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Gluconolactone induces cellulase gene expression in cellulolytic filamentous fungus *Trichoderma reesei*

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Keywords: Gluconolactone; *Trichoderma reesei*; cellulase; β-glucosidase; transporter

Abstract

Filamentous fungus *Trichoderma reesei* is well known for its high capacity of producing cellulases for industrial application. Besides crystalline cellulose, several soluble sugars including cellobiose can effectively induce cellulase formation. In this study, gluconolactone, previously reported as a β-glucosidase inhibitor, was demonstrated to be capable of inducing cellulase gene expression at a level equivalent to that induced by cellobiose. Gluconolactone-induced formation of cellulase was abolished in *T. reesei* strain lacking Xyr1 or Crt1, two key regulators for cellulase gene expression. The induced expression of cellulase gene *cbh1* was eliminated in the absence of intracellular β-glucosidase Cel1a on gluconolactone while it was hardly affected by the absence of extracellular β-glucosidase Bgl1. We further found that the absence of a cellobiose/glucose transporter Stp1 compromised cellulase production and led to a lower consumption of extracellular gluconolatone. These results suggest that gluconolatone-derived inducing signal involves both its sensing at the membrane and its intracellular delivery for further processing to initiate cellulase formation.

Introduction

Filamentous fungus *Trichoderma reesei* (telemorph *Hypocrea jecorina*) is one of the most prolific cellulase producers in industry due to its excellent capacity to secret large amounts of cellulases, which consist of at least three types of enzymes including endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21)[1]. The synergistic action of endoglucanases and exoglucanases can achieve the efficient degradation of crystalline cellulose with cellobiose as the major product, which is further hydrolyzed to glucose by β-glucosidases[2]. Cellobiohydrolase 1 (CBH1) is the most abundant cellulase secreted by *T. reesei*, which accounts for approximate 60% of the total extracellular cellulases[3]. Bgl1(Cel3a) and Cel1a are the two major extracellular and intracellular β-glucosidases respectively[4]. Deletion of *cel1a* significantly compromised expression of cellulase gene *cbh1* induced by cellulose[4].

Besides insoluble cellulose, several soluble disaccharides can also effectively induce cellulase formation in *T. reesei*. Specifically, both cellobiose and its transglycosylating derivative, sophorose, have been shown to provoke cellulase induction[5-7]. Besides, lactose is actually one of the most important economic soluble carbon sources for industrial production of cellulases by *T. reesei*. It has been reported that when cellobiose was applied as an inducer for cellulase production, its hydrolysis by extracellular β-glucosidases declined cellulase formation[8]. Addition of a strong competitive inhibitor of β-glucosidase—D-glucono-δ-lactone, also known as gluconolactone, can effectively facilitate the cellulase formation[8]. Here, we present evidence that gluconolactone alone can induce cellulase formation in *T. reesei* and compared its cellulase-inducing ability with that from cellobiose. We show that the cellulase gene induction by gluconolactone relies on two key cellulolytic regulators

Xyr1 and Crt1. We further show the effects of the absence of extracellular β-glucosidase Bgl1 and intracellular β-glucosidase Cel1a as well as a cellobiose/glucose transporter Stp1 in gluconolactone-induced expression of cellulase gene.

Experimental section

Strains, medium and cultivation

The wild type strain *Trichoderma reesei* TU-6 (ATCC MYA-256)[9], a uridine-auxotrophic derivative of *T. reesei* QM9414 (ATCC 26921), was used as the parental strain in this work. *T. reesei* strains with targeted mutations used in this study were listed in Table 1. Strains were grown on a rotary shaker with 200 rpm at 30 ^{o}C in the minimal medium as described by Mandels and Andreotti[10] with glycerol (0.25% or 1%, w/v), Avicel (1%, w/v), cellobiose (0.5%, w/v) and gluconolactone (0.1 - 0.75%, w/v) as the sole carbon source as indicated respectively. The above carbon sources were all purchased from Sigma-Aldrich. *Escherichia coli* DH 5α was used for routine plasmid construction and amplification.

Table 1 *Trichoderma reesei* strains used in this study

Name	Reference
TU-6	(9)
Δ*xyr1*	(12)
Δ*crt1*	(13)
Δ*cel1a*	(4)
Δ*stp1*	(13)

For the cellulase production analysis, strains were precultured with 1% (w/v) glycerol for 36 h and transferred to the same fresh medium for another 12 h growth.

After harvested by filtration，mycelia were washed thrice with medium without carbon sources and finally equal amount of mycelia were transferred to a fresh medium containing respective carbon sources as indicated.

Enzymatic activity measurements

The exoglucanase activity assay was performed in 200 μL of reaction mixture containing 100 μL of sodium acetate buffer (50 mM, pH 5.0), 50 μL of culture supernatant and 50 μL of 5 mM p-nitrophenol-D-cellobioside (pNPC) plus 0.1% gluconolactone as the substrates. The reaction was incubated for 30 min at 45 ℃. One unit of exoglucanase activity (U) was defined as the amount of enzyme that releases 1 mM of pNP per minute.

