Microbial Cell Factories

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Metformin regulates cellulase production in *Trichoderma reesei* via calcium signaling and mitochondrial function

Jiajia Wang[1†], Yumeng Chen[1†], Jiawei Cong[1] and Wei Wang[1*]

Abstract

Background *Trichoderma reesei* is renowned for its cellulase-producing ability and is used for biofuel production from lignocellulose. In plants and fungi, cellulase production is induced by cellulose and suppressed by glucose; however, whether metformin can enhance cellulase production and mitochondrial function in *T. reesei* remains unclear. Metformin reduces blood glucose levels by inhibiting hepatic gluconeogenesis; therefore, it is worth investigating whether metformin transmission modulates cellulase biosynthesis in *T. reesei*.

Results Metformin increased cellulase production and the transcription of cellulase-related genes. It also enhanced the concentrations of Ca^{2+} in the cytosol and mitochondria and regulated the transcription levels of cellulase-related genes by modulating calcium homeostasis in *T. reesei* QM6a. In addition, metformin was identified as an antioxidant that can enhance cellulase activity by reducing reactive oxygen species (ROS). Our results demonstrated that metformin influences the state of mitochondria by enhancing mitochondrial activity and membrane potential to promote cellulase production.

Conclusion Collectively, these results indicate that metformin enhances cellulase production, scavenges ROS, and protects mitochondrial activity in *T. reesei*.

Keywords *Trichoderma reesei*, Metformin, Cellulase, Calcium signaling, ROS, Mitochondria

Background

Naturally occurring cellulose is abundant in various waste materials originating from wood and corn stover, households, and other agricultural byproducts. Through the action of cellulase, an extracellular enzyme, cellulose can be converted into valuable resources, such as biofuels and other bio-based products, using cost-effective and environmentally friendly bioprocesses [1]. Cellulase also has extensive applications in the detergent, papermaking, fruit juice, and beverage industries [2]. *Trichoderma reesei* can produce substantial amounts of cellulase and thus could be used to facilitate the conversion of biowaste into biofuels [3, 4]. Exploiting the cellulase expression mechanism in *T. reesei* has long been a topic of great interest. This study provides valuable insights for discovering new strategies to enhance cellulase production and to develop *T. reesei* as a microbial host for heterologous gene expression.

In recent years, microbial cell research has focused on identifying novel strategies that promote cellulase activity in *T. reesei* and elucidating its underlying induction

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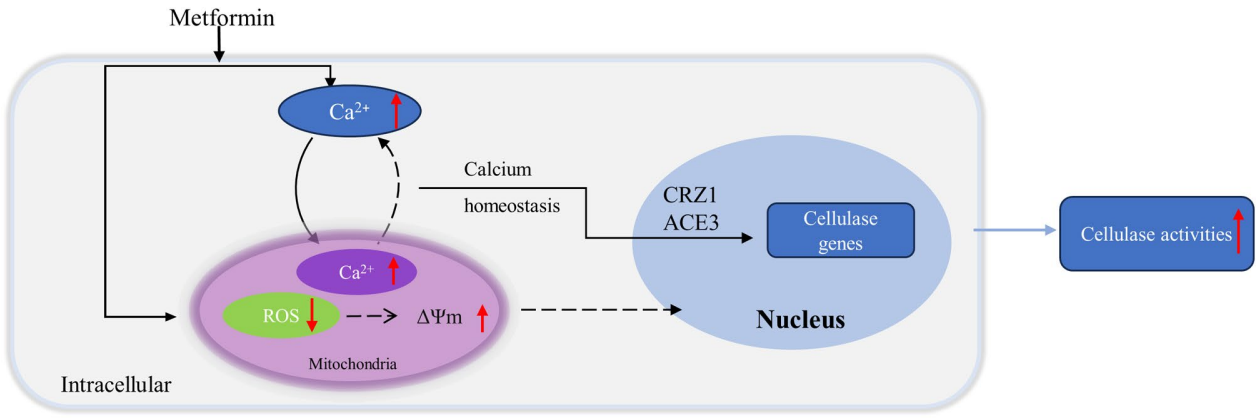
Graphical Abstract



mechanisms. Previous studies have shown a relationship between Mn^{2+} and cellulase activity in *T. reesei* and demonstrated that *N, N*-dimethylformamide can enhance cellulase production in *T. reesei* by triggering Ca^{2+} signaling [5, 6]. Furthermore, Sr^{+}, Zn^{2+}, and PEG8000 may enhance cellulase activity in *T. reesei* [7, 8]. These findings highlight the significance and necessity of selecting appropriate strategies that promote cellulase activity and may improve our understanding of the cellulase production mechanism of *T. reesei*.

Cellulase production is modulated by several factors [9], including transcription, secondary-messenger-mediated signaling pathways involving Ca^{2+}, and reactive oxygen species (ROS). Ca^{2+} participates in many different intracellular signaling pathways [10, 11], and calcium signaling components, such as free Ca^{2+}, calmodulin (CAM), calcineurin (CNA), and calcineurin-responsive zinc finger transcription factor 1 (CRZ1), which coalesce to form a signaling cascade [11]. For example, intracellular Ca^{2+} can trigger CRZ1, which regulates its downstream target gene *XYR1*, to enhance cellulase activity [12].

ROS originate from physiological processes such as aerobic respiration and are indispensable signaling molecules that can regulate developmental and physiological processes in fungi [13, 14] and the induction of exogenous substances [15]. Mitochondria are important organelles that contribute to cellular energy supply metabolism [16] and signal transduction pathway regulation and act as a major source of ROS [17]. In fungi, ROS have many biological functions; however, high ROS concentrations are harmful [18]. Wu et al. suggested that putrescine may indirectly affect the production of ganoderic acid (GA) biosynthesis by regulating ROS concentrations, which may influence the expression of key genes associated with GA biosynthesis [19]. Gao et al. also reported that mycelial branching and secondary metabolism were dependent on intracellular ROS [20]. ROS have an essential regulatory role during the treatment of mammalian diseases and in plant responses to environmental stress; however, ROS have been relatively poorly studied in filamentous fungi.

Metformin, also called metformin hydrochloride, is used to treat type 2 diabetes owing to its potent hypoglycemic effects [21]. In patients with type 2 diabetes, metformin can reduce blood glucose levels by inhibiting hepatic gluconeogenesis in a redox-dependent manner [22]. Metformin not only influences glucose metabolism, but is also beneficial in treating other underlying conditions, such as aging, cardiovascular diseases, cancer, and neurodegenerative diseases [23]. Studies have demonstrated that metformin can stimulate mitochondrial fission, enhance mitochondrial respiration, aid in the recovery of the mitochondrial life cycle, and stabilize mitochondrial function [24]. Moreover, metformin reduces oxidative stress, inhibits chronic inflammation, and boosts antioxidant defenses [25]. In plants and fungi, cellulase production is induced by cellulose and suppressed by glucose [9]; however, whether metformin can enhance cellulase expression in *T. reesei* remains unclear.

