Overexpression of nicotinamide mononucleotide adenyltransferase (nmnat) increases growth rate, Ca2+ content and cellulase production in *Ganoderma lucidum*

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Abstract

Background: There is an urgent need to search for new and economical ways to utilize diverse lignocellulose. *Ganoderma lucidum* is well-known edible medicinal fungus that has a strong ability to degrade a wide variety of cellulosic biomass and its nutrient utilization is closely related to extracellular cellulase. Nicotinamide adenine dinucleotide (NAD+), a nutritional sensor molecule, can respond to nutritional states and regulate cellular metabolism. Nicotinamide mononucleotide adenyltransferase (nmnat) is the key enzyme that catalyses the biosynthesis of NAD+.

Result: In this study, a homologue of the gene encoding nmnat was cloned from *G. lucidum*. The Agrobacterium tumefaciens -mediated transformation (ATMT) method was used to construct the two overexpression strains OE:: *nmnat4* and OE:: *nmnat19* in *G. lucidum*. In the overexpression strains, the transcript levels of the *nmnat* gene and the NAD+ content were significantly increased. *Gl nmnat* overexpression strains showed dramatically stronger colony growth on different carbon sources, and the intracellular Ca 2+ concentration increased 3.95-fold and 2.10-fold in OE:: *nmnat4* and OE:: *nmnat19* , respectively, compared with WT strains. The CMCase activity increased by approximately 2.8-fold and 3-fold, and *p* NPGase activity increased by approximately 1.9-fold and 2.1-fold in OE:: *nmnat4* and OE:: *nmnat19* strains compared with the WT strains, respectively. Furthermore, it was found that NAD+ might induce cellulase production by regulating cytosolic Ca 2+ concentration.

Conclusions: Taken together, our results revealed for the first time that NAD + could stimulate cellulase production and demonstrated that NAD + could increase the transcript levels of cellulase genes via the intracellular Ca 2+ concentration in *G. lucidum*. This research also provides a theoretical basis for conducting cellulase-related work on other basidiomycetes.

Background

Worldwide annual production of dry plant material in the form of lignocellulosic waste was estimated to be approximately 100 billion tons [1], but lignocellulosic biomass, as a cheap, renewable, and abundant carbon source, is normally used for land filling or is simply incinerated. Therefore, it is urgent to search for cost-effective and economical approaches to utilize lignocellulosic biomass. In nature, fungi have the ability to secrete large amounts of lignocellulolytic enzymes and are considered the principal agents involved in the degradation of lignocelluloses. Currently, relevant research has studied primarily a small number of ascomycetes fungi for industrial applications [2–4], and the types and quantity of industrial strains are limited. Meanwhile, many basidiomycetes need to be exploited, and cellulase related work in basidiomycetes is lacking. *Ganoderma lucidum* is a representative medical basidiomycetes and has a long history of use as a remedy in traditional medicine in Asia because of its pharmacological activities [5, 6]. *G. lucidum* is highly adaptable to grow on and degrade a wide variety of lignocellulosic biomass, so many lignocellulosic materials (such as agricultural residues, forestry wastes and thinnings) are used as culture medium in the process of cultivation. The substrate-degrading ability of *G. lucidum* could be

related to its growth [7]. Therefore, it is of practical and economic value to carry out cellulase-related work in *G. lucidum.*

To improve the utilization of cellulose, the processes of nutrient sensing and metabolism modulation needs to be explored. While there are a number of pathways involved in cellulase regulation in filamentous fungi, previous research has focused on three of these regulatory mechanisms. The first mechanism involves the direct transcriptional regulation of genes encoding cellulase. Cellulase gene expression is regulated directly by the action of transcription factors. So far, several transcription factors involved in the regulation of cellulase expression in filamentous fungi have been identified, including XYR1/XlnR, CLR–1/ClrA, CLR–2/ClrB, ace1/ace2/ace3 and so on [8]. The second mechanism is "signal transduction pathway". For example, Ca^{2+} signal transduction pathways can regulate the expression and secretion of cellulases in *Trichoderma reesei* [9]. In addition, cAMP signaling also can participate in the light-mediated regulation of cellulase gene in *Trichoderma reesei* [10]. The third mechanism is that regulation of nutrient sensing pathways. The best-studied mechanism is CreA/CRE1/CRE–1 mediated carbon catabolite repression (CCR). In addition, there are several highly conserved nutrient-sensing pathways implicated in the utilization of cellulose, including PKA (the cyclic AMP-protein kinase A), SNF1 (sucrose non-fermenting 1) complex and TOR (target of rapamycin) pathway [11–13]. For example, our pervious study showed SNF1 could regulate cellulose degradation by inhibiting CreA during the utilization of cellulose in *Ganoderma lucidum* [14].

Since its initial discovery more than a century ago as a coenzyme, a cofactor in fermentation, nicotinamide adenine dinucleotide (NAD+) has received abundant attention in research, among others, from four Nobel Prize laureates [15]. As a coenzyme, it has been reported that NAD+ could be involved in changes in metabolic flow [16, 17]. NAD+ also acts as a substrate regulating post-translational protein modifications, such as NAD-dependent deacetylation and ADP-ribosylation, which have a decisive impact on vital processes, including gene expression, genome stability, life span regulation, secondary metabolite production and many others [18–21]. In addition, NAD+ and its metabolites also have an important function in the cytoplasm as signalling molecules. For example, NAD+ is the biosynthetic precursor of metabolites that mediate intracellular calcium mobilization and is closely related to calcium signaling [22]. Recent studies have shown that many environmental stresses, including calorie restriction [23], could significantly regulate the intracellular NAD+ content in *Saccharomyces cerevisiae.* Meanwhile, several nutrient-sensing pathways have also been associated with NAD+ metabolism [20, 24, 25]. Currently, NAD+ is synthesized by three pathways: the de novo pathway, the Preiss–Handler pathway, and the salvage pathway [26, 27]. In these pathways, nicotinamide mononucleotide adenyltransferase (nmnat) is the key enzyme [28]. So far, the function of NAD+ in various physiological processes (such as nutritional responses and signalling pathways) has been observed in various species [20, 29]. However, the functions of NAD+ need to be further developed and have not been reported to date in macro-basidiomycetes.