Western blot analysis

Western blot analysis was performed according to standard protocols[11]. Detection of the cellulase CBH1 was performed by immunoblot using a polyclonal antibody raised against amino acids (426-446) of CBH1 as previously described[4]. Equal amount of culture supernatant relative to biomass was loaded for strains tested.

Nucleic acid isolation and Quantitative RT-PCR

Fungal mycelia was filtered and washed twice with water before frozen in liquid nitrogen and then stored in ultra-low temperature freezer. Total RNA was extracted according to the Trizol reagent (Invitrogen, USA) following the manufacturer's protocol and subsequently treated with TURBO DNA-free kit (Ambion) to remove DNA. The PrimeScript RT reagent Kit (Takara, Japan) was used for reverse transcription. Quantitative RT-PCR was performed on a Bio-Rad myIQ2 thermocycler (Bio-Rad) with a total reaction volume of 20 μL using the SYBR Green Supermix (Takara) with 250 nM each of forward and reverse primers and template cDNA of appropriate concentration. Each reaction was repeated for three times. Data

analysis was performed using the Relative Quantitation/Comparative CT (ΔΔCT) method and was normalized to the endogenous control actin with expression on glycerol as the reference sample. Primers used in quantitative RT-PCR were listed in Table S1.

HPLC analysis

Analysis of the amount of residual gluconolactone in culture supernatant was performed by HPLC (High Performance Liquid Chromatography) as follows. After centrifuged for 10 min at 12000 g and filtered with a 0.22 μm membrane to remove microparticulate interfused, the supernatants were applied onto a Bio-Rad Aminex HPX-42A carbohydrate column and analyzed by LC-10AD HPLC (Shimadzu, Japan) with a RID-10A refractive index detector equipped. The mobile phase was distilled water at a flow rate of 0.4 mL/min and column temperature was 75 ^{o}C.

Biomass determination. The dry weight of mycelium was recorded by first collecting mycelium from 250 mL medium cultivated for the indicated time, and then centrifuging at 10, 000 g for 10 min, and final drying to a constant weight at 80 ^{o}C.

Results

Gluconolactone can induce cellulase gene expression in *T. reesei*

We originally employed gluconolactone as an inhibitor of β-glucosidase to reduce extracellular hydrolysis when culturing *T. reesei* strain with cellobiose, a potential inducer of cellulase formation in response to cellulose. In order to determine the influence of gluconolactone on cellulase production, we cultured *T. reesei* with medium containing gluconolactone at concentrations of 0.1 - 0.75% (w/v) and performed western blot analysis to detect CBH1 production in the culture supernatant. Unexpectedly, gluconolactone alone is capable of inducing cellulase formation, with maximum CBH1 production detected at the concentration of 0.25% (Fig. 1a).

Analysis of the extracellular hydrolytic activity toward pNPC confirmed that optimal cellulase production with the highest hydrolytic activity occurred in the presence of 0.25% gluconolactone (Fig. 1b). Quantitative RT-PCR analysis indicated a significant induced transcription of *cbh1*, as well as *bgl1* and *cel1a* that encodes the major extracellular and intracellular β-glucosidase respectively (Fig. 1c). In order to assess the inducing potency of gluconolactone, the relative level of endogenous *cbh1* mRNA in *T. reesei* cultured with 0.25% gluconolactone versus 0.5% (w/v) cellobiose was analyzed. We chose 0.5% cellobiose due to its relatively appreciable ability to induce cellulase formation as compared with other concentrations of cellobiose (data not shown). After 24 h induction by gluconolactone, the relative induced transcription of *cbh1* reached a level about ten-fold as high as that by cellobiose (Fig. 1d), indicating that gluconolatone is a more efficient inducer for cellulase production. In addition, gluconolactone also served as a kind of carbon source for *T. reesei* since a moderate growth was observed, although the final biomass yield is about 30% lower than that cultured with glycerol (Fig. 1e).

Cellulase induction by gluconolactone requires the presence of two key cellulolytic regulators, Xyr1 and Crt1

The transcription activator Xyr1 is essential for cellulase gene expression in *T. reesei*, whose absence abolished transcription of most cellulase genes on cellulose or lactose[12]. In our previous study, we identified a "transceptor" Crt1 that is probably involved in signal sensing and transmitting during induction on crystalline Avicel[13]. Deletion of *crt1* resulted in complete loss of cellulase production on cellulose or lactose or sophorose[13]. The *T. reesei* strain lacking Xyr1 or Crt1 lost the capability to form cellulases on gluconolactone, with neither secreted CBH1 nor induced transcription of *cbh1* detected (Fig. 2a and 2b). Besides *cbh1*, induced transcription of

bgl1 or *cel1a* was also abolished (Fig. 2c and 2d), demonstrating that the cellulolytic response to gluconolatone is also regulated by these two regulators Xyr1 and Crt1. Moreover, we observed that in comparison with wild type strain (WT), relative transcription of *crt1* in strain Δ*xyr1* and the relative transcription of *xyr1* in Δ*crt1* were both abrogated (Fig. 2e and 2f), indicating that each of them plays an indispensable role in gluconolactone induction for cellulase expression.

