Overexpression of nicotinamide mononucleotide adenylyltransferase (nmnat) increases the growth rate, Ca^{2+} content and cellulase production in *Ganoderma lucidum*

Shengli Wang

Nanjing Agricultural University

Jing Han

Nanjing Agricultural University

Jiale Xia

Nanjing Agricultural University

Yanru Hu

Nanjing Agricultural University

Liang Shi

Nanjing Agricultural University

Ang Ren

Nanjing Agricultural University

Jing Zhu

Nanjing Agricultural University

Mingwen Zhao (✉ mwzhao@njau.edu.cn)

Nanjing Agricultural University

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Abstract

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

Results: 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 two *G. lucidum* overexpression strains, OE::nmnat4 and OE::nmnat19, in which the nmnat gene transcript levels and the NAD⁺ content were significantly increased. *Glnmnat* overexpression strains showed dramatically increased colony growth on different carbon sources, and the intracellular Ca²⁺ concentration was increased by 3.95- and 2.10-fold in the *G. lucidum* OE::nmnat4 and OE::nmnat19 strains, respectively, compared with that observed in the WT strain. The CMCase activity increased by approximately 2.8- and 3-fold, and that of pNPGase increased by approximately 1.9- and 2.1-fold in the *G. lucidum* OE::nmnat4 and OE::nmnat19 strains, respectively, compared with that observed in the WT strain. Furthermore, NAD⁺ was observed to potentially induce cellulase production by regulating the cytosolic Ca²⁺ concentration.

Conclusions: Taken together, for the first time, our results revealed that NAD⁺ can stimulate cellulase production and increase the transcript levels of cellulase genes via increasing the intracellular Ca²⁺ concentration in *G. lucidum*. This research also provides a theoretical basis for conducting cellulase-related work in other basidiomycetes.

Background

The worldwide annual production of dry plant material in the form of lignocellulosic waste is estimated to be approximately 100 billion tons [1], but lignocellulosic biomass, a cheap, renewable, and abundant carbon source, is typically disposed of in landfills or is simply incinerated. Therefore, there is an urgent need to identify cost-effective and economical approaches for the utilization of lignocellulosic biomass. In nature, fungi have the ability to secrete large amounts of lignocellulolytic enzymes and are considered to be the principal agents involved in the degradation of lignocelluloses. At present, relevant research has primarily focused on a small number of ascomycetes fungi for industrial applications [2-4], and the types and quantity of industrial strains are limited. However, many basidiomycetes could be exploited, but cellulase-related work in basidiomycetes is lacking. *Ganoderma lucidum* is a representative medical basidiomycete that has a long history of use as a remedy in traditional medicine in Asia because of its pharmacological activities [5, 6]. Because *G. lucidum* is highly adaptable and is able to grow on and degrade a wide range of lignocellulosic biomass, many lignocellulosic materials (such as agricultural residues, forestry wastes and thinnings) are used as culture medium in the process of its cultivation. The

substrate-degrading ability of *G. lucidum* may be related to its growth [7]. Therefore, it is of practical and economic value to perform cellulase-related work in *G. lucidum*.

To improve the utilization of cellulose, the processes of nutrient sensing and metabolic modulation should 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 directly regulated by the action of transcription factors. To date, 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, and ace1/ace2/ace3 [8]. The second mechanism involves signal transduction pathways. For example, Ca²⁺ signal transduction pathways can regulate the expression and secretion of cellulases in *Trichoderma reesei* [9]. In addition, cAMP signalling can also participate in the light-mediated regulation of cellulase gene expression in *Trichoderma reesei* [10]. The third mechanism is the 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 the cyclic AMP-protein kinase A (PKA), sucrose non-fermenting 1 (SNF1) complex and target of rapamycin (TOR) pathways [11-13]. For example, our previous study showed that SNF1 can regulate cellulose degradation by inhibiting CreA during cellulose utilization in *G. lucidum*. [14].