Therefore, this study aimed to investigate the effect of metformin on cellulase production in *T. reesei* QM6a. Changes in cellulase activity and cellulase regulatory mechanisms were analyzed using 60 mM of metformin. In addition, calcium signaling, the direction of calcium ion flow, antioxidant capacity, and mitochondrial function after metformin treatment were examined, and a potential mechanism to elucidate how metformin enhances cellulase production in *T. reesei* QM6a was proposed. This study offers a new perspective on cellulase production and the underlying regulatory mechanisms influenced by metformin.

Materials and methods

Strains and culture conditions

The experiments were conducted using *Trichoderma reesei* QM6a (ATCC 13631) purchased from ATCC. Fresh conidia of *T. reesei* were inoculated on potato dextrose agar (PDA) medium and incubated at 28 °C in the dark. Thereafter, the universal fungal medium, Mandels (MA), was used to germinate conidia in all subsequent experiments [26]. Metformin's effect on cellulase production was assessed using minimal medium (MM) with 1% (w/v) Avicel (#C104842, Aladdin, Shanghai, China) [7]. Conidia were grown in MA medium (100 mL) with 2% glucose (28 °C for 36 h), then, the mycelia were centrifuged at 12,000 × *g* for 10 min, washed with carbon-free MM, and transferred to 50 mL of fresh MM containing 1% (w/v) Avicel (pH-101; Sigma-Aldrich, St. Louis, MO, USA) supplemented with different concentrations of metformin (0, 20, 40, 60, and 80 mM).

Enzyme activity analysis

To assess enzymatic activity, we performed cultivations (1 mL) at various time intervals and centrifuged the biomass at 12,000 × *g* at 4 °C for 5 min. Cellulase activity was determined using the supernatant. The activities of *p*NPCase, FPase, and CMCase were determined as previously described [27].

cDNA extraction and RT-qPCR

Gene target expression levels were tested using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. Mycelial RNA was extracted using the Fast-Pure Plant DNA Isolation Mini Kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. RT-qPCR was performed using an ABI Step One thermocycler (Applied Biosystems, Foster City, CA, USA). Data was normalized using sar1 as an internal reference, and the $2-\Delta\Delta Ct$ method was used for analysis. The RT-qPCR primers are listed in Table S1.

Detection of cytosolic Ca^{2+} and ROS

The levels of cytosolic Ca^{2+} and ROS were evaluated using the fluorescent probes Fluo-4 AM and DCHF-DA, respectively (Beyotime, Shanghai, China). The assays were conducted according to the manufacturer's instructions, with minor modifications. Briefly, the hyphae and fluorescent probes were maintained at 28 °C for 30 min and then the hyphae were rinsed in Hank's Balanced Salt Solution (HBSS; no calcium, magnesium, and phenol red) to reduce noise [28]. In this study, all fluorescent detection was performed using an inverted microscope (Eclipse Ti-E, Nikon, Tokyo, Japan). The ImageJ-win 64 software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the average fluorescence intensity of all probes in mycelia.

Detection of mitochondrial Ca^{2+}

Rhod-2 AM is a calcium indicator with high affinity and mitochondrial localization. Rhod-2 AM was used to determine the source of metformin-activated Ca^{2+} in mitochondria [29] according to the manufacturer's instructions (#40776ES50, YEASEN, Shanghai, China), with minor modifications. The mycelium was incubated with fluorescent probes for 30 min at 28 °C in the dark and then washed with HBSS to remove the dye and reduce background noise.

Mitochondrial activity and membrane potential detection

MitoTracker® Red CMXRos was used to visualize the number of active mitochondria in cells according to the manufacturer's instructions (#40741ES50, YEASEN, Shanghai, China), with slight modifications. JC-1 is a commonly used fluorescent probe for detecting mitochondrial membrane potential ($\Delta\Psi m$) [30]. To explore the changes in mitochondrial function after metformin treatment, mycelia were stained with JC-1, and the ratio of red/green fluorescence intensity was calculated to determine mitochondrial health. MitoTracker® Red CMXRos was pre-warmed at 37 °C before incubation and JC-1 labeling was increased by adding 1% Tween-20 during dissolution. The labeling compounds were subsequently transferred to the mycelia and cultivated at 28 °C before the hyphae were washed thrice with HBSS.

Chemical treatments

Different concentrations of metformin were added during the transfer experiments. N-Acetyl-L-cysteine (NAC) and L-ascorbic acid (VC) were used as ROS scavengers, H_2O_2 was used as an ROS generator, and $LaCl_3$ was used as a plasma membrane Ca^{2+} channel repressor. In the transfer experiment, fresh MM was added directly to NAC and VC (mycelium transferred) at the time of mycelial transfer, and $LaCl_3$ and H_2O_2 were added 24 h after mycelial transfer.

Statistical analysis

To ensure reproducibility, experimental data was obtained from three independent experiments and independent sets of experiments. The error bars represent the standard deviation of the mean of the samples taken in triplicate. Statistical significance was determined using the Student's *t*-test. Significance was set at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

Results

Cellulase production increases after metformin treatment in *T. reesei* QM6a

To determine the effect of metformin on cellulase production, the mycelia of *T. reesei* QM6a were precultured to equal biomass before being transferred into liquid

MM containing 1% (w/v) Avicel and different concentrations of metformin (0, 20, 40, 60, and 80 mM). The addition of metformin at different concentrations significantly improved cellulase activity compared to the control group; in particular, 60 mM metformin markedly increased the activities of *p* NPCase, CMCase, and FPase by 90, 67, and 95%, respectively, after 4 days of cultivation (Fig. 1a–c). Metformin addition did not significantly alter the strain biomass quantity (Fig. S1); thus, enzyme activity is reported in units per milliliter (U/mL). These findings indicated that a range of 20–80 mM metformin promoted cellulase activity after 4 days of cultivation, with 60 mM being the optimal concentration for augmenting cellulase production in *T. reesei* QM6a. Results of an SDS-PAGE analysis (Fig. S2) of the extracellular proteins secreted by QM6a supplemented with 0 and 60 mM metformin agreed with the cellulase activity results presented above.