The intracellular β-glucosidase Cel1a plays an essential role in cellulase gene expression on gluconolactone

As described above, gluconolactone is a strong inhibitor of β-glucosidase probably by competing its substrate-binding site with cellobiose[14]. In order to determine the effects of β-glucosidases on gluconolactone induction, we test the effect of the absence of two major β-glucosidases, extracellular Bgl1 and intracellular Cel1a, on gluconolactone-induced cellulase production respectively. The absence of Bgl1 hardly affected the induced expression of *cbh1* though a relatively lower transcriptional level was observed as compared with WT strain at a later stage of induction (Fig. 3a and 3b). In contrast, the absence of intracellular Cel1a severely compromised the cellulase formation since neither extracellular CBH1 secretion nor notable induced transcription of *cbh1* was detected in the Δ*cel1a* strain (Fig. 3a and 3b). These results indicated that, as compared with Bgl1, intracellular Cel1a plays an essential role in cellulase gene expression when induced by gluconolactone. In accordance with the serious defect in cellulase induction on gluconolactone in Δ*cel1a*, a drastic decrease occurred in the relative transcriptional level of *xyr1* and *crt1* (Fig. 3c and 3d).

Cellulase production induced by gluconolactone is compromised in the absence of a cellobiose/glucose transporter Stp1

It is reasonable to speculate that the induction of cellulase genes by gluconolactone

incurs either gluconolactone sensors or uptake transporters for further intracellular processing. We previously identified a cellobiose transporter named Stp1 from *T. reesei*, which is also capable of transporting glucose[13]. Transcription of *stp1* is upregulated on induction by gluconolactone, with a 7-fold elevation after 12 h cultivation (Fig. 4a). The absence of Stp1 in *T. reesei* compromised cellulase formation in response to gluconolactone, as demonstrated by western blot analysis of the extracellular CBH1 (Fig. 4b). Examination of endogenous *cbh1* mRNA by quantitative RT-PCR indicated that the defective cellulase gene induction occurred at a transcriptional level 24 h after induction. The induced transcription of β-glucosidase genes *bgl1* and *ce1a*, as well as two regulator genes *xyr1* and *crt1* were also compromised (Fig. 4c, 4d and 4e). We reasoned that the initial elevated transcription of *cbh1*, *cel1a* and *bgl1* in Δ*stp1* relative to WT after 12 h cultivation on gluconolactone was attributed to the relative higher background expression of *crt1* that occurred during cultivation on glycerol (Fig. 4c and 4d), which also led to enhanced cellulase production on crystalline Avicel, lactose and sophorose[13]. Analysis of the amount of residual gluconolactone in the culture supernatant by HPLC showed that, in comparison with WT strain, Δ*stp1* displayed a much slower rate in consumption of extracellular gluconolactone (Fig. 5a), which correlated well with the defective growth on gluconolactone (Fig. 5b). All together, the above results indicated that Stp1 participated in the cellulase gene expression on induction by gluconolactone and probably functions via affecting its intracellular delivery.

Discussion

In this study, we presented evidence that gluconolactone, an inhibitor for extracellular β-glucosidase applied to facilitate cellulase production on cellobiose, is itself capable of inducing cellulase gene expression. Although the cellulase-inducing

potency of gluconolactone is not as potent as that of cellulose (Fig. S1), it can provoke cellulase gene induction at a level equivalent to that from cellobiose, which is considered as the potential inducer for cellulase formation in response to cellulose. The oxidized disaccharide cellobiono-1,5-1actone (CBL) has also been shown to be capable of inducing cellulase formation[8]. CBL may well arise from the action of cellobiohydrolase 1 (CBH1) and cellobiohydrolase 2 (CBH2) by splitting non-reducing ends of cellulose rather than the action of an oxidase[8]. Therefore, although CBL is a low-V_{max} substrate of *T. reesei* β-glucosidases[8], gluconolactone is supposed to be present in the hydrolysate of cellulose. We have also observed that gluconolactone as the sole carbon source can promote moderate growth of *T. reesei*. HPLC analysis of residual gluconolactone in culture supernatant also confirmed that gluconolactone can be assimilated in *T. reesei*. Similar to many other inducing substrates including cellulose, lactose, cellobiose, and sophorose, we found that cellulolytic response to gluconolactone relies on the two general regulators for cellulase production, Xyr1 and Crt1. However, unlike the case for cellobiose whereas the absence of β-glucosidases Bgl1 and Cel1a facilitates the induced expression of cellulase genes[4], the absence of Cel1a significantly compromised cellulase production in response to gluconolactone. Since gluconolactone can not be hydrolyzed by Cel1a, the exact mechanism involved certainly warrants further study.