Collectively, NAD+ serves an important function in calcium signaling and nutrient sensing pathways, which are related with cellulase regulation. To study the functions of NAD+ in cellulase regulation of *G.*

lucidum, the *Glnmnat* gene was cloned, and overexpression strains were constructed utilizing the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method. In our study, it was first identified that NAD+ could regulate cellulase production, and further analysis showed that NAD+ might induce cellulase improvement via the Ca^{2+} concentration in *G. lucidum.* This work encourages us to carry out cellulase-related work with other basidiomycetes.

Results

Cloning and sequence analysis of the *nmnat* gene

Based on the amino acid similarity to *Dichomitus squalens nmnat* (NCBI Reference Sequence: XP_007369986.1), only the gene *Glnmnat* (GenBank Accession Number: MH394247) was screened from the *G. lucidum* genome database (http://www.herbalgenomics.org/galu/). The length of the *nmnat* cDNA was 891 bp, and predictions indicated that this cDNA could encode a protein of 297 amino acids with a molecular mass of 32.89 kDa and an isoelectric point of 6.05. Analysis of the predicted amino acid sequence of *Glnmnat* identified the two highly conserved ATP-binding domains SxTxxR motif and GxxxPx[T/H]xxH motif, which can be easily detected in multiple sequence alignments and are commonly found in the *nmnat*-like proteins [30] (Fig. 1). These results implied that the *Glnmnat* gene that was cloned could belong to the *nmnat* family.

Construction of *G. lucidum nmnat* overexpression strains and measurement of NAD+ content

To explore the function of NAD+ in *G. lucidum,* the OE::*nmnat* vector was constructed (Fig. S1A), and *Agrobacterium tumefaciens*-mediated transformation (ATMT) was performed to construct *OEnmnat*mutant strains. The PCR results showed that the putative transformants presented the hygromycin B phosphotransferase (*hph)* gene and the *gpd* promoter-*nmnat* gene (Fig. S1B). Quantitative reverse-transcription PCR (qRT-PCR) analysis was utilized to detect the transcription level of *nmnat* in the selected mutant strains. Thirty positive transformants were selected, and seven of them had a higher transcript levels, with the relative expression of *nmnat* approximately 3–6-fold higher than the WT strains (Fig. S1C). OE::*nmnat4* and OE::*nmnat19* strains were randomly selected for further analysis. (Fig. 2A). The *nmnat* gene is a key gene in the synthesis of NAD+; therefore, the NAD+ content in the two *nmnat* overexpression strains was measured. The NAD+ content in *G. lucidum* was increased 1.35-fold and 1.38-fold in the OE::*nmnat4* and OE::*nmnat19* strains, respectively, compared with the WT strains (Fig. 2B). This result was consistent with the trend of *nmnat* gene expression at the transcript level. The increase in the NAD+ content further indicated that the cloned gene was a functional *nmnat* gene.

G. lucidum nmnat overexpression strains displayed better growth characteristics

Early studies have shown that the content of NAD+ might play an important role in response to nutritional conditions [31]. To investigate the influence of NAD+ on the mycelial growth of *G. lucidum* on different carbon sources, all tested strains were inoculated on MM (minimal medium) agar plates with 1% carbon sources, namely, glucose, sucrose, lactose, glycerol, xylan, CMC-Na and lignin. *Glnmnat* overexpression strains showed dramatically stronger colony growth on these carbon sources than WT strains (Fig. 3A and B). The results implied that NAD+ might be involved in the process of utilizing nutrition.

Effects of NAD+ on cellulase production of *G. lucidum*

To further investigate the role of NAD+ in nutrient utilization in *G. lucidum,* the same weight of precultured mycelia of *G. lucidum* in CYM liquid was transferred to liquid MM containing 1% Avicel as the sole carbon source and for the determination of cellulase activity. As shown in Fig. 4A and B, CMCase activity (representing endo-*β*-glucanase activity) in OE::*nmnat4* and OE::*nmnat19* strains approximately increased by 2.8-fold and 3-fold, respectively, compared with that of the WT strain. *p*NPGase activity (representing *β*-glucosidase activity) in OE::*nmnat4* and OE::*nmnat19* strains increased by approximately 1.9-fold and 2.1-fold compared with that of the WT strain, respectively. Meanwhlie, the total protein concentrations of all strains also was determined by a BCA Protein Assay Kit. As shown in Fig. 4C, there were no significant differences in total protein concentrations between WT strains and overexpression strains. The protein secretion is not directly correlated with the growth of the mycelium, and may be a result of complex inducing factors.

In addition, the level of transcription of the genes encoding major cellulases and transcription factors in *G. lucidum* was evaluated. Three putative endoglucanase coding genes (GL24196, GL29421, and GL28282), three putative cellobiohydrolase coding genes (GL18725, GL 29727, and GL30351) and three putative beta-glucosidase coding genes (GL27550, GL20743, and GL24911), respectively, were selected from the genome of *G. lucidum.* As shown in Fig. 4D, the gene expression of the major cellulases was significantly upregulated in the *Glnmnat* overexpression strains compared with that in the WT strains. Then, two negative transcription regulators for producing cellulase, creA (GL19424) and ace1 (GL15296), and two positive transcription regulators for producing cellulase, clr–1 (GL26482) and clr–2 (GL15667), were obtained. These results showed that the expression of clr–1 and clr–2 were significantly upregulated in the *Glnmnat* overexpression strains compared with the WT strains, while the expression of creA and ace1 were significantly downregulated in the *Glnmnat* overexpression strains compared with the WT strains (Fig. 4E). These results were consistent with the increasing of cellulase activity and implied that NAD+ might influence cellulase production in *G. lucidum.*

Effect of NAD+ on the accumulation of Ca^{2+} in *G. lucidum*

Previous studies have demonstrated that the calcium signal transduction pathway can upregulate cellulase gene expression [9]. Therefore, Fluo–3-pentaacetoxymethyl ester (Fluo–3AM), an acetoxymethyl

ester of a Ca^{2+}-specifc probe, was used to detect relative amounts of free intracellular Ca^{2+}. As shown in Fig. 5A and B, the fluorescence intensity of OE::*nmnat4* and OE::*nmnat19* strains was significantly higher than that of the WT strain, and the Ca^{2+} fluorescence value was upregulated by approximately 3.95-fold and 2.10-fold, respectively, compared with the WT strain. To investigate whether the increased levels of cytosolic Ca^{2+} can trigger calcium signal transduction pathways in *G. lucidum,* qRT-PCR was carried out to analyse the transcriptional levels of calcium signalling-related genes in *G. lucidum,* including calmodulin (*cam),*, Ca^{2+} and cam-dependent protein kinase genes (*camk1, camk2* and *camk3),*, *calreticulin [*regulatory] gene, *calcineurin* [catalytic] genes (*cna1* and *cna2),*, and calcineurin-responsive zinc finger transcription factor gene (*crz).*. As shown in Fig. 5C, the expression of most genes were increased to varying degrees in *G. lucidum nmnat* overexpression strains compared with the WT strain. These results implied that NAD+ might increase the concentration of cytosolic Ca^{2+}, thus stimulating the calcium signal transduction pathway in *G. lucidum.*