We analyzed the expression levels of three key cellulase genes (*cbh1*, *cbh2*, and *egl1*) to investigate the effect of metformin on cellulase synthesis. Moreover, after 72 and 84 h of culture supplemented with 0 and 60 mM metformin, we assessed the expression of *xyr1* and *ace3* (two critical cellulase transcription activators) using RT-qPCR [26]. Of note, 60 mM metformin significantly increased the transcription levels of *cbh1*, *cbh2*, and *egl1* after 72 and 84 h, which is consistent with the observed increases in cellulase activity shown in Fig. 1a–c. Metformin supplementation increased the transcription of *ace3*, which is a pivotal transcription factor, by 180.06% (Fig. 1g). In contrast, the expression of another cellulase activator, *xyr1*, was upregulated by 22.18% at 84 h after metformin supplementation (Fig. 1h). These findings indicate that *ace3* has a more pronounced influence than *xyr1* in regulating cellulase-related gene expression after metformin treatment.

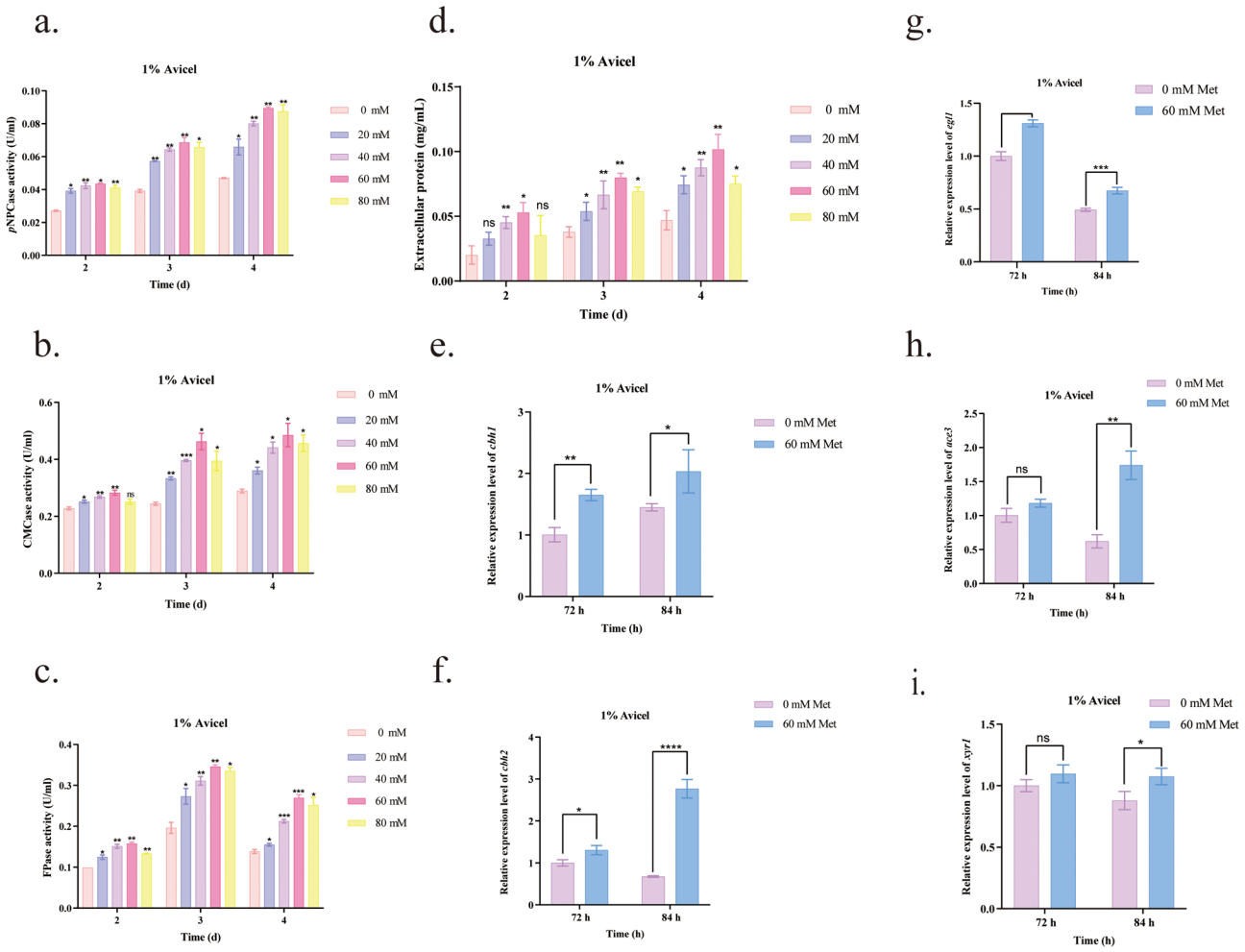


Fig. 1 Effects of different metformin concentrations on cellulase production in *T. reesei*. (**a**) *p* NPCase, (**b**) CMCase, (**c**) FPase activity and (**d**) protein concentration in *T. reesei* QM6a detected 2, 3, or 4 days after treatment with 0–80 mM metformin. The effects of 0 mM Met (not metformin-treated) and 60 mM Met (medium included 60 mM Met) on the expression of different cellulase-related genes (**e**) *cbh1*, (**f**) *cbh2*, (**g**) *egl1*, (**h**) *ace3*, and (i) *xyr1* in *T. reesei* QM6a (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

Calcium signaling pathways in *T. reesei* are induced by metformin treatment

We evaluated how the addition of metformin could upregulate cellulase-related gene expression. The fluorescent probe Fluo-4 AM was used to evaluate the concentrations of Ca^{2+} in the cytosol; the substance emits green fluorescence upon crossing the cell membrane and interacting with cellular Ca^{2+} [31]. The relative quantity of free Ca^{2+} within the cells was characterized by the green fluorescence intensity. Fluorescence intensity in the experimental group (with 60 mM metformin) increased by 2.36 times compared to the control group (Fig. 2b), indicating that metformin elevated the level of cytosolic Ca^{2+} in *T. reesei* QM6a.

Calcium serves as a pivotal secondary messenger in the activation of calcium signaling pathways. Variations in calcium levels can stimulate the production of transcription factors, such as *crz1*, which orchestrates the regulation of downstream genes. To explore the potential link between metformin-induced elevation of intracellular calcium levels and augmentation of cellulase activity via *crz1* activation, the expression levels of genes associated with the regulation of calcium signaling were analyzed using RT-qPCR. Metformin supplementation markedly increased the expression of *crz1* after 60 and 72 h of treatment (Fig. 2c), indicating that metformin may enhance cellulase activity by modulating calcium signaling and upregulating *crz1* expression.

