesei* WT, Δ*cel1a*, Δ*cel1a* CE*cel1a*, and Δ*cel1a* CE*cel1a* (E367A) strains after transfer to a lactose (1% [wt/vol])-containing medium. (D) Transcript levels of *lac4* in the Δ*cel1a* CE*lac4* strain induced by 1% (wt/vol) lactose were analyzed by quantitative RT-PCR. Copy numbers of *lac4* transcripts relative to those of the actin gene are shown. (E) Exoglucanase activity analysis of the culture supernatants of the WT, Δ*cel1a*, Δ*cel1a* CE*cel1a*, Δ*cel1a* CE*cel1a* (E367A), and Δ*cel1a* CE*lac4* strains grown on 1% (wt/vol) lactose. pNPC was used as the substrate. (F) Western blot analysis of extracellularly secreted CBH1 proteins of the WT, Δ*cel1a*, Δ*cel1a* CE*cel1a*, Δ*cel1a* CE*cel1a* (E367A), and Δ*cel1a* CE*lac4* strains grown on 1% (wt/vol) lactose. Equal amounts of culture supernatant relative to biomass were loaded for all strains. (G) Transcript levels of *cbh1* in *T. reesei* WT, Δ*cel1a*, Δ*cel1a* CE*cel1a*, Δ*cel1a* CE*cel1a* (E367A), and Δ*cel1a* CE*lac4* strains after transfer to a lactose (1% [wt/vol])-containing medium. Data shown are the means of results from three independent experiments. Error bars show the SD for these replicates.

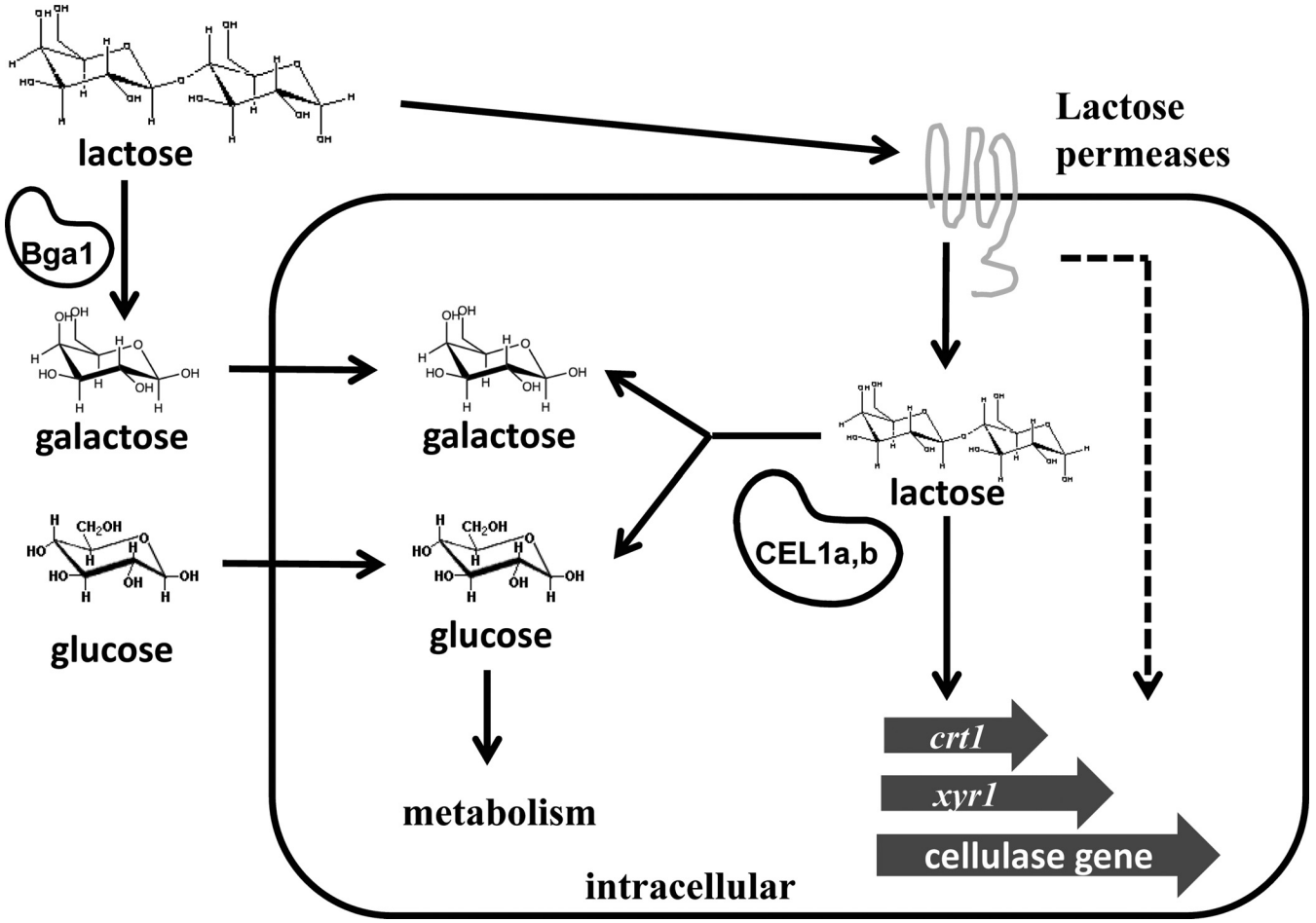

FIG 9 Model of the role of intracellular β-glucosidases in cellulase induction by lactose in *T. reesei*. Upon growth of cells on lactose, lactose is either hydrolyzed extracellularly by BGA1 or transported intracellularly by lactose permeases. Further processing of lactose by the intracellular β-glucosidases CEL1a and CEL1b is necessary to initiate the transcriptional induction of genes, including *xyr1*, *crt1*, and cellulase genes. A possible signaling cascade may exist that initiates from CRT1 and converges on the β-glucosidase-mediated pathway to ensure efficient induction (dashed line).