Since its initial discovery more than a century ago as a coenzyme used in fermentation, nicotinamide adenine dinucleotide (NAD⁺) has received a great deal of research, including from four Nobel Prize laureates, among others [15]. As a coenzyme, NAD⁺ has been reported to potentially be involved in changes in metabolic flow [16, 17]. NAD⁺ also acts as a substrate that regulates post-translational protein modifications, such as NAD-dependent deacetylation and ADP-ribosylation, which have a major impact on crucial 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 associated with calcium signalling [22]. Recent studies have shown that many environmental stresses, including calorie restriction [23], can significantly regulate the intracellular NAD⁺ content in *Saccharomyces cerevisiae*. Furthermore, several nutrient-sensing pathways have also been shown to be associated with NAD⁺ metabolism [20, 24, 25]. Currently, NAD⁺ can be synthesized by three pathways: the de novo pathway, the Preiss-Handler pathway, and the salvage pathway [26, 27]. In these pathways, nicotinamide mononucleotide adenylyltransferase (nmnat) is the key enzyme [28]. To date, 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 signalling and nutrient sensing pathways, which are associated with cellulase regulation. To study the functions of NAD⁺ in cellulase regulation in

G. lucidum, the *Glnmnat* gene was cloned, and overexpression strains were constructed utilizing the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method. In our study, for the first time, it was shown that NAD⁺ can regulate cellulase production, and further analysis showed that NAD⁺ may induce cellulase production in response to the Ca²⁺ concentration in *G. lucidum*. The results of this study should encourage additional 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), 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 encodes a 297-amino acid protein 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 with the motifs SxTxxR and GxxxPx[T/H]xxH, which can be easily detected in multiple sequence alignments and are commonly present in *nmnat*-like proteins [30] (Fig. 1). These results suggested that the *Glnmnat* gene that was cloned may belong to the *nmnat* family.

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

To explore the function of NAD⁺ in *G. lucidum*, an 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 harboured the hygromycin B phosphotransferase (*hph*) gene and the *gpd* promoter-*nmnat* gene (Fig. S1B). Quantitative reverse-transcription PCR (qRT-PCR) analysis was performed to detect the transcription level of *nmnat* in the selected mutant strains. Thirty positive transformants were selected, seven of which had a high transcript levels, with relative *nmnat* expression that was approximately 3-6-fold higher than that of the WT strain (Fig. S1C). The *G. lucidum* strains OE::*nmnat4* and OE::*nmnat19* were randomly selected for further analysis. (Fig. 2A). Because the *nmnat* gene is a key gene in the synthesis of NAD⁺, the NAD⁺ content in the two *nmnat* overexpression strains was measured. Compared with the WT strain, the NAD⁺ content in *G. lucidum* the *G. lucidum* OE::*nmnat4* and OE::*nmnat19* strains was increased by 1.35- and 1.38-fold, respectively (Fig. 2B). This result was consistent with the observed 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 display better growth characteristics

Previous studies have shown that the NAD⁺ content in cells may play an important role in the 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 minimal medium (MM) agar plates with

supplemented with 1% carbon sources, namely, glucose, sucrose, lactose, glycerol, xylan, CMC-Na or lignin. The *Glnmnat* overexpression strains showed dramatically increased colony growth on these carbon sources than the WT strains (Fig. 3A and B), indicating that NAD⁺ may be involved in the process of nutrition utilization.

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

To further investigate the role of NAD⁺ in nutrient utilization in *G. lucidum*, the same weight of precultured *G. lucidum* mycelia in CYM liquid medium were 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, the CMCase activity (representing endo- β -glucanase activity) in the *G. lucidum* OE::*nmnat4* and OE::*nmnat19* strains increased by approximately 2.8- and 3-fold compared with that observed in the WT strain, respectively. In addition, pNPGase activity (representing β -glucosidase activity) in the *G. lucidum* OE::*nmnat4* and OE::*nmnat19* strains increased by approximately 1.9- and 2.1-fold compared with that of the WT strain, respectively. Furthermore, the total protein concentrations of all strains was also determined using a BCA Protein Assay kit. As shown in Fig. 4C, no significant differences in total protein concentrations were observed between the WT and overexpression strains. Protein secretion is not directly correlated with mycelial growth and may be affected by complex inducing factors.