$LaCl_3$ is an antagonist of plasma membrane calcium channels that inhibits the influx of extracellular Ca^{2+}, and Ca^{2+} serves as a critical checkpoint at the boundary between life and death in cells [32]. Thus, the effect of metformin on cytoplasmic Ca^{2+} spikes with $LaCl_3$ as a modulatory agent was investigated. The metformin-induced increase in intracellular Ca^{2+} levels was significantly attenuated by the addition of $LaCl_3$ (Fig. 2a). The addition of 5 mM $LaCl_3$ resulted in a reduction in the fluorescence intensity of metformin-induced mycelia compared with samples without $LaCl_3$. These findings suggest that metformin rapidly increases cytosolic calcium levels

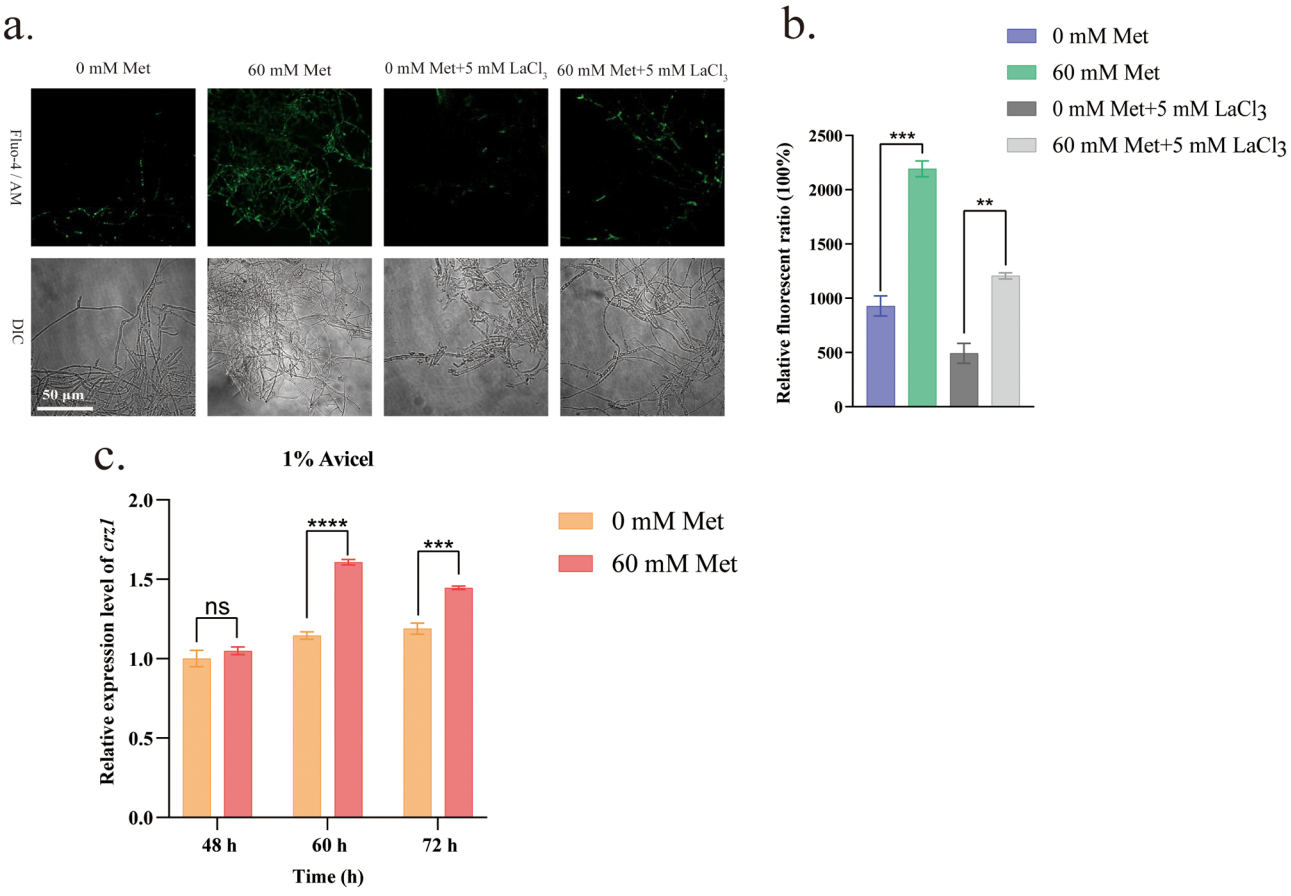


Fig. 2 Effects of different metformin concentrations on cytosolic Ca^{2+} concentration and calcium signaling. (**a**) Cytosolic Ca^{2+} levels were assessed using Fluo-4 AM. *Trichoderma reesei* QM6a was cultivated in an MM medium for 2 days and subsequently treated with $LaCl_3$, 60 mM metformin, or 0 mM metformin. Hyphae were stained with 4 Fluo-4 AM to monitor the Ca^{2+} concentrations. (**b**) Comparative fluorescence ratios demonstrating the effect of metformin and $LaCl_3$ on cytosolic Ca^{2+}. The *x*-axis depicts the various treatments with metformin or $LaCl_3$, while the y-axis depicts the Ca^{2+} fluorescence ratio as detected by CLSM. (**c**) Transcriptional levels of *crz1* after 48, 60, or 72 h of 0 mM or 60 mM metformin treatment (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

and subsequently activates the calcium signaling pathway (Fig. 2a, b).

Furthermore, the impact of this intervention on metformin-induced cellulase overexpression was evaluated, including *p* NPCase and CMCase activity, and the expression levels of key cellulase-related genes, such as *cbh1* and *egl1*.

At 60 mM, metformin did not induce cellulase overproduction in *T. reesei* QM6a (with 5 mM $LaCl_3$). The addition of $LaCl_3$ led to a reduction in *p* NPCase and CMCase activity, respectively, after 3 days of incubation compared with samples without $LaCl_3$, and the expression levels of *cbh1* and *egl1* reflected these findings (Fig. 3a, b). Metformin-induced upregulation of *cbh1* and *egl1* was inhibited following the introduction of $LaCl_3$ (Fig. 3c).

Treatment with $LaCl_3$ significantly attenuated metformin-induced cellulase overexpression, indicating that $LaCl_3$ obstructs the surge of cytosolic Ca^{2+} that is essential for metformin-induced cellulase production. These results demonstrate that metformin increases cellulase activity by activating Ca^{2+} signaling pathways.