NAD+ regulates cellulase activity via intracellular Ca^{2+}

$LaCl_3$, a plasma membrane Ca^{2+} channel blocker, was used to prevent influx of external Ca^{2+}. As shown in Fig. 6A and B, the cytosolic Ca^{2+} concentration could be effectively attenuated in *G. lucidum nmnat* overexpression strains after the addition of $LaCl_3$. Furthermore, CMCase and *p*NPGase activities and transcription of related genes were analysed. As expected, after adding 5 mM $LaCl_3$ to *G. lucidum nmnat* overexpression mutants, both the CMCase and *p*NPGase activities decreased significantly (Fig. 7A and B), and the transcription of genes encoding the major cellulases also exhibited a similar tendency (Fig. 7C~K). Similarly, the transcription of clr–1 and clr–2 was markedly reduced in *G. lucidum nmnat* overexpression mutants with 5 mM $LaCl_3$ treatment, while the transcription of creA and ace1 was markedly improved (Fig. 7L~O). Taken together, these data indicated that NAD+ might induce cellulase improvement via the Ca^{2+} concentration in *G. lucidum.*

Discussion

NAD+ is important in responding to nutrient states and regulating cellular metabolism. For example, Qiao et al. [32] reported that NAD+ could regulate metabolic hepatic functions during different nutrient states in mice. In *Saccharomyces cerevisiae,* NAD+ can respond to calorie restriction, leading to an increased life span [33]. In the process of cultivation, *Ganoderma lucidum* also has to encounter various nutritional conditions. In our study, *Glnmnat* overexpression strains showed dramatically stronger colony growth in different carbon source conditions. This phenomenon might be caused by the fact that NAD+ can affect multiple metabolism and signalling pathways. Additionally, it was determined that cellulase activity and the expression levels of the main cellulase genes were significantly increased in *Glnmnat* overexpression strains compared with WT strains. These results showed that NAD+ can regulate cellulases of *G. lucidum.* Currently, limited work regarding cellulases has been carried out in *G. lucidum.* For example, previous work on the characterization of endoglucanases has been reported in *G. lucidum* [34]. In

addition, it was reported that genomic, transcriptomic and secretomic analyses were used to find that the overall expression of cellulases was higher than that of hemicellulases and lignin-modifying enzymes in *G. lucidum [7*]. The genetic method was further used to extend the regulation mechanism of cellulases in *G. lucidum* in our study.

Ca^{2+} is widely used as a secondary messenger in prokaryotic and eukaryotic cells. Recently, NAD+ has also gained much attention as an extracellular molecule that is involved in calcium signalling [37]. NAD+ can be transformed into many derivatives, including nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic ADP-ribose (cADPR), and adenosine diphosphoribose (ADPR). Furthermore, cADPR and NAADP can evoke Ca^{2+} release from endogenous Ca^{2+} stores, while ADPR stimulates Ca^{2+} entry from the extracellular space, and the mechanism by which NAD+ and its derivatives regulate calciumsignal transduction has been comprehensively discussed in the review [35]. In this work, *Glnmnat* overexpression strains increased Ca^{2+} concentrations and the transcriptional levels of calcium signalling-related genes. Similar results have been reported in other works. For example, extracellular NAD+ regulates intracellular free calcium concentration in humans [36]. These results suggest that NAD+ is involved in regulating the content of Ca^{2+}. However, the mechanism that NAD+ utilizes to participate in this process is not very clear and merits additional research in *G. lucidum.*

Ca^{2+}, the most important secondary messenger, can regulate many biological functions in filamentous fungi. For example, Ca^{2+} might be involved in the heat stress-mediated regulation of hyphal branching and ganoderic acid biosynthesis in *G. lucidum* [37]. It was also reported that Ca^{2+} could have different effects on cellulase production under diverse conditions. In *Botrytis cinerea* cultivated in medium containing calcium, the intracellular polygalacturonase and CMCase had low activities [38]. Upon the addition of Ca^{2+} under cellulose culture conditions, the signalling pathway for extracellular Ca^{2+} significantly stimulated extracellular cellulase activities in *Trichoderma reesei [*9]. Chen et al. [39] further reported that Mn^{2+} could modulate the expression of cellulase genes in *T. reesei*Rut-C30 strains via Ca^{2+}. In our work, a similar result was found as NAD+ could induce cellulase improvement via the Ca^{2+} concentration in *G. lucidum.* Currently, selecting new transcription factors and engineering the activity of transcription factors have been the main efficient strategies to enhance the production of cellulase enzymes in industrial strains. For example, recent work showed that overexpression of a novel regulatory gene, *Trvib−1,* could improve cellulase production in *T. reesei*40]. Because *G. lucidum,* as a medicinal mushroom, is easily influenced by various environmental conditions, the regulatory mechanism of cellulases of *G. lucidum* is more meaningful. In our work, we first reported that NAD+ could induce cellulase production in *G. lucidum.* As a multifunctional molecule, NAD+ may regulate cellulase through multiple signalling pathways. Further studies showed that NAD+ might regulate the production of cellulase via calcium signalling. However, other mechanisms of how NAD+ regulates cellulase are still worthy of further investigation.

Conclusions

In summary, OE::*nmnat4* and OE::*nmnat19* strains were constructed to characterize the functions of NAD+ in macro-basidiomycetes. The CMCase activity and *p*NPGase activity notably increased in OE::*nmnat4* and OE::*nmnat19.* In addition, NAD+ can also regulate intracellular Ca^{2+} levels, and Ca^{2+} might influence cellulase production and the gene expression of cellulases. Thus, an investigation of the detailed molecular mechanism of cellulases in other basidiomycetes merits further study.

Methods

Strains and growth conditions.