In addition, the transcription levels of the genes encoding major cellulases and transcription factors in *G. lucidum* were evaluated. Three putative endoglucanase-encoding genes (GL24196, GL29421, and GL28282), three putative cellobiohydrolase-encoding genes (GL18725, GL 29727, and GL30351) and three putative beta-glucosidase-encoding genes (GL27550, GL20743, and GL24911) 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 observed in the WT strain. Subsequently, two negative transcriptional regulators for cellulase production, *creA* (GL19424) and *ace1* (GL15296), and two positive transcriptional regulators for cellulase production, *clr-1* (GL26482) and *clr-2* (GL15667), were evaluated. These results showed that the expression of *clr-1* and *clr-2* was significantly upregulated in the *Glnmnat* overexpression strains compared with that observed in the WT strains, whereas that of *creA* and *ace1* was significantly downregulated (Fig. 4E). These results were consistent with the observed increase in cellulase activity and indicated that NAD⁺ may influence cellulase production in *G. lucidum*.

Effect of NAD⁺ on the accumulation of Ca²⁺ 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 Ca²⁺-specific probe, was used to detect relative amounts of free intracellular Ca²⁺. As shown in Fig. 5A and B, the fluorescence intensity of the *G. lucidum* OE::*nmnat4* and OE::*nmnat19* strains was significantly higher than that observed in the WT strain, and the Ca²⁺ fluorescence value was upregulated by approximately 3.95- and 2.10-fold compared with the WT strain, respectively. To investigate whether

increased levels of cytosolic Ca^{2+} can trigger calcium signal transduction pathways in *G. lucidum*, qRT-PCR was performed 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*), the *calreticulin* [regulatory] gene, the *calcineurin* [catalytic] genes (*cna1* and *cna2*), and the calcineurin-responsive zinc finger transcription factor gene (*crz*). As shown in Fig. 5C, the expression of most of these genes was increased to varying degrees in the *G. lucidum nmnat* overexpression strains compared with that observed in the WT strain. These results suggest that NAD^+ may increase the concentration of cytosolic Ca^{2+} , thereby 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 the influx of external Ca^{2+} . As shown in Fig. 6A and B, the cytosolic Ca^{2+} concentration could be effectively attenuated in the *G. lucidum nmnat* overexpression strains after the addition of LaCl_3 . Furthermore, CMCase and *p*NPGase activities and the transcription of related genes were analysed. As expected, after the addition of 5 mM LaCl_3 to the *G. lucidum nmnat* overexpression mutants, both the CMCase and *p*NPGase activities significantly decreased (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 the *G. lucidum nmnat* overexpression strains treated with 5 mM LaCl_3 , while that of *creA* and *ace1* was markedly improved (Fig. 7L~O). Taken together, these data indicate that NAD^+ may induce cellulase induction by altering the Ca^{2+} concentration in *G. lucidum*.

Discussion

NAD^+ is important in the ability of cells to respond to different nutrient states and in the regulation of cellular metabolism. For example, Qiao et al. [32] reported that NAD^+ can 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]. During the cultivation process, *Ganoderma lucidum* also has to encounter various nutritional conditions. In our study, the *Glnmnat* overexpression strains showed dramatically increased colony growth under different carbon source conditions. This phenomenon may be due to NAD^+ being able to affect multiple metabolism and signalling pathways. Additionally, cellulase activity and the expression levels of the primary cellulase genes were shown to be significantly increased in the *Glnmnat* overexpression strains compared to that observed in the WT strain. These results showed that NAD^+ can regulate cellulases in *G. lucidum*. Currently, limited work regarding cellulases has been performed in *G. lucidum*. For example, previous work on the characterization of endoglucanases has been reported in *G. lucidum* [34]. In addition, the results of genomic, transcriptomic and secretomic analyses showed that the overall expression of cellulases was higher than that of hemicellulases and lignin-modifying enzymes in *G. lucidum* [7]. In our study, genetic methods were further used to extend the regulatory mechanism of cellulases in *G. lucidum*.