Our result showed that the induced cellulase gene expression by gluconolactone was affected by the absence of Stp1, which is a cellobiose/glucose transporter identified previously[13]. The background expression level of cellulolytic regulator Crt1 in Δ*stp1* is higher than that in WT, which may lead to the increased transcription of cellulase gene *cbh1* during the initial stage (12 h) of induction by gluconolactone. However, the induced transcriptional level of *cbh1* was apparently compromised after

24 h cultivation, indicating the absence of Stp1 indeed affected cellulase gene expression. Given that the absence of Stp1 compromised extracellular consumption of gluconolactone, and that intracellular Cel1a is essential for the induction, it can be anticipated that intracellular delivery of gluconolactone constitutes an important step in initiating the induction cascade. Possibility can not be excluded that other sensing and signaling pathways may exist, one of which may be mediated by Crt1, since cellulase formation in Δ*stp1* strain was not completely compromised. Moreover, gluconolactone recognition by Stp1 may depend on its specialty for cellobiose transportation since in contrast to Stp1, absence of a hexose-specific transporter, Tr22912, that can not transport cellobiose in *T. reesei* did not compromise but rather facilitated cellulase gene expression (data not shown).

According to our results, we proposed a model for the possible roles of various proteins involved in the induced cellulase gene expression by gluconolactone (Fig. 6). Upon gluconolactone induction, the transcription of cellulase genes including *cbh1*, *cel1a* and *bgl1* were upregulated to produce cellulases. Two key cellulolytic regulators including the transcription activator Xyr1 and the membrane sensor Crt1, are indispensable for induced cellulase gene expression on gluconolactone. Besides Crt1, another protein located on plasma membrane, the cellobiose/glucose transporter Stp1 plays an important role in gluconolactone induction probably by importing extracellular gluconolactone, which may be further processed by intracellular β-glucosidases to initiate the induction cascade. Our study thus provides a foundation to understand the mechanism of action by gluconolactone in inducing cellulase formation that would contribute to its application in industrial production of cellulases.

Conclusions

We reported that gluconolactone alone can effectively provoke cellulase gene expression in cellulolytic fungus *T. reesei*. Two general cellulolytic regulators Xyr1 and Crt1 as well as the major intracellular β-glucosidase Cel1a are essential in the gluconolactone-induced cellulase gene expression . A cellobiose/glucose transporter Stp1 also plays an important role in cellulolytic response to gluconolactone and extracellular consumption of gluconolactone. With the advantage of being hydrolyzed at a much lower velocity, gluconolactone as well as other sugar lactones may serve as promising inducers for cellulase production in industry.

Acknowledgements

This work is supported by grants from the National Basic Research Program of China (2011CB707402), the National Natural Science Foundation (31300059 to Zhang W and 31270116 to Liu W), New Century Excellent Talents in University (NCET-10-0546), Shandong Provincial Funds for Distinguished Young Scientists (JQ201108), and the Scientific Research Foundation for Excellent Young and Middle-Aged Scientists of Shandong Province of China (BS2013NJ021). We thank Prof. Robert L. Mach from Vienna University of Technology for kindly providing Δ*xyr1* strain.

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Figure legends

Figure 1. Gluconolactone can induce cellulase gene expression in *T. reesei* and support moderate growth as the sole carbon source. (a) and (b), western blot analysis of extracellular CBH1 and exoglucanase activity of the culture supernatant from *T. reesei* cultivated with gluconolactone at concentrations of 0.1 – 0.75% (w/v) for the indicated time period. Equal control protein was loaded to assess the difference of protein transfer efficiency on each PVDF membrane in western blot analysis. (c) Quantitative RT-PCR analysis of gene expression of *cbh1*, *bgl1* and *cel1a* induced by 0.25% gluconolactone. (d) Quantitative RT-PCR analysis of relative transcription of *cbh1* on induction by 0.25% (w/v) gluconolactone versus 0.5% (w/v) cellobiose. (e) Growth curves of *T. reesei* with 0.25% (w/v) glycerol and 0.25% (w/v) gluconolactone as the sole carbon source respectively. In all panels, values are the mean of three biological replicates. Error bars are the SD from these replicates.