Escherichia coli DH5α was grown in Luria Broth (LB) medium that contained kanamycin (50 μg mL^{-1}) or ampicillin (100 μg mL^{-1}) to amplify the plasmid. The *G. lucidum* ACCC53264 strain (dicaryon) was obtained from the Agricultural Culture Collection of China. The WT (wild-type) and *Glnmnat* overexpression strains were inoculated on CYM liquid medium (2% glucose, 1% maltose, 0.05% $MgSO_4.7H_2O$, 0.2% yeast extract, 0.46% KH_2PO_4 and 0.2% tryptone) shaking for 5 days at 28 °C. Subsequently, mycelia were washed with sterile water and then cultured in MM liquid medium plus 1% Avicel (1% w/v Avicel, nitrate salts, trace elements, pyridoxine μg $μl^{-1}$, pH 6.5) shaking for 2 days at 28 °C. The culture supernatants were subjected to testing for the CMCase and *p*NPGase activity and protein concentration analysis. The harvested mycelia were used for the transcript analyses of the genes and transcription factors related to cellulases and of calcium signalling-related genes. To assess the effect of Ca^{2+} on the regulation of cellulase production of *G. lucidum,* 5 mM (final concentration) $LaCl_3$ (Aladdin, Shanghai, China) was added after 1 day of culture in MM liquid medium plus 1% Avicel [39].

Gene cloning and bioinformatics sequence analysis

The entire sequence of *G. lucidum nmnat* (*Glnmnat)* was obtained by polymerase chain reaction (PCR) using *G. lucidum* cDNA as templates with the primers listed in Table S1. The fragments were inserted into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. The National Center for Biotechnology Information (NCBI) was used for BLAST similarity searches. The theoretical molecular weight of the protein was predicted utilizing the online ExPASY tool (http://expasy.org/tools/pi_tool.html). The conserved motifs were determined from the NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd). Multiple sequence alignments were performed utilizing the DNAMAN program.

Construction of overexpression plasmids and strains

The construction of a fungal overexpression vector, *pGlgpd,* has been described in our previous study [41]. This vector was used for the construction of *G. lucidum nmnat* overexpression strains, which were transformed by *Agrobacterium tumefaciens*-mediated transformation (ATMT) [42]. The *nmnat* gene was

amplified with the primers listed Table S1. The detection of a fusion fragment containing the glyceraldehyde–3-phosphate dehydrogenase promoter and the *nmnat* gene was performed using the primers listed Table S1. These plasmids were used to transform the *G. lucidum* strains and were named *OE::nmnat.*

Extraction of genomic DNA

Following growth on CYM medium for 7 days at 28 °C, the mycelia of each strain were harvested and then ground into powder in liquid nitrogen. Total DNA was extracted from the fungus by a method adapted from the CTAB method, as previously described [42]. Multiplex PCR analysis was used to confirm the presence of a fusion fragment containing the *gpd* promoter– *nmnat* gene in genomic DNA isolated from putative transformants.

qRT- PCR analysis of gene expression

Total RNA was extracted utilizing an RNA Isolation Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. A 5× All-In-One RT MasterMix kit (with AccuRT Genomic DNA Removal Kit, ABM, Canada) was used to obtain cDNA for gene expression analysis. Subsequently, qRT-PCR-amplified fragments were detected using SYBR Green qPCR SuperMix. Gene expression was evaluated by calculating the difference between the threshold cycle (CT) value of the analyzed gene and the CT value of the 18S rRNA housekeeping gene. Quantitative revers-transcriptase PCR (qRT-PCR) calculations analyzing the relative gene expression levels were performed according to the $2^{-\Delta\Delta CT}$method described by Livak and Schmittgen [43]. The levels of gene-specific messenger RNA (mRNA) expressed by the WT and *Glnmnat* overexpression strains were evaluated by quantitative real-time PCR as described previously [44]. All of the strains for qRT-PCR analysis were inoculated on liquid CYM medium at 28 °C for 5 days. Subsequently, mycelia were washed with sterile water and then incubated in MM plus 1% Avicel at 28 °C shaking for 2 days, after that the mycelia was collected and frozen in liquid nitrogen. In addition, the expression patterns of the *nmnat* genes in mycelia were analysed utilizing the same method. The primers are displayed in Supplementary Table 1.

Determination of NAD+

The nicotinamide adenine dinucleotide was measured by the cycling assay as described previously [45]. NAD+ was extracted with 0.1 M HCl. The assays require the phenazine ethosulfate-catalysed reduction of thiazolyl blue tetrazolium bromide (MTT) in the presence of ethanol and alcohol dehydrogenase for NAD+. The rate of reduction of MTT was monitored at 570 nm.

Vegetative growth assays

A 6-mm-diameter hyphal tip plug of all of the strains was cultured on minimal medium (MM) agar plates containing different carbon sources (1% glucose, 1% sucrose, 1% lactose, 1% glycerol, 1% xylan, 1% CMC-Na and 1% lignin) for 5 days. All of the strains were generated in triplicate, and the experiment was repeated twice.

Enzyme activity assays and total protein contents

All of the strains were inoculated on liquid CYM medium at 28 °C for 5 days. Subsequently, mycelia were washed with sterile water and then incubated in 1% Avicel liquid medium at 28 °C shaking for 2 days. For the cellulase activity assay and protein concentrations detection assay, the culture supernatants of fungal strains were collected by centrifugation at 4 °C and 12,000×g for 10 min. The protein concentrations were determined by a BCA Protein Assay Kit (sigma). The endoglucanase (CMCase) activity was measured using the dinitrosalicylic acid (DNS) method [46]. A total of 200 µl of the culture supernatants was mixed with 200 µl of sodium carboxymethylcellulose (1%, soluble in citrate-phosphate buffer (50mM, pH 4.0)), followed by incubation at 50℃ for 30 min for CMC. The reaction was terminated by the addition of 400 µl DNS reagent and boiled for 10 min. The absorbance at 540 nm was measured. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of reducing sugar per min. *β*-glucosidase was assayed by determining the release of *p*-nitrophenol from *p*-nitrophenyl-*β*-D-glucopyranoside (*p*NPG). For this, 100 µl the culture supernatants was mixed with 100 µl *p*NPG (5mM) and incubated at 50℃ for 30 min. The reaction was stopped by adding 200 µl of 1 M sodium carbonate (pH 11.5), and the released 4-nitrophenol was quantified at 410 nm using a 4-nitrophenol standard curve. One unit of enzyme activity was defined as the amount of protein that released 1 µmol of *p*-nitrophenol per min.