CEL1b abolishes *cbh1* gene expression on lactose. We further presented evidence that both the CEL1a protein and its hydrolytic activity are responsible for the efficient cellulase induction by lactose. The intracellular β-glucosidase-mediated induction pathway may act downstream of intracellular delivery of lactose to activate XYR1, ensuring the successfully induced expression of cellulase genes.

While several lines of evidence support the notion that catabolism of lactose in *T. reesei* is realized only by extracellular hydrolysis and subsequent uptake of the resultant monomers (4), the most recent research identifies several MFS transporters that behave as lactose permeases and are involved in cellulase induction (9–11). In these scenarios, it is plausible to speculate that the intracellular presence of lactose may represent a key point in generating an inducing signal for cellulase gene expression. In the present report, we provide evidence supporting such an assumption. First, our data revealed that in addition to β-glucosidase activity, recombinant CEL1a also possesses weak hydrolytic activity toward lactose and ONPG. It is known that quite a few β-glucosidases belonging to glycosyl hydrolase (GH) family 1 also display β-galactosidase activity (23). Second, the absence of CEL1a and CEL1b almost abolishes cellulase gene induction by lactose, and catalytically inactive CEL1a is incapable of restoring efficiently induced cellulase gene expression. On the other hand, intracellular hydrolysis of lactose does not seem to fully account for the CEL1a- and CEL1b-mediated induction, since expression of a different GH2 galactosidase is incapable of restoring cellulase gene induction by lactose. Indeed, it has been shown that intracellular β-galactosidase activity is negligible, and slow metabolism of D-galactose alone is not sufficient to account for the efficient cellulase induction on lactose (8). Our result showing that galactose is not able to restore efficient induction in the absence of CEL1a and CEL1b reinforces such a point. However, the possibility that the subtle difference in the rates at which these two glycosidases catalyze the hydrolysis of lactose represents a very critical parameter for the efficient induction by lactose cannot be excluded. The possibility also exists that CEL1a and CEL1b could otherwise possess hydrolysis-associated activities, such as transglycosylation, to process lactose beyond hydrolysis. In this respect, it has been demonstrated that intracellular accumulation of galacto-oligosaccharides correlates with the level of cellulase formation during growth of *T. reesei* on lactose (24). Although the exact mechanism for how intracellular β-glucosidases contribute to the formation of inducer for cellulase gene expression warrants further study, the

relatively earlier induction and higher level of cellulase gene expression conveyed by the constitutively expressed CEL1a protein are industrially relevant in view of cellulase production on lactose.

The lactose metabolism pathway has been shown to affect cellulase induction, though the involved mechanism is still elusive. An interesting point that somewhat contrasts with what has been reported for wild-type *T. reesei* can be made from our data. While the efficient transcription of cellulase genes is compromised in the Δ*cel1a* strain, we found that full restoration or even a higher level of cellulase gene expression in the Δ*cel1a* strain by adding galactose to the lactose medium was not influenced by further deletion of *xyl1*, whose absence results in a severe reduction in cellulase gene expression in WT *T. reesei* (7). This finding indicates that the restored cellulase gene expression can proceed independently of the second D-galactose-catabolizing pathway in the absence of CEL1a. On the other hand, transcription of *gal1*, the first gene of the Leloir pathway, is not affected during the growth of the Δ*cel1a* strain on lactose. Although the possibility that GAL1 contributes to the restored induction upon galactose addition in the absence of CEL1a cannot be excluded at present, it is clear that intracellular β-glucosidases act independently of galactose metabolism in mediating the inducing cascade, considering that their absence has no effect on the growth on galactose. Finally, the induced cellulase gene expression in the Δ*cel1a* strain upon galactose addition is not due to the restored extracellular hydrolysis of lactose, since the simultaneous absence of BGA1 has no effect on the recovered induction. The finding that addition of galactose is not able to restore the induction in the Δ*cel1a* Δ*cel1b* strain, although it restores its growth on lactose, further supports such a conclusion.

Our previous work and that of others have shown that CRT1, an MFS transporter protein, is critical for cellulase induction by lactose and cellulose (9). While transcription of *crt1* is downregulated in the absence of intracellular glucosidases, constitutively expressing *crt1* in the Δ*cel1a* Δ*cel1b* strain is not sufficient to recover its ability to respond to lactose induction. The failure to achieve efficient induction in these strains does not seem to result from the decreased expression of XYR1, since overexpression of XYR1 in the Δ*cel1a* Δ*cel1b* strain failed to initiate induction. We therefore hypothesize that intracellular processing of lactose by CEL1a and CEL1b acts downstream of intracellular delivery of lactose mediated by lactose permeases to achieve the efficient induction of cellulase genes (Fig. 9). The possibility cannot be excluded, however, that a potential signaling cascade initiated by CRT1 may exist and run relatively independent of β-glucosidase-mediated processing of lactose. Inducing signals transmitted by intracellular glucosidases and CRT1 both are thus necessary and may converge on XYR1 to ensure the successful cellulase gene expression on lactose.

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