Ca^{2+} is widely used as a secondary messenger in prokaryotic and eukaryotic cells. Recently, NAD^+ has also gained a great deal of 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 promote 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 calcium signal transduction has been comprehensively reviewed [35]. In this work, the *Glnmnat* overexpression strains exhibited increased Ca^{2+} concentrations and transcriptional levels of calcium signalling-related genes. Similar results have been reported in other studies. For example, extracellular NAD^+ has been shown to regulate intracellular free calcium concentration in humans [36]. These results suggest that NAD^+ is involved in regulating the content of Ca^{2+} . However, the mechanism by which NAD^+ participates in this process remains unclear 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+} may 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+} can have different effects on cellulase production under diverse conditions. In *Botrytis cinerea* cultivated in medium containing calcium, low activities of intracellular polygalacturonase and CMCase were observed [38]. In another study, after 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+} can modulate the expression of cellulase genes in *T. reesei* Rut-C30 strains via Ca^{2+} . A similar result was observed in our study, as NAD^+ could induce cellulase production by regulating the Ca^{2+} concentration in *G. lucidum*. Currently, selecting new transcription factors and engineering the activity of transcription factors have been the primary strategies used to efficiently enhance the production of cellulase enzymes in industrial strains. For example, recent work showed that the 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 production in *G. lucidum* is more important. In this study, for the first time, we reported that NAD^+ can induce cellulase production in *G. lucidum*. As a multifunctional molecule, NAD^+ may regulate cellulase through multiple signalling pathways. Further studies showed that NAD^+ may regulate the production of cellulase via calcium signalling. However, other mechanisms regarding how NAD^+ regulates cellulase are still worthy of further investigation.

Conclusions

In summary, in this study, the *G. lucidum* OE::*nmnat4* and OE::*nmnat19* strains were constructed to characterize the functions of NAD^+ in macro-basidiomycetes. The CMCase and pNPGase activities notably increased in the *G. lucidum* OE::*nmnat4* and OE::*nmnat19* strains. In addition, NAD^+ was also shown to regulate intracellular Ca^{2+} levels, and Ca^{2+} may influence cellulase production and the

expression of cellulase-encoding genes. Thus, detailed studies to elucidate the molecular mechanisms of cellulases in other basidiomycetes merits further study.

Methods

Strains and growth conditions

Escherichia coli DH5 α was grown in Luria Broth (LB) medium that contained kanamycin (50 $\mu\text{g mL}^{-1}$) or ampicillin (100 $\mu\text{g mL}^{-1}$) to promote plasmid propagation. The *G. lucidum* ACCC53264 strain (dikaryon) was obtained from the Agricultural Culture Collection of China. The WT (wild-type) and *Glnmnat* overexpression strains were inoculated in CYM liquid medium (2% glucose, 1% maltose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% yeast extract, 0.46% KH_2PO_4 and 0.2% tryptone) and cultured with shaking for 5 days at 28 °C. Subsequently, the mycelia were washed with sterile water and then cultured in MM liquid medium plus 1% Avicel (1% w/v Avicel, nitrate salts, trace elements, and pyridoxine $\mu\text{g }\mu\text{L}^{-1}$, pH 6.5) with shaking for 2 days at 28 °C. The culture supernatants were then assayed for CMCase and *p*NPGase activity and used for protein concentration analysis. The harvested mycelia were used for transcript analyses of the genes and transcription factors related to cellulases and calcium signalling-related genes. To assess the effect of Ca^{2+} on the regulation of cellulase production in *G. lucidum*, 5 mM LaCl_3 (final concentration) (Aladdin, Shanghai, China) was added after 1 day of culturing 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 template with the primers listed in Table S1. The fragments were inserted into the vector pMD18-T (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 using 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 program DNAMAN.

Construction of overexpression plasmids and strains

The construction of a fungal overexpression vector, *pGlgpd*, was described in our previous study [41]. This vector was used for the construction of *G. lucidum nmnat* overexpression strains, which were transformed via *Agrobacterium tumefaciens*-mediated transformation (ATMT) [42]. The *nmnat* gene was amplified using 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*, and the resulting strains 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 ground into powder in liquid nitrogen. Total DNA was extracted from the fungus using 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 the 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 analysed gene and the CT value of the 18S rRNA housekeeping gene. Quantitative reverse-transcriptase PCR (qRT-PCR) calculations analysing 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 used for qRT-PCR analysis were inoculated in liquid CYM medium and cultured at 28 °C for 5 days. Subsequently, mycelia were washed with sterile water and then incubated in MM plus 1% Avicel at 28 °C with shaking for 2 days, after which the mycelia were 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 for these analyses are provided in Supplementary Table 1.

Determination of NAD⁺

The nicotinamide adenine dinucleotide levels were measured using the cycling assay as described previously [45], where 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 each strain 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 or 1% lignin) for 5 days. All of the strains were assayed in triplicate, and the experiment was repeated twice.