Ca^{2+} detection assay

The free cytosolic Ca^{2+} in the related media was monitored with the aid of Fluo–3-pentaacetoxymethyl ester (Fluo–3AM), an acetoxymethyl ester of a fluorescent calcium indicator dye (Invitrogen). Fluo–3AM was loaded into the cells by incubation at 37 ℃ for 30 min, and the cells were then washed three times with phosphate-buffered saline. Images of Ca^{2+} green fuorescence were observed using a fluorescence microscope (BX53F; Olympus). The ZEN lite (Zeiss software) was used to analyze the average of the fluorescence intensity, and the detailed method was described previously [47].

Statistical analysis

The data from at least three independent sample measurements were averaged to ensure that the trends and relationships observed in the cultures were reproducible. The mean values of each experiment were expressed as the mean ± SD. Appropriate statistical tests were used in each figure, and Student's t-test

was used to compare two samples. Duncan's multiple range tests were used for multiple comparisons. A P-value < 0.05 was considered to be significant.

Abbreviations

nmnat: nicotinamide mononucleotide adenyltransferase; NAD+: nicotinamide adenine dinucleotide; CMCase: endo-*β*-glucanase activity; *p*NPGase: *β*-glucosidase activity; ATMT: *Agrobacterium tumefaciens*-mediated transformation; qRT-PCR: quantitative reverse-transcription PCR; Fluo–3AM: Fluo–3-pentaacetoxymethyl ester; $LaCl_3$: Lanthanum chloride.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information flies.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JH, JX, YH, LS, AR and ZJ performed the experiments. SW planned and carried out experiments and measurements and interpreted experimental data. MZ conceived of the study. MZ coordinated the project All authors read and approved the final manuscript.

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Supplemental Material

FIG. S1 The construction and characterization of *OE::nmnat* strains. Construction of OE::*nmnat* In these plasmids, transcription of *hph* and the target genes is driven by the gpd promoter. (B) The amplification pattern obtained with primers for the gpd promoter-*nmnat* fusion fragment using genomic DNA isolated from the *G. lucidum* transformants. Lane M: DL 2000 DNA marker; Lane P: p*Gl-nmnat* as a positive control; Lane 1: WT strain as a negative control; Lanes 2-8 (top): randomly chosen transformants containing hph; Lanes 2-8 (bottom): randomly chosen transformants containing a gpd promoter-*nmnat* fusion fragment. (C) The relative mRNA levels of *nmnat* in *G. lucidum*. The expression level of the *nmnat* gene in the WT strain was arbitrarily set to 1. The values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the WT strains (**, $P < 0.01$, Student's t-test).

Figures

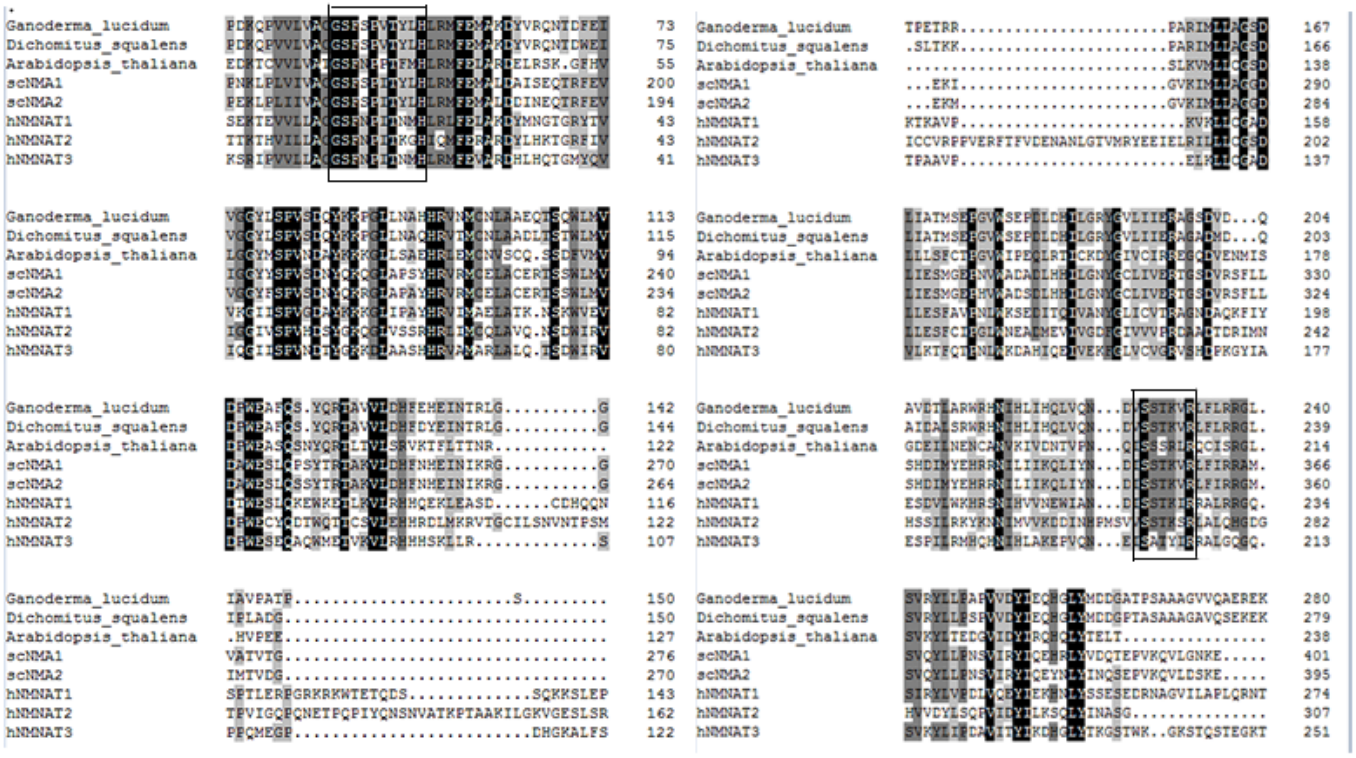

Figure 1

Amino acid sequence alignments of the conserved domain in nmnat. The residues included in the alignment are indicated to the left. The alignment was generated with DNAMAN software. Dichomitus squalens (XP_007369986); Arabidopsis thaliana (NP_200392.3); Saccharomyces cerevisiae S288C (NP_013432.1), (NP_011524.1); and Homo sapiens (NP_073624.2), (NP_055854.1), (AAK52726.1). Black shading indicates invariant residues, and the two additional shades of grey represent at least 80% conservation and at least 60% conservation, respectively.