Figure 2. The individual absence of the two key cellulolytic regulators Xyr1 and Crt1 abolished cellulase gene expression on induction by gluconolactone. Western blot analysis of CBH1 secreted in the culture supernatant (a) and quantitative RT-PCR analysis of relative transcription of *cbh1* (b), *bgl1* (c) and *cel1a* (d) in WT, Δ*xyr1* and Δ*crt1* strains in the presence of 0.25% gluconolactone. (e) and (f), the relative transcriptional level of *crt1* in Δ*xyr1* strain and the relative transcriptional level of *xyr1* in Δ*crt1* strain cultured by 0.25% gluconolactone. Values are the mean of three

biological replicates. Error bars are the SD from these replicates.

Figure 3. The absence of the major intracellular β-glucosidase Cel1a but not extracellular Bgl1 resulted in a drastic loss of gluconolactone-induced expression of cellulase gene *cbh1*. (a) and (b), quantitative RT-PCR analysis of relative transcription of *cbh1* and western blot analysis of CBH1 secreted in the culture supernatant from WT, Δ*cel1a* and Δ*bgl1* respectively cultured with 0.25% gluconolactone for the indicated time period. The values below the panels in (b) indicate the ratio of the intensity of CBH1 signal as measured by densitometry to that of the positive control protein. (c) and (d), quantitative RT-PCR analysis of relative transcription of *xyr1* and *crt1* in WT and Δ*cel1a* strains on induction by 0.25% gluconolactone. Values are the mean of three biological replicates. Error bars are the SD from these replicates.

Figure 4. The absence of Stp1 compromised cellulase gene expression induced by gluconolactone. (a) Analysis of the relative transcription of *stp1* in *T. reesei* cultured in 0.25% gluconolactone for the indicated time period by quantitative RT-PCR. Western blot analysis of CBH1 secreted in the culture supernatant (b) and quantitative RT-PCR analysis of relative transcriptional level of genes including *cbh1*, *cel1a* and *bgl1* (c) as well as *crt1* (d) and *xyr1* (e) in WT and Δ*stp1* strains in the presence of 0.25% gluconolactone.

Figure 5. The absence of Stp1 compromised extracellular consumption of gluconolactone and vegetative growth with gluconolactone as the sole carbon source in *T. reesei*. (a) HPLC analysis of the residual gluconolactone in the culture supernatant of *T. reesei* when cultured with 0.25% gluconolactone. Control sample represented the medium supernatant without mycelium inoculation. (b) dry weight of mycelium of Δ*stp1* and WT strains cultured with 0.25% **gluconolactone as the sole**

carbon source for the indicated time period.

Figure 6. Model for the possible roles of various proteins involved in cellulase-gene expression induced by gluconolactone. Gluconolactone-induced cellulase gene expression requires the presence of two cellulolytic regulators Xyr and Crt1, as well as the intracellular β-glucosidase Cel1a. The cellobiose/glucose transporter Stp1 plays an important role in gluconolactone induction and is probably responsible for cellular uptake of gluconolactone.