Metformin increases mitochondrial calcium

Our findings revealed that metformin promoted the accumulation of cytoplasmic calcium ions; however, after the addition of $LaCl_3$, cytosolic Ca^{2+} was significantly but not completely inhibited after metformin treatment. Metformin has been reported to target mitochondria, thus playing a crucial role in maintaining calcium homeostasis and regulating normal cellular metabolism [33]. In addition, other potential mechanisms for Ca^{2+} activation by metformin have been reported, such as the modulation of the calcium ion pool within mitochondria. Rhod-2 AM, a positively charged fluorescent probe, was employed to specifically accumulate in mitochondria through potential-driven uptake, producing a distinct red, dot-like staining pattern under fluorescence microscopy [34]. To test our hypothesis, Rhod-2 AM was used to identify Ca^{2+} changes within mitochondria. The intensity of Rhod-2 AM fluorescence was markedly higher in metformin-treated cells than in untreated cells (Fig. 4a). Quantitative analysis revealed an 80.7% increase in fluorescence intensity in the experimental group (60 mM metformin) than in the control group (Fig. 4b). In conclusion, metformin not only increased the intracellular Ca^{2+} concentration but also induced mitochondrial calcium ion uptake, which may have contributed to the upregulation of cellulase-related genes in *T. reesei* QM6a.

Metformin reduces ROS generation

ROS are natural byproducts of aerobic respiration primarily generated in the mitochondria. ROS accumulation can have harmful effects on cells, and metformin is reported to possess antioxidant properties [35]. Li et al. reported the negative effect of ROS on cellulase production in *T. reesei* RUT-C30 [7]; therefore, we aimed to investigate the effect of metformin on the cellular redox system under ROS stress and its impact on cellulase production. To measure the cellulase activity in *T. reesei* QM6a, the intracellular ROS concentration was manipulated using NAC and VC as ROS scavengers and H_2O_2 as a ROS-generating agent. Treatment with 4 mM H_2O_2 led to a reduction in both *p* NPCase and CMCase activity (by approximately 17.27% and 18.41%, respectively), compared with control (QM6a without 4 mM H_2O_2) (Fig. 5). Conversely, when treated with 1 mM NAC, there was a noticeable increase in *p* NPCase and CMCase activity (21.56% and 16.14%, respectively), indicating the positive effect of ROS scavenging on cellulase production (Fig. 5). Enzyme activity was enhanced after the treatment of *T. reesei* QM6a with VC, which was consistent with previous findings and suggests that high ROS concentrations negatively affect cellulase production by *T. reesei* QM6a (Fig. 5) Additionally, the cellulase activity was higher after treatment with 60 mM metformin than that in the

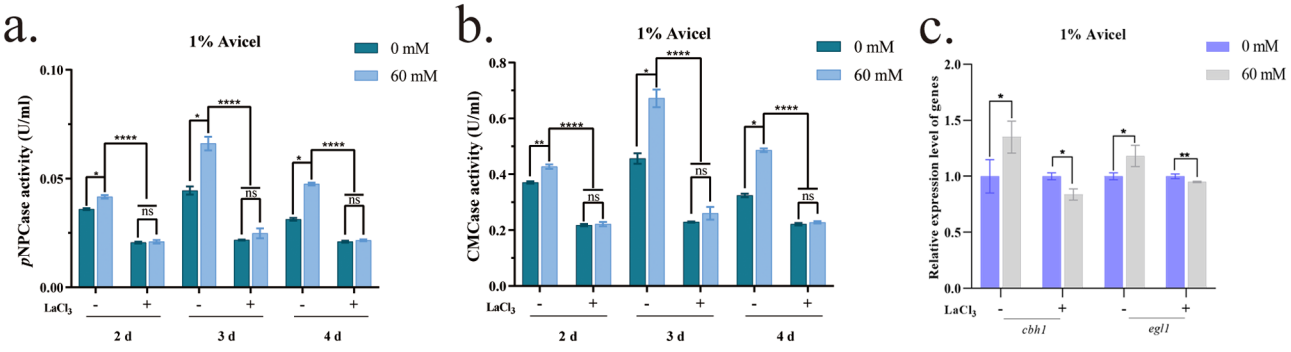


Fig. 3 Effect of LaCl3 on the production of cellulase after metformin treatment. The T. *reesei* QM6a strain, which was grown in a medium enriched with and without 60 mM metformin and in a medium enriched with (+) or without (–) 5 mM $LaCl_3$, exhibited (**a**) increased *p* NPCase and (**b**) CMCase activity. Transcription levels of *cbh1* and *egl1* (**c**) were measured 72 h after treatment (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). Figure 3c shows the relative expression ratio of 60 mM metformin compared to no metformin addition (set as 1)