Enzyme activity assays and total protein contents

All of the strains cultured in liquid CYM medium at 28 °C for 5 days. Subsequently, the mycelia were washed with sterile water and then incubated in 1% Avicel liquid medium at 28 °C and with shaking for 2 days. For the cellulase activity assay and protein concentrations determinations, 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 using a BCA Protein Assay kit (Sigma). The endoglucanase (CMCase) activity was measured using the dinitrosalicylic acid (DNS) method [46]. For each sample, 200 µl of culture supernatants was mixed with 200 µl of sodium carboxymethylcellulose [1%, soluble in citrate-phosphate buffer (50 mM, pH 4.0)] and then incubated at 50°C for 30 min to assess CMC activity. The reaction was terminated by the addition of 400 µl of DNS reagent and boiled for 10 min. The absorbance at 540 nm was measured, and one unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of reducing sugar per min. β -Glucosidase activity was assayed by determining the release of *p*-nitrophenol from *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG). For this assay, 100 µl of the culture supernatants were mixed with 100 µl of *p*NPG (5 mM) and incubated at 50°C for 30 min. The reaction was stopped by the addition of 200 µl 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.

Ca²⁺ detection assay

The free cytosolic Ca²⁺ in the related media was monitored with using 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 °C for 30 min, and the cells were then washed three times with phosphate-buffered saline. Images of Ca²⁺ green fluorescence were observed using a fluorescence microscope (BX53F; Olympus). ZEN lite (Zeiss software) was used to determine 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 means $\pm$ SD. Appropriate statistical tests were used for the data presented 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 of < 0.05 was considered to be significant.

Abbreviations

nmnat: nicotinamide mononucleotide adenylyltransferase; 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; LaCl₃: 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 files.

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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Figures

Ganoderma_lucidum	EDCQPVVIVAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	73	Ganoderma_lucidum	TFETRR.....FARIMLPAGSD	167
Dichomitus_squalens	EDCQPVVIVAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	75	Dichomitus_squalens	.SLTKK.....FARIMLPAGSD	166
Arabidopsis_thaliana	EDITCVVIVAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	55	Arabidopsis_thaliana	SLKVMILPAGSD	138
scNMA1	ENELPVIIVAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	200	scNMA1	...EKI.....GVKIMLPAGSD	290
scNMA2	ENELPVIIVAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	194	scNMA2	...EKI.....GVKIMLPAGSD	284
hNMNAT1	SETEVIVILAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	43	hNMNAT1	KTRAVP.....KVLICGAD	158
hNMNAT2	TTTTHVILLAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	43	hNMNAT2	ICCVRFVVERSTFVDENANLGTVMRYEEIEELICGSD	202
hNMNAT3	KSIIPVIVILAGGSFSPVYUHLRMFEVAFYVVRQNTDFEI	41	hNMNAT3	TPAAVP.....ELPILCGAD	137
Ganoderma_lucidum	VGCYHSFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	113	Ganoderma_lucidum	LIATMSEGVVSEFELCHILGRMGVLIIRASISVD...Q	204
Dichomitus_squalens	VGCYHSFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	115	Dichomitus_squalens	LIATMSEGVVSEFELCHILGRMGVLIIRASISVD...Q	203
Arabidopsis_thaliana	LGCMYFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	94	Arabidopsis_thaliana	ELLSECTHGVVSEFELCHILGRMGVLIIRASISVD...Q	178
scNMA1	IGCMYFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	240	scNMA1	LIESMGEHNVVSEFELCHILGRMGVLIIRASISVD...Q	330
scNMA2	VGCYHSFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	234	scNMA2	LIESMGEHNVVSEFELCHILGRMGVLIIRASISVD...Q	324
hNMNAT1	VRCIHSFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	82	hNMNAT1	LIESMGEHNVVSEFELCHILGRMGVLIIRASISVD...Q	198
hNMNAT2	IGCMYFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	82	hNMNAT2	LIESMGEHNVVSEFELCHILGRMGVLIIRASISVD...Q	242
hNMNAT3	IGCMYFVSDNYKQGLAFSYHRYRMGLACERTESWIMV	80	hNMNAT3	LIESMGEHNVVSEFELCHILGRMGVLIIRASISVD...Q	177
Ganoderma_lucidum	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	142	Ganoderma_lucidum	AVETLARWRHHLHLHQLVCH...DSETHVRLFLRRL	240
Dichomitus_squalens	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	144	Dichomitus_squalens	AIDALSRWRHHLHLHQLVCH...DSETHVRLFLRRL	239
Arabidopsis_thaliana	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	122	Arabidopsis_thaliana	GDEILNENCAVHIVDNTVFN...DSETHVRLFLRRL	214
scNMA1	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	270	scNMA1	SHDINYEHRHHLHLHQLVCH...DSETHVRLFLRRL	366
scNMA2	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	264	scNMA2	SHDINYEHRHHLHLHQLVCH...DSETHVRLFLRRL	360
hNMNAT1	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	116	hNMNAT1	ESDVINKHRSRHLHLHQLVCH...DSETHVRLFLRRL	234
hNMNAT2	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	122	hNMNAT2	HSSILRKYRHLHLHLHQLVCH...DSETHVRLFLRRL	282
hNMNAT3	DEWEAFQS.YQRDAVVLCHFEHEINTRLG.....G	107	hNMNAT3	ESPIIRHCHHLHLHQLVCH...DSETHVRLFLRRL	213
Ganoderma_lucidum	IAVPAF.....S.....	150	Ganoderma_lucidum	SVRYILRAHVDYIECHGLMDDGATPSAAGVQAESEK	280
Dichomitus_squalens	IELADG.....S.....	150	Dichomitus_squalens	SVRYILRAHVDYIECHGLMDDGATPSAAGVQAESEK	279
Arabidopsis_thaliana	.HVPDE.....S.....	127	Arabidopsis_thaliana	SVRYILRAHVDYIECHGLMDDGATPSAAGVQAESEK	238
scNMA1	VAVITG.....S.....	276	scNMA1	SVQYILNSVIRVIECHGLMDDGATPSAAGVQAESEK	401
scNMA2	IMTVDS.....S.....	270	scNMA2	SVQYILNSVIRVIECHGLMDDGATPSAAGVQAESEK	395
hNMNAT1	SETLERGRKRKWTETQDS.....SQKKSLEP	143	hNMNAT1	STRIVEDLVCEYIECHGLMDDGATPSAAGVQAESEK	274
hNMNAT2	TEVIGCFQNETPQPIYQNSNVATKTAAILGKVGESLSR	162	hNMNAT2	HVVYIESQFVEDYIECHGLMDDGATPSAAGVQAESEK	307
hNMNAT3	PECMGEF.....DHGKALFS	122	hNMNAT3	SVRYILRAHVDYIECHGLMDDGATPSAAGVQAESEK	251