Figure 1

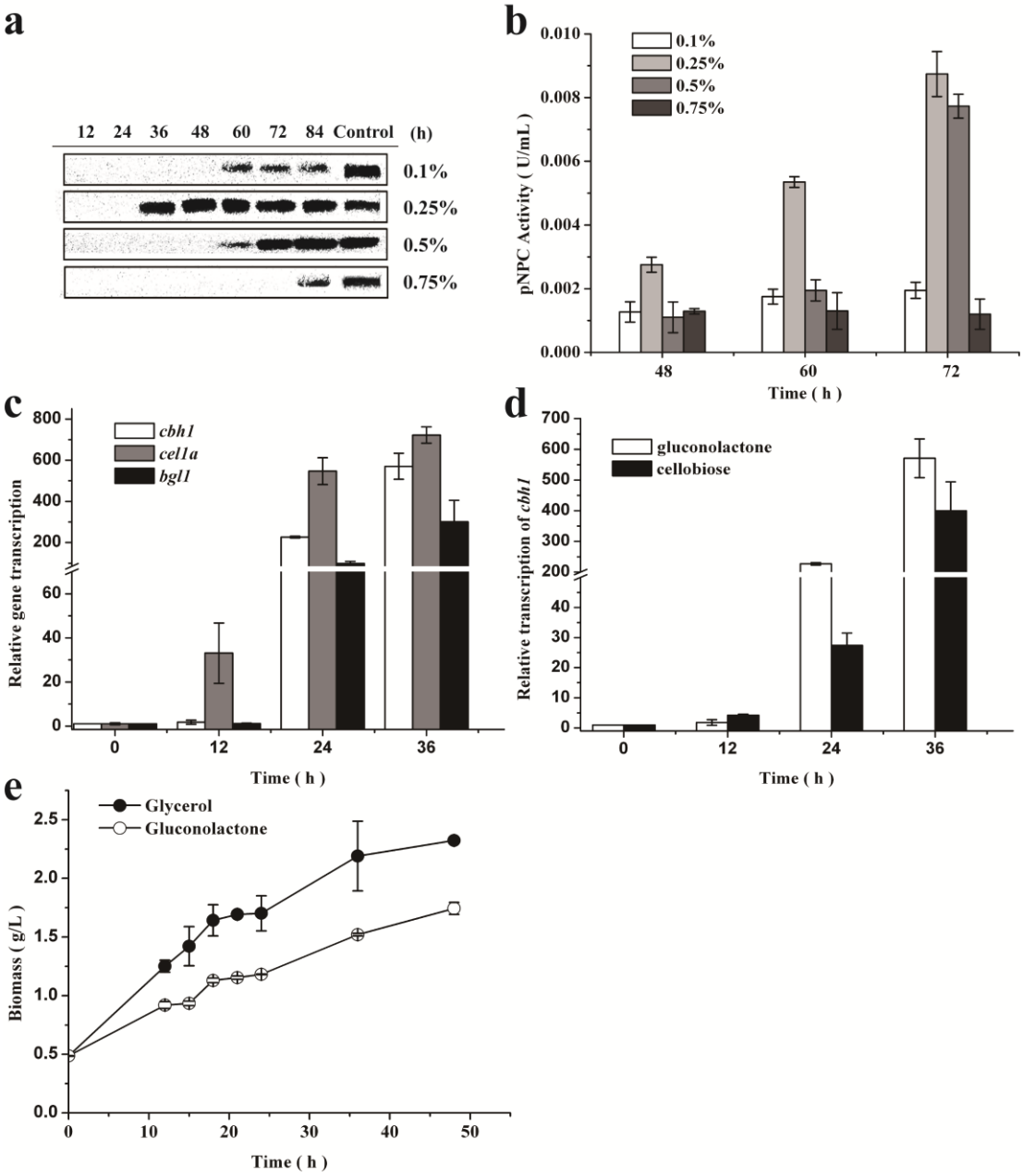

Figure 2

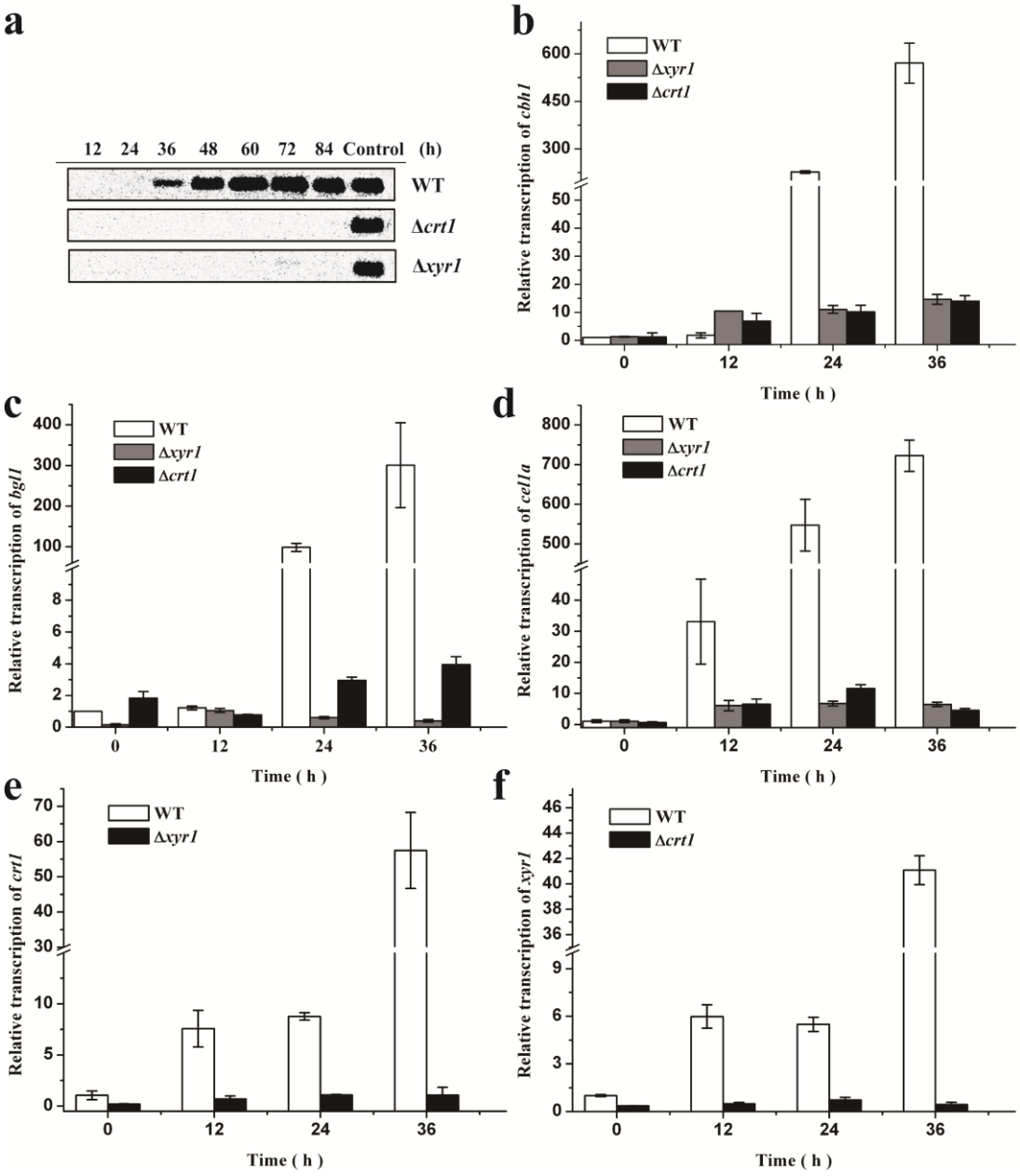