Figure 2

Detection of nmnat transcript levels and NAD+ content in Glnmnat overexpressed strains. (A) All of the strains for qRT-PCR analysis were inoculated on liquid CYM medium at 28 °C for 5 days. qRT-PCR analysis of the expression of Glnmnat in the tested strains. The relative mRNA levels of Glnmnat were calculated as the ratio of Glnmnat mRNA to endogenous 18S rRNA. The expression level of the Glnmnat gene in the WT strains was arbitrarily set to 1. (B) Five-day-old liquid mycelia from tested strains were collected to measure the NAD+ content. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines (**, P < 0.01, Student's t-test).

Figure 3

Phenotypic characterization of the growth of Glnmnat overexpression strains. (A) G. lucidum strains were inoculated on minimal medium plates containing 1% different carbon sources at 28 °C for 5 days. (B) Measurement of colony diameter. Three individual replicates of each experiment were performed. The values are the mean ± standard deviation (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines (**, P < 0.01, Student's t-test).

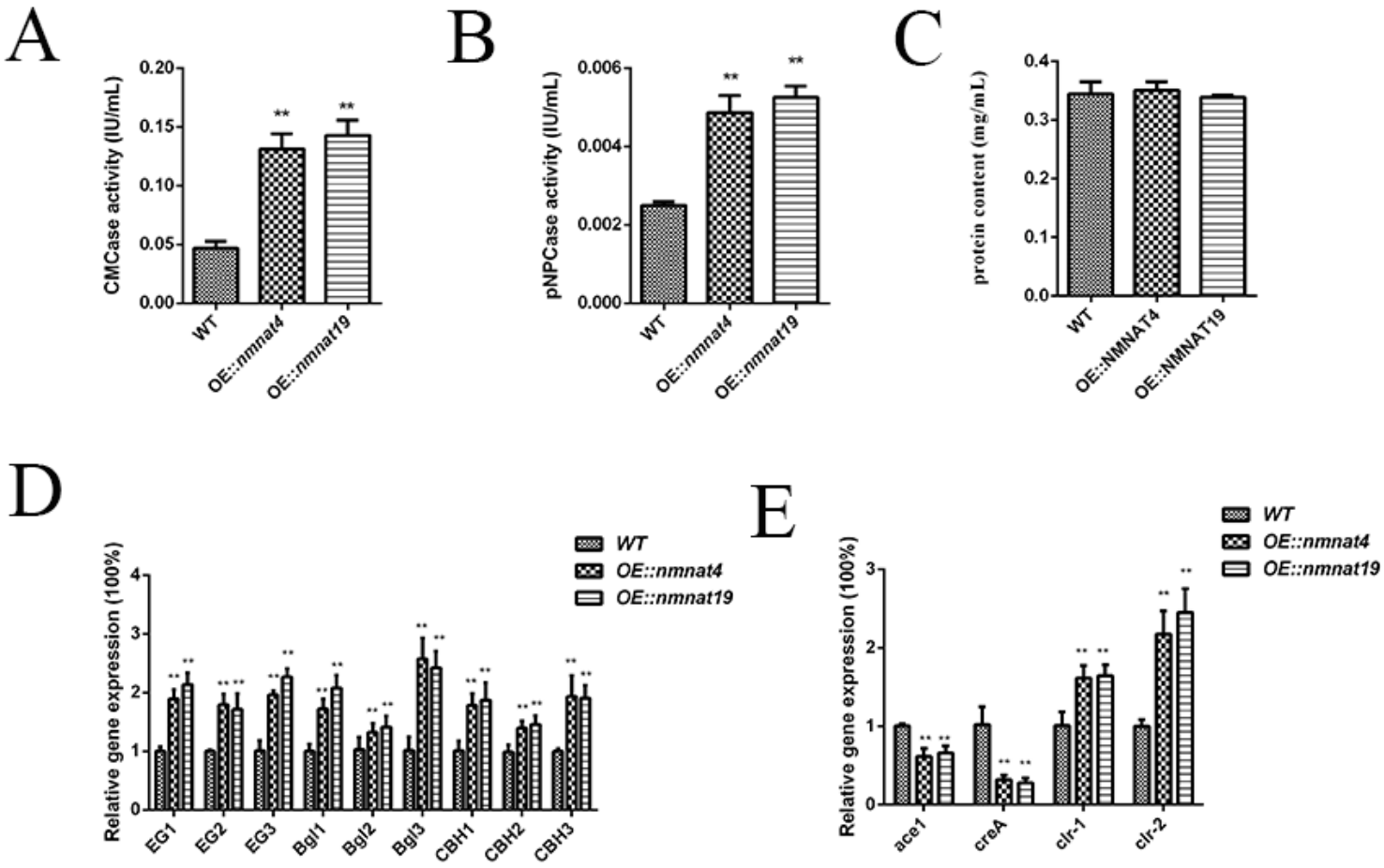

Figure 4

Effects of NAD+ on cellulase production of G. lucidum All tested strains were cultured at 28 °C on CYM liquid medium for 5 days, and then transferred to MM liquid medium plus Avicel 1% for two days. (A) Measurement of the CMCase activity in the strains tested. (B) Measurement of the pNPGase activity in the strains tested. (C) Measurement of the protein concentrations in the strains tested. (D) The transcriptional levels of genes encoding the major cellulases in the strains tested. (E) The transcriptional levels of transcription factors for cellulase production in the tested strains. Three independent biological replicates were performed for all experiments. The values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the WT strains (**, P < 0.01, Student's t-test).