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 using DNAMAN. 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% and 60% conservation, respectively.

Figure 4

Detection of nmnat transcript levels and NAD⁺ content in the Glnmnat overexpression strains. (A) All of the strains used for qRT-PCR analysis were cultured in 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 the Glnmnat mRNA level to that of the endogenous 18S rRNA gene. The expression level of the Glnmnat gene in the WT strains was arbitrarily set to 1. (B) Five-day-old liquid mycelia from the tested strains were collected to measure the NAD⁺ content. The values are the means ± 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 7

Phenotypic characterization of the growth of the *Glnmnat* overexpression strains (A) *G. lucidum* strains were cultured on minimal medium plates containing different carbon sources (1%) at 28 °C for 5 days. (B) Measurement of colony diameter. Three individual replicates of each experiment were performed. The values are the means $\pm$ 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 10

Effects of NAD⁺ on cellulase production in *G. lucidum*. All of the tested strains were cultured at 28 °C in 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 tested strains. (B) Measurement of the pNPCase 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 tested strains. (E) The transcriptional levels of transcription factors associated with cellulase production in the tested strains. Three independent biological replicates were performed for all experiments. The values are the means $\pm$ 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 13

Ca²⁺ concentration and the transcriptional levels of calcium signalling-related genes in the Glnmnat overexpression strains (A) All of the 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 16

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 before being 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 means ± 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 19

Effects of LaCl₃ treatment on cellulase production in *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 before being treated with or without 5 mM LaCl₃ for one day in MM plus 1% Avicel. (A) Measurement of CMCase activity in the tested strains. (B) Measurement of the pNPGase activity in the tested strains. (C~O) The transcriptional levels of the genes encoding the major cellulases and transcription factors for cellulase production in the tested strains. Three independent biological replicates were performed for all experiments. The values are the means ± 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).

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