Figure 3

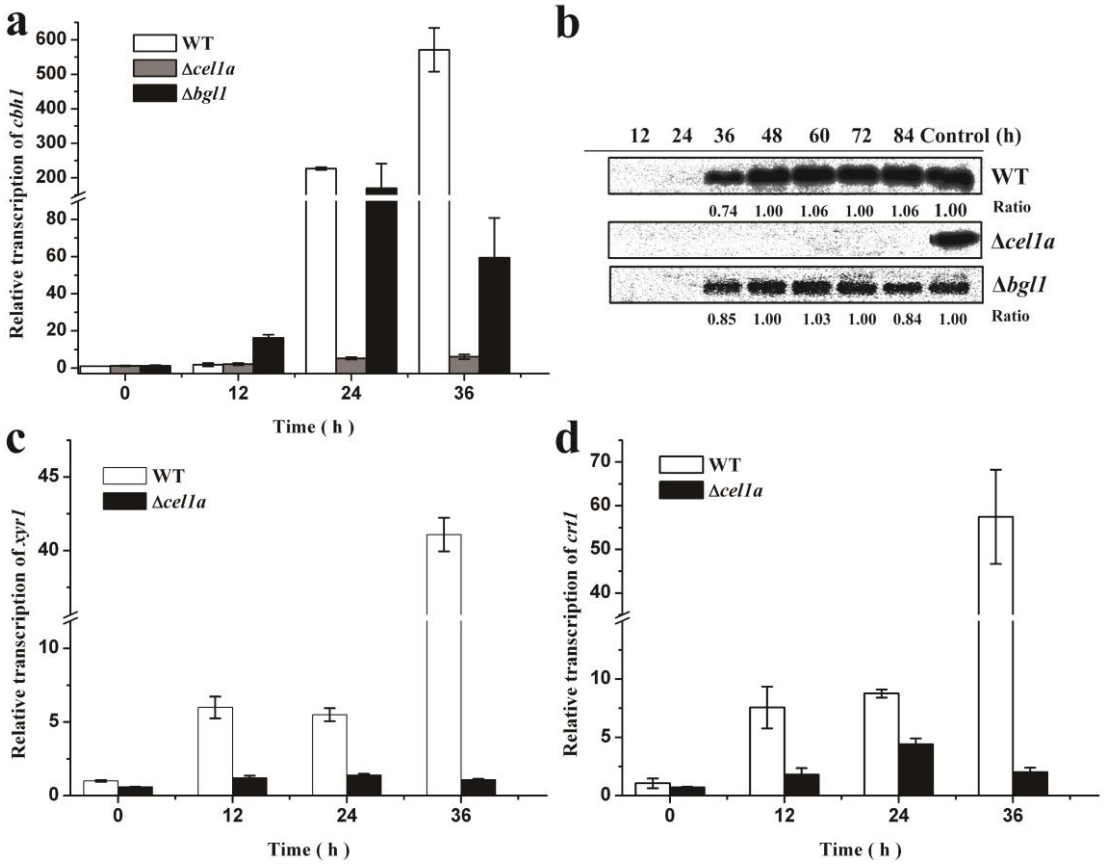

Figure 4