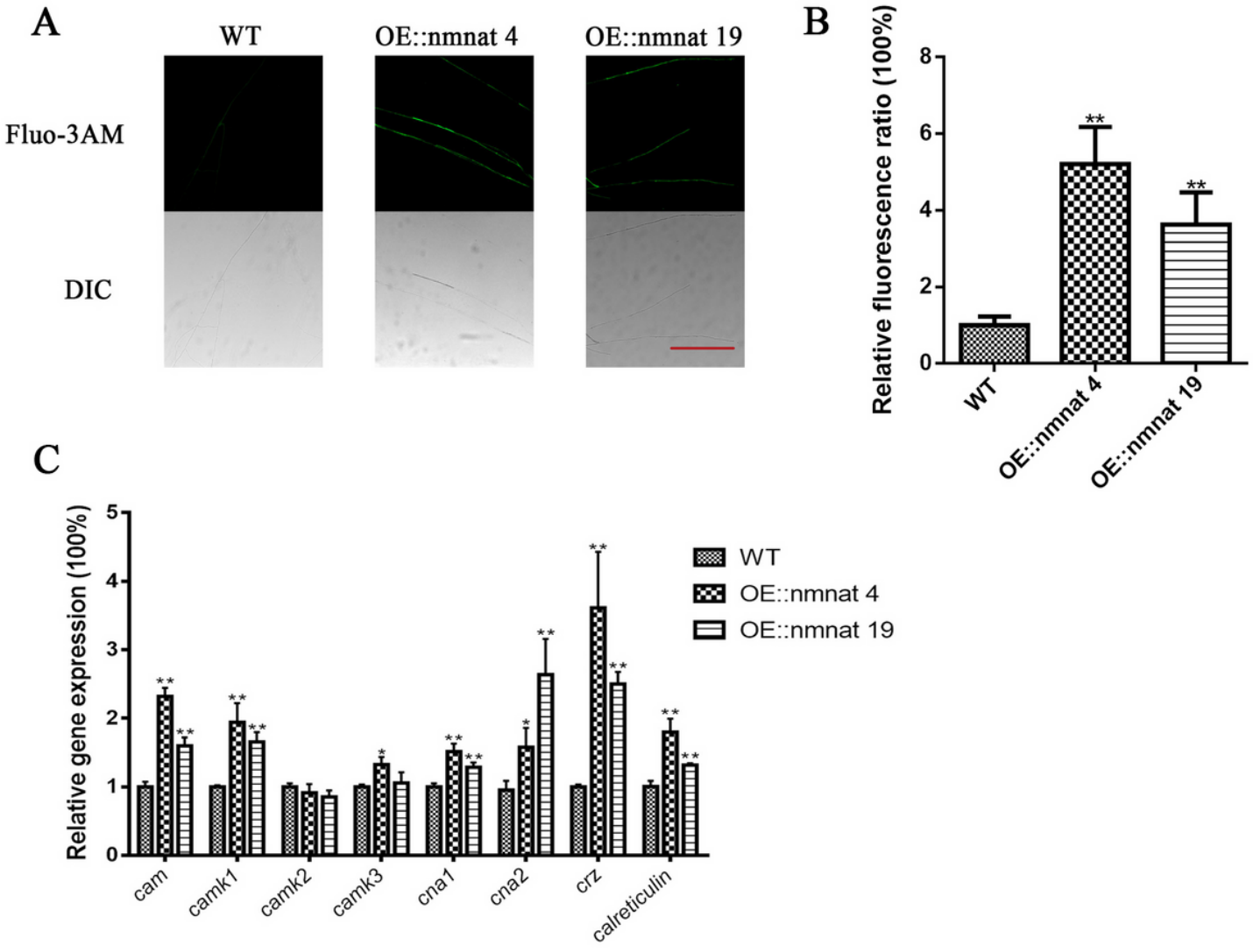

Figure 5

Ca2+ concentration and the transcriptional levels of calcium signalling-related genes in Glnmnat overexpression strains. (A) All tested strains were cultured at 28 °C on CYM solid agar medium for 5 days, and then transferred to MM plus 1% Avicel for two days. The change in the Ca2+ concentration was measured by Fluo-3AM fluorescence staining. Scale bar = 100 μm. (B) Changes in the Ca2+ fluorescence ratio in the hyphal regions. The y-axis represents the Ca2+ fluorescence ratio as measured by CLSM, and the x-axis represents the different strains. The values in each column represent the captured fluorescence ratio in each observation. (C) All tested strains were cultured at 28 °C on CYM liquid medium for 5 days, and then transferred to MM liquid medium plus 1% Avicel for two days. Measurement of the transcriptional levels of calcium signalling-related genes. The values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the WT strains (**, $P < 0.01$, Student's t-test).