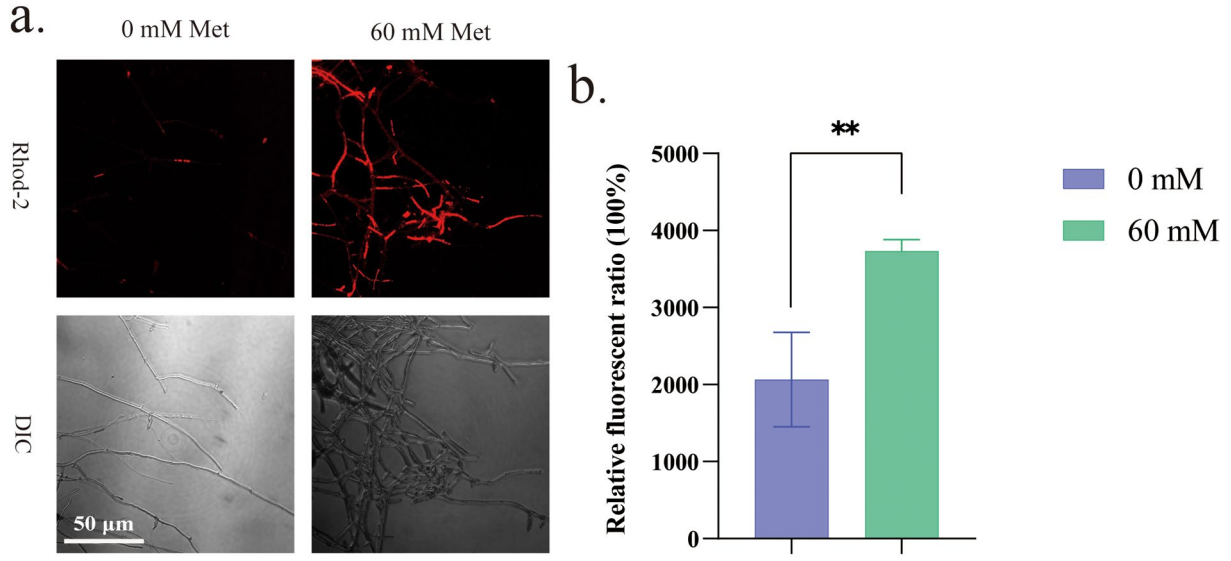


Fig. 4 Mitochondrial Ca^{2+} levels increase after metformin treatment. (**a**) Mitochondrial Ca^{2+} levels were measured using the specific fluorescent probe Rhod-2 AM. The *T. reesei* QM6a strain was cultured in MM for 2 days with 0 mM or 60 mM metformin. Hyphae were treated with Rhod-2 AM and the relative fluorescence was microscopically monitored, where stronger red fluorescence indicated a higher mitochondrial Ca^{2+} concentration. (**b**) Comparative fluorescence ratios demonstrating the effects of metformin on mitochondrial Ca^{2+}. The x-axis represents the different metformin treatments, while the y-axis represents the Ca^{2+} fluorescence ratio as detected by CLSM (**$p < 0.01$)

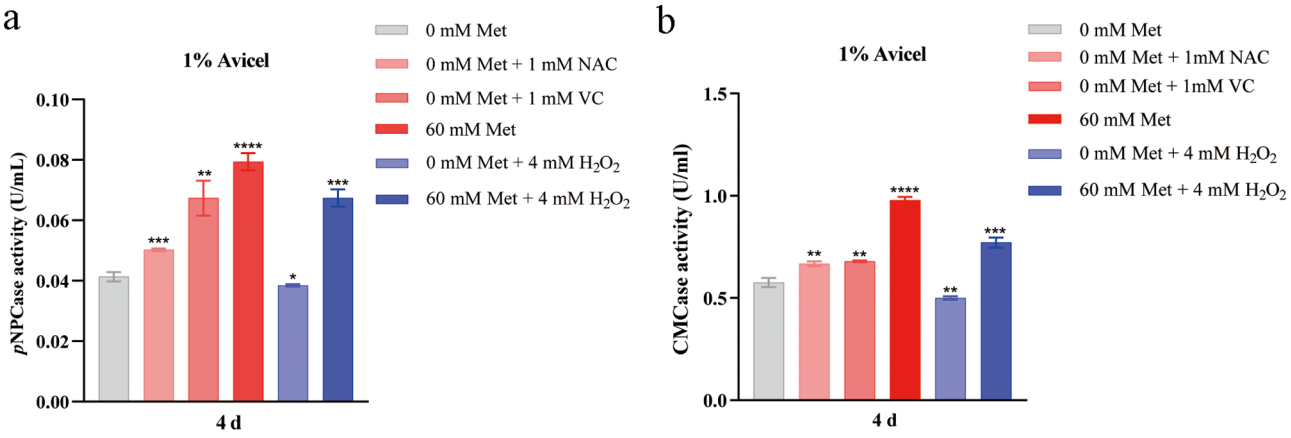


Fig. 5 Effect of reactive oxygen species (ROS) on metformin-induced cellulase production. Activities of *p* NPCase (**a**) and CMCase (**b**) in *T. reesei* QM6a strain with or without 60 mM metformin treatment, 4 mM H_2O_2, 1 mM NAC, or 1 mM VC in MM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

control (no treatment), although it was slightly reduced by the addition of 4 mM H_2O_2. Notably, *p* NPCase and CMCase activity were 92% and 70.03% higher in the presence of 60 mM metformin than those with NAC or VC treatments, respectively. NAC and VC are widely used antioxidants, and VC is commonly used in the nutraceutical, medical, and beauty industries. Several studies have demonstrated that metformin has antioxidant properties and scavenges ROS to reduce inflammation in plants and animals [36]. Our data suggest that metformin may enhance cellulase activity by scavenging ROS and is a suitable antioxidant for *T. reesei* QM6a.

To validate this hypothesis, the ROS levels in *T. reesei* mycelia were assessed using DCFH-DA (S0033S, Beyotime, Beijing, China) [37]. The findings indicated a marked increase in ROS levels upon 4 mM H_2O_2 treatment in the control group, whereas the metformin-treated strains exhibited significantly lower ROS levels, indicating metformin's ROS-scavenging capabilities and its role in enhancing cellulase production (Fig. 6).

In summary, high ROS concentrations have a detrimental effect on cellulase production in *T. reesei* QM6a. However, metformin functions as an antioxidant in QM6a cells by scavenging ROS, thereby enhancing cellulase activity.

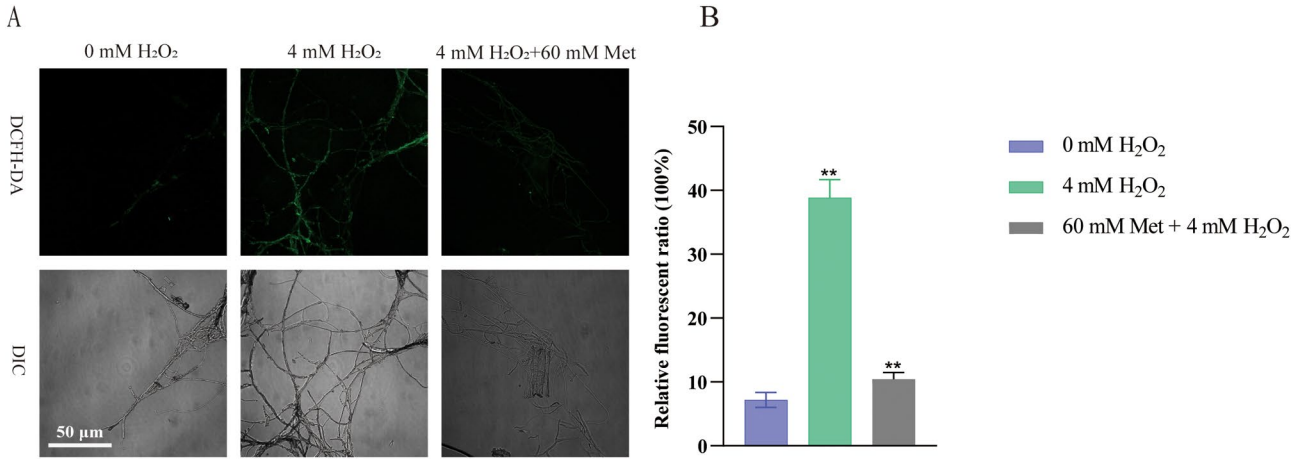


Fig. 6 Metformin reduces ROS levels. To measure intracellular ROS levels, the fluorescent probe DCFH-DA was utilized. *Trichoderma reesei* QM6a was grown in liquid MM with or without metformin and H_2O_2. (**a**) Hyphae were treated with DCFH-DA, and the fluorescence intensity was continuously monitored using automatic inverted fluorescence microscopy. (**b**) Higher green fluorescence intensity indicates increased levels of intracellular ROS. Differential interference contrast (DIC) microscopy was also employed (**$p < 0.01$)