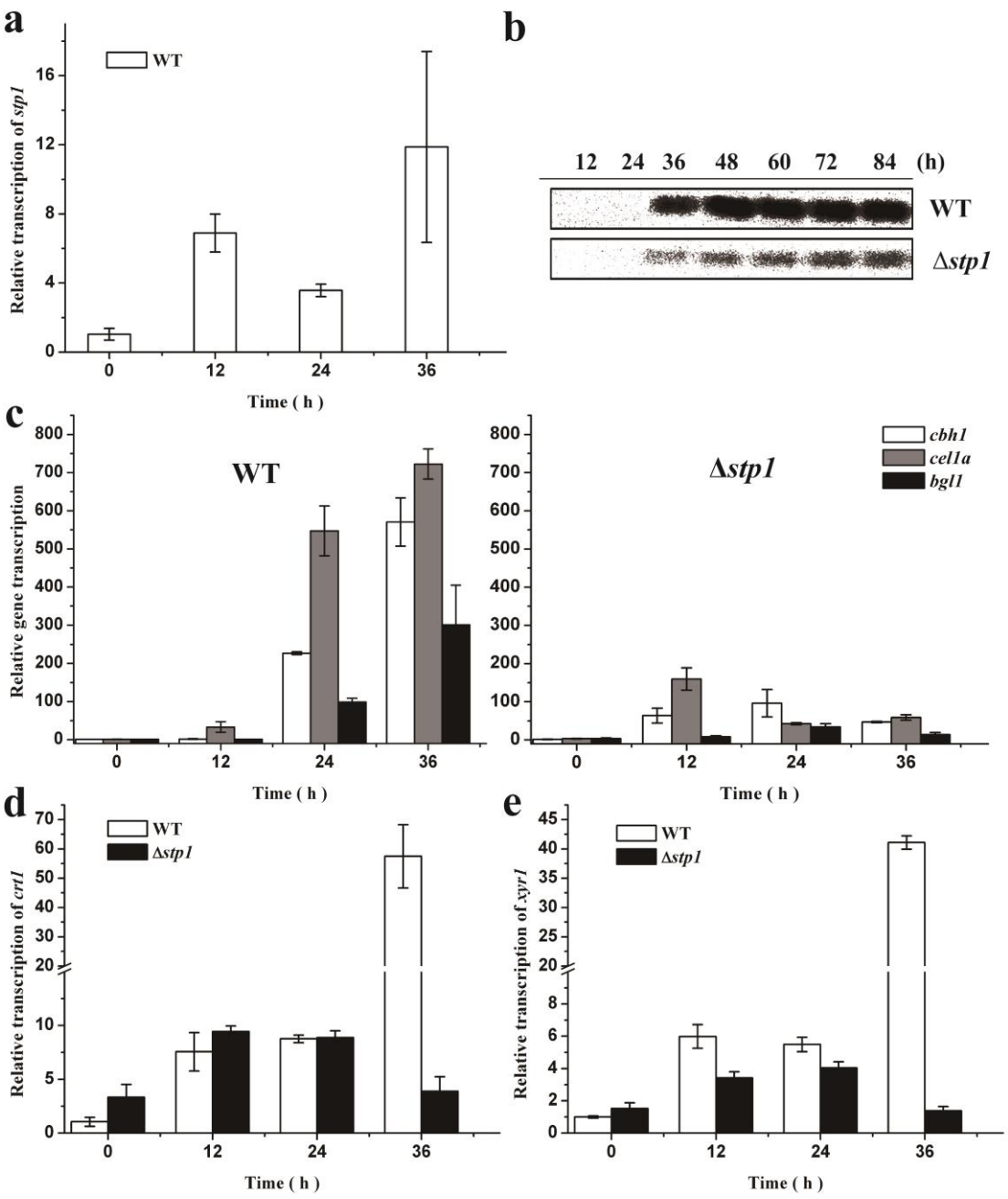

Figure 5

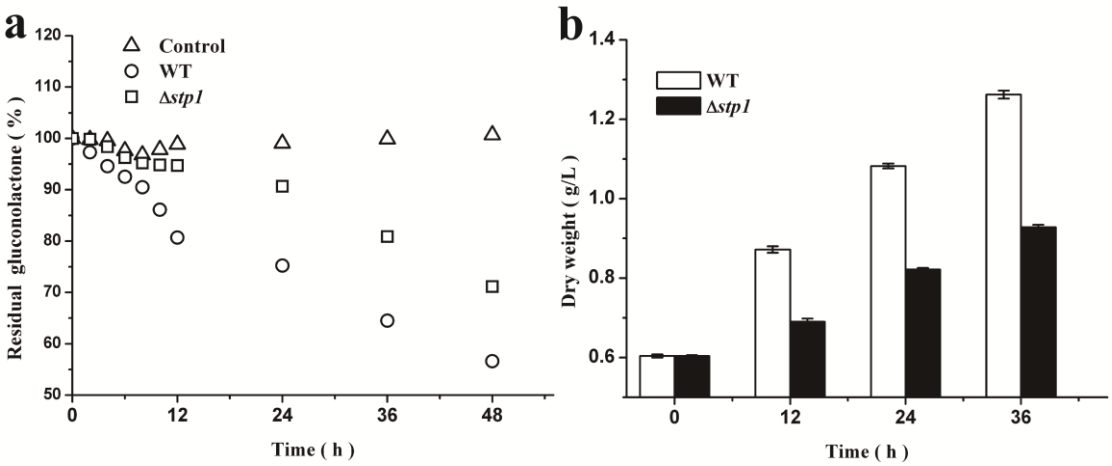

Figure 6