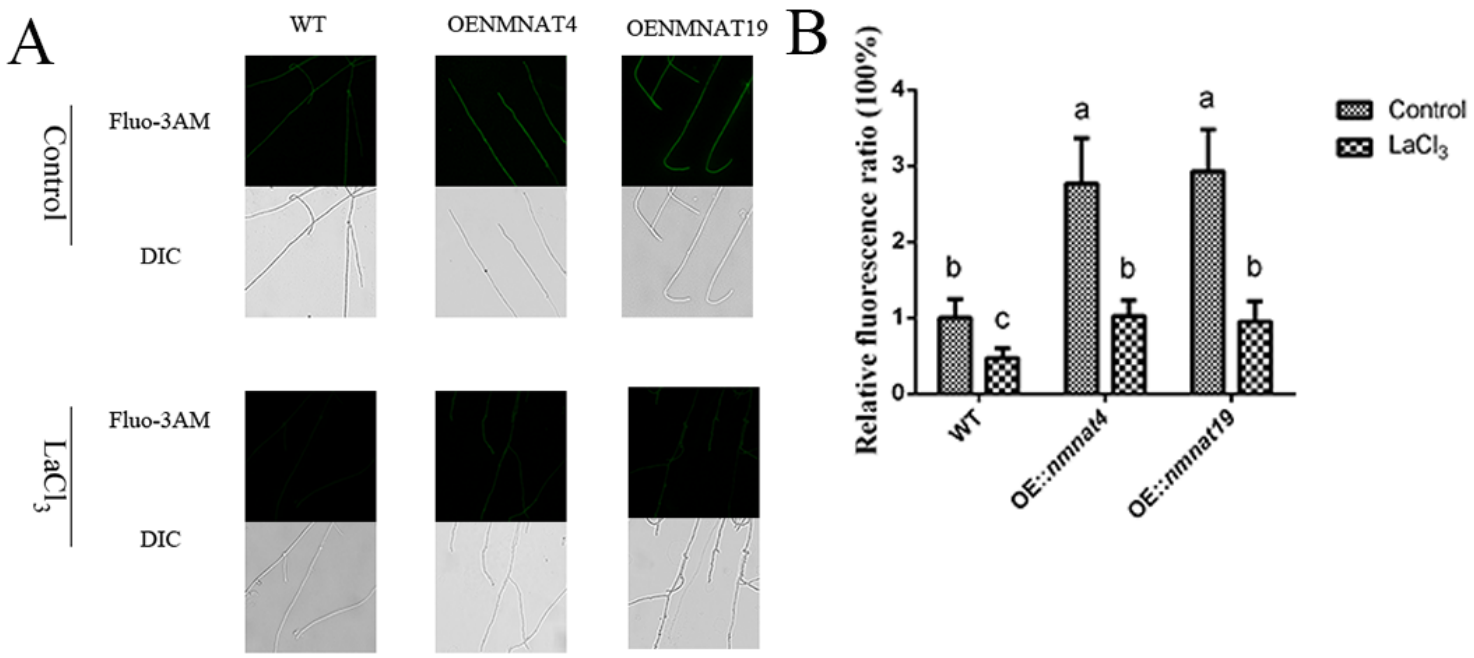

Figure 6

The Ca2+ concentration in G. lucidum after the Ca2+ channel blocker (LaCl3) treatment (A) All tested strains were cultured at 28 °C on CYM solid agar medium for 5 days, and then transferred to MM solid agar medium plus 1% Avicel for one day and then treated with or without 5 mM LaCl3 for one day in MM plus 1% Avicel. The change in the Ca2+ concentration was measured by Fluo-3AM fluorescence staining. Scale bar = 100 μm. (B) Changes in the Ca2+ fluorescence ratio in the hyphal regions. The y-axis represents the Ca2+ fluorescence ratio as measured by CLSM, and the x-axis represents the different strains. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$ according to Duncan's multiple range test).

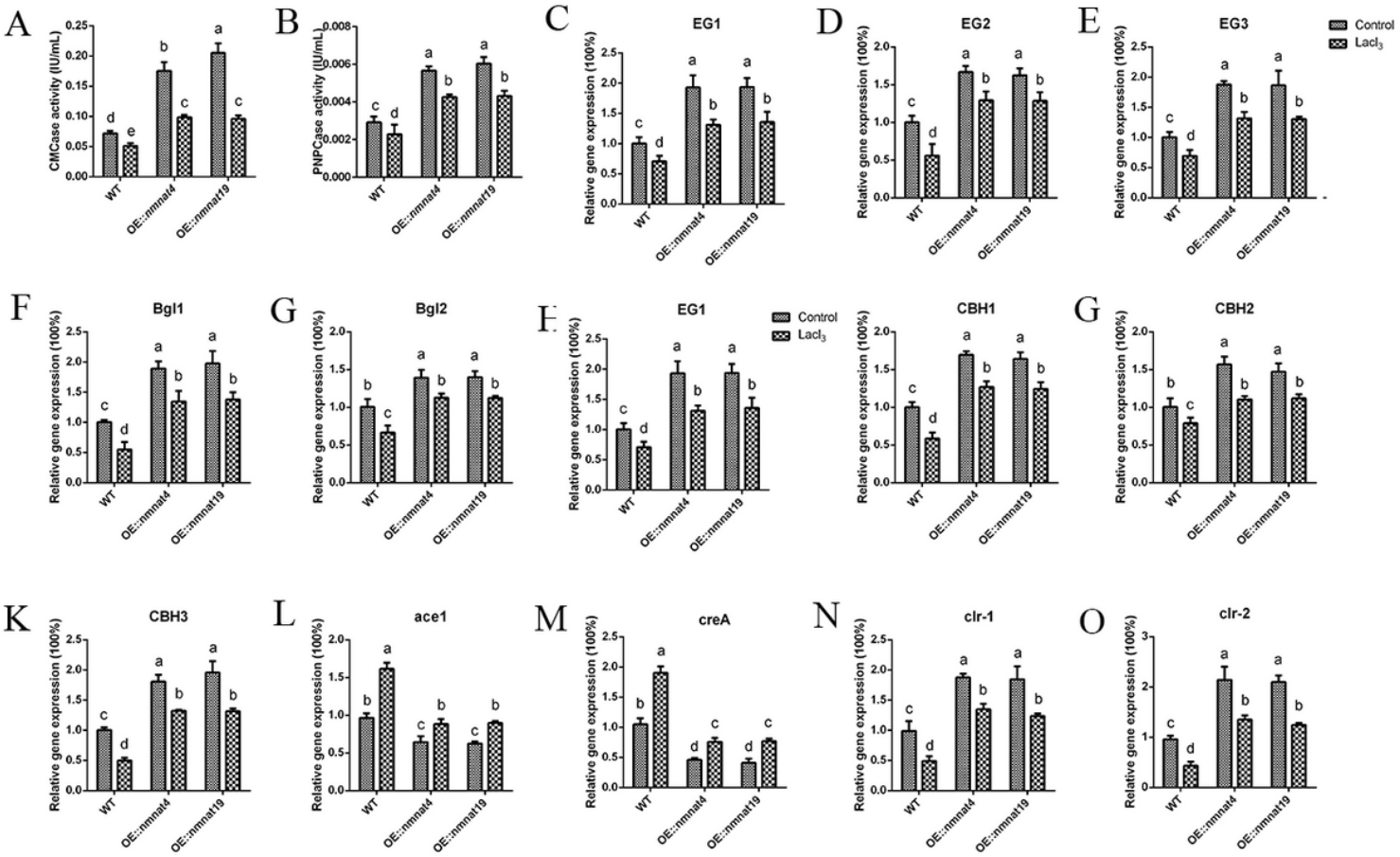

Figure 7

Effects of LaCl3 treatment on cellulase production of G. lucidum All tested strains were cultured at 28 °C on CYM liquid medium for 5 days, and then transferred to MM liquid medium plus 1% Avicel for one day and then treated with or without 5 mM LaCl3 for one day in MM plus 1% Avicel. (A) Measurement of CMCase activity in the strains tested. (B) Measurement of the pNPGase activity in the strains tested. (C~O) The transcriptional levels of the genes encoding the major cellulases and transcription factors for cellulase production in the strains tested. Three independent biological replicates were performed for all experiments. The values are the mean ± standard deviation (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$ according to Duncan's multiple range test).

Supplementary Files

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- FIG.S1.tif