Metformin promotes cellulase enzyme activity by regulating mitochondrial health

Mitochondria are colloquially referred to as the "powerhouse of the cell" and their biological activities play an important role in several different signal transduction pathways, including biosynthetic, calcium homeostasis, and redox control-related pathways [38]. Bharath et al. found that metformin enhanced mitochondrial activity and functional integrity in humans [29]. To explore the relationship between increased cellulase activity in metformin-treated *T. reesei* QM6a and mitochondria, MitoTracker® Red CMXRos dye was used to characterize the mycelial mitochondrial activity [39]. Fluorescence intensity notably increased following supplementation with 60 mM metformin, exhibiting a 46.35% increase in the treated strain compared with that of the untreated control (Fig. 7b). These results suggested that the heightened mitochondrial activity induced by metformin was linked to the elevated cellulase activity in *T. reesei* QM6a.

Mitochondrial membrane potential (ΔΨm) is a universal selective indicator used to evaluate the health of mitochondrial functions; when mitochondria are damaged, the membrane potential decreases, and mitochondrial activity is reduced [40]. To further investigate the integrity of mitochondrial function after the addition of metformin, a JC-1 fluorescent probe was used to label the strains before and after metformin induction to detect mitochondrial membrane potential. The red fluorescence intensity of the control group (without metformin) was markedly weaker than that of the group treated with 60 mM metformin. The green fluorescence intensity was considerably higher, while the orange fluorescence intensity was also significantly enhanced in the merged images of red–green fluorescence (Fig. 7c). Quantification of the fluorescence signals revealed a 26% increase in red fluorescence intensity, a 10.23% decrease in green fluorescence intensity, and a 40% increase in red/green fluorescence intensity in metformin-treated strains (Fig. 7d). These results suggest that 60 mM metformin effectively enhanced the mitochondrial membrane potential (ΔΨm) of *T. reesei* QM6a, which promoted mitochondrial function and increased cellulase activity.

Discussion

Cellular metabolism is significantly affected by the induction of metal ions and exogenous compounds, such as Mn^{2+}, Sr^{2+}, and Zn^{2+} [5, 7, 41], which can trigger the calcium signaling pathway to increase cellulase production. In the present study, cellulase production and the transcription of cellulase-related genes (e.g., *cbh1*, *cbh2*, *egl1*, and *ace3*) were enhanced by metformin application. We also found that the concentrations of extracellular metformin were almost constant in the fermentation broth of *T. reesei*, which is worthy of further investigation.

Of note, the transcription factor ACE3 is essential in cellulase production in *T. reesei*. Ca^{2+} is a crucial secondary messenger that plays a pivotal role in the biological functionality of cells and is activated by several external stimuli, including changes in temperature, pH, light conditions, and drug application [42]. Our findings demonstrate that metformin boosts cellulase production by activating the calcium signaling pathway. Fungi can regulate various cellular metabolic processes by sensing changes in intracellular Ca^{2+} concentrations, which activate the expression of target genes [43]. Our study demonstrated that *crz1* responds to rapid increases in Ca^{2+} induced by metformin and regulates cellulase production. The plasma membrane Ca^{2+} inhibitor $LaCl_3$ was used to further demonstrate that metformin activates the calcium signaling pathway to promote cellulase activity.

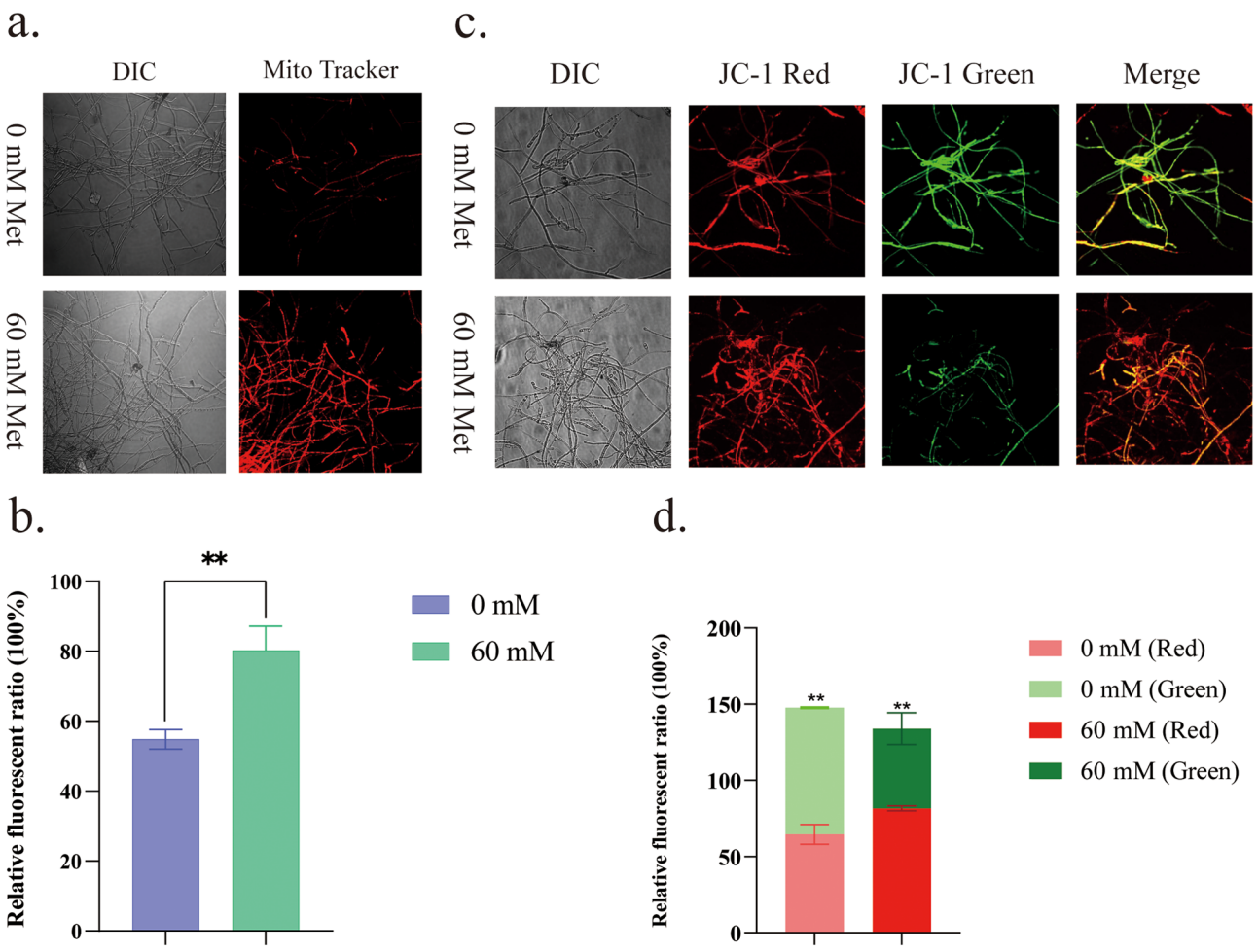


Fig. 7 Impact of metformin on cellulase production in relation to mitochondrial health. (**a**) Mitochondrial activity in *T. reesei* QM6a with or without metformin treatment at a concentration of 100 nM was assessed using MitoTracker® Red CMXRos. (**b**) Higher levels of red fluorescence intensity are associated with improved mitochondrial health, followed by (**c**) measurement of mitochondrial membrane potential (ΔΨm) with JC-1 dyes. Fluorescence intensity was monitored using automatic inverted fluorescence microscopy. (**d**) Ratio of red to green fluorescence intensity is indicative of mitochondrial function integrity (**$p < 0.01$)

Maintenance of intracellular Ca^{2+} homeostasis depends on various intracellular stores, including the mitochondria, endoplasmic reticulum, and Golgi apparatus; however, the regulation of Ca^{2+} homeostasis in *T. reesei* QM6a remains unclear. Besides being the "powerhouse of the cell," mitochondria also regulate Ca^{2+} homeostasis [33]. Mitochondrial response signals rely on increased concentrations of intracellular Ca^{2+} and store Ca^{2+}, stabilizing the new homeostasis of intracellular Ca^{2+} [44]. In addition, increased mitochondrial Ca^{2+} partly compensates for impaired carbohydrate-mediated mitochondrial respiration [45]. Our study found that mitochondrial Ca^{2+} is released into the cytoplasm when intracellular Ca^{2+} is blocked (Fig. 2). Previous reports on calcium transport between mitochondria and the cytoplasm are consistent with our data, indicating that mitochondria regulate intracellular Ca^{2+} homeostasis.

We labeled intracellular calcium ions with a fluorescent probe and found that 60 mM metformin increased both cytoplasmic and mitochondrial Ca^{2+} concentrations. The calcium signaling transcription factor, CRZ1, was also upregulated in response to metformin treatment, suggesting that metformin affects cytoplasmic and mitochondrial Ca^{2+} concentrations by regulating Ca^{2+} homeostasis in the cytoplasm and mitochondria. Calcium signaling also regulates the transcriptional level of downstream CRZ1, increasing the transcriptional level of the cellulase synthesis gene in *T. reesei*.

ROS are produced during various physiological processes, such as exposure to drugs, xenobiotics, and metal ions. They can impair mitochondrial function and directly or indirectly damage DNA and proteins [46]. High levels of ROS negatively impact cellulase activity in *T. reesei* RUC-30, and significant increases in ROS induced by PEG8000 inhibited mycelial growth and

cellulase expression [7, 8]. Our results indicate that high concentrations of ROS negatively affect cellulase activity and that metformin acts as a ROS scavenger. Our findings suggest that metformin has anti-inflammatory properties, which may be due to the inhibition of ROS production. These results are consistent with those of previous studies, which have demonstrated that metformin treatment can decrease blood oxidative stress and cholesterol levels [47]. Metformin may also inhibit mitochondrial dysfunction and modulate antioxidant protection to promote the clearance of damaged mitochondria [48]. Remarkably, the increase in cellulase activity induced by metformin was equally effective to that induced by NAC or VC, further suggesting that metformin acts as an antioxidant, promoting cellulase activity and protecting *T. reesei* QM6a from cellular damage.

Metformin promotes mitochondrial activity and health, which may alleviate age-associated inflammation [29]. Yao et al. demonstrated that healthy mitochondrial transplantation is an effective strategy for engineering stem cells for tissue regeneration [49]. Gorospe et al. demonstrated that experimentally increasing mitochondrial membrane potential is sufficient to restore timely cell cycle progression in *Saccharomyces cerevisiae* [50]. These reports indicate that the physiological health of mitochondria is closely associated with cell health. In this study, we indicated that the mitochondrial activity of *T. reesei* QM6a was promoted by 60 mM metformin, which significantly improved mitochondrial membrane potential. Metformin enhances mitochondrial respiration, membrane potential, and ATP in liver cells [25]. Our results are similar to those previously reported, and we hypothesized that metformin increases membrane potential and promotes ATP production to enhance cellulase production in *T. reesei* QM6a. Additionally, we hypothesized that metformin improves mitochondrial function and enhances the expression levels of cellulase-related genes.

Conclusions

In this study, we identified that metformin enhances cellulase expression in *T. reesei* QM6a and proposed a potential mechanism by which metformin modulates cellulase production (see Graphical abstract). Metformin induces rapid increases in intracellular Ca^{2+} and increases mitochondrial Ca^{2+} levels. These elevated Ca^{2+} levels activate the signal transduction pathway of the *crz1* transcription factor, which in turn induces cellulase production. Moreover, high ROS stress negatively affects cellulose production in *T. reesei*, while metformin scavenges ROS to promote cellulase production. This research reveals metformin's ability to target mitochondria to increase cellulase gene transcription by boosting mitochondrial activity and function, providing new insights into signal transduction mechanisms during cellulase synthesis.

Abbreviations

CMCase	Endo-β-glucanase activity
crz1	Calcineurin-responsive zinc finger transcription factor 1
FPase	Filter paper activity, representing total extracellular cellulase activity
PNPCase	Exo-β-glucanase activity
ROS	Reactive oxygen species
RT	Qpcr-Real-time quantitative PCR

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

JW planned and conducted the experiments, interpreted the experimental data, and wrote the manuscript. YC analyzed the data, reviewed the manuscript, and supported the research funding. JC analyzed partial data. WW directed and coordinated the study and reviewed the manuscript. All authors have read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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