Mn$^{2+}$ modulates the expression of cellulase genes in *Trichoderma reesei* Rut-C30 via calcium signaling

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Abstract

**Background:** The filamentous fungus *Trichoderma reesei* Rut-C30 is one of the most vital fungi for the production of cellulases, which can be used for biofuel production from lignocellulose. Nevertheless, the mechanism of transmission of external stimuli and signals in modulating cellulase production in *T. reesei* Rut-C30 remains unclear. Calcium is a known second messenger regulating cellulase gene expression in *T. reesei*.

**Results:** In this study, we found that a biologically relevant extracellular Mn$^{2+}$ concentration markedly stimulates cellulase production, total protein secretion, and the intracellular Mn$^{2+}$ concentration of Rut-C30, a cellulase hyper-producing strain of *T. reesei*. Furthermore, we identified two Mn$^{2+}$ transport proteins, designated as TPHO84-1 and TPHO84-2, indicating that they are upstream in the signaling pathway that leads to cellulase upregulation. We also found that Mn$^{2+}$ induced a significant increase in cytosolic Ca$^{2+}$ concentration, and that this increased cytosolic Ca$^{2+}$ might be a key step in the Mn$^{2+}$-mediated regulation of cellulase gene transcription and production. The utilization of LaCl$_3$ to block plasma membrane Ca$^{2+}$ channels, and deletion of *crz1* (calcineurin-responsive zinc finger transcription factor 1) to interrupt calcium signaling, showed that Mn$^{2+}$ exerts the induction of cellulase genes via calcium channels and calcium signaling. To substantiate this, we identified a Ca$^{2+}$/Mn$^{2+}$ P-type ATPase, TPMR1, which could play a pivotal role in Ca$^{2+}$/Mn$^{2+}$ homeostasis and Mn$^{2+}$ induction of cellulase genes in *T. reesei* Rut-C30.

**Conclusions:** Taken together, our results revealed for the first time that Mn$^{2+}$ stimulates cellulase production, and demonstrates that Mn$^{2+}$ upregulates cellulase genes via calcium channels and calcium signaling. Our research also provides a direction to facilitate enhanced cellulase production by *T. reesei*.

**Keywords:** *Trichoderma reesei*, Mn$^{2+}$ stimulation, Cellulase, Mn$^{2+}$ transport, Calcium signaling, TPMR1

Background

Lignocellulosic biomass, the most abundant renewable energy source, can be hydrolyzed to sugars for bioethanol production. A common host cited for the production of cellulases and hemicellulases is the saprotrophic, filamentous fungus *Trichoderma reesei*, which is well known for its excellent ability to secrete a broad range of cellulases at very high levels [1–4]. Due to its ability to degrade and thrive on cellulose-containing fabrics, *T. reesei* has attracted attention and was consequently studied in detail [5, 6]. However, compared with the energy-efficient production of cellulases and hemicellulases, the induction and regulation of the expression of genes encoding these enzymes in *T. reesei* are still not completely understood. Additionally, the induction of high-level cellulase production is dependent on inducers such as cellulose, d-xylose, lactose, or sophorose [3, 7–9], increasing the costs for the application of produced enzymes. Due to the extensive applications of cellulases and hemicellulases, the induction and regulation of the expression of genes encoding these enzymes have drawn significant attention.

Recent studies have demonstrated that cellulase production is regulated in response to environmental stress,
such as light [10, 11], organic solvents [12], and metal ions [13–15]. A great example was shown by Chen and co-workers, who illustrated that Ca}\textsuperscript{2+} plays an important role in the production of cellulase or hemicellulase in T. reesei Rut-C30 [13]. Chen et al. [13] suggested that external Ca}\textsuperscript{2+} stimulated hyphal growth, growth-independent cellulase production, and total protein secretion of T. reesei Rut-C30 through the Ca}\textsuperscript{2+}/(calmodulin)–calcineurin–CRZ1 signal transduction pathway.

Intracellular accumulation of Mn\textsuperscript{2+} can interfere with calcium metabolism [16]. In Ganoderma lucidum, one of the most well-known medicinal basidiomycetes producing many bioactive compounds such as ganoderic acids, Mn\textsuperscript{2+} is thought to enhance cytosolic Ca\textsuperscript{2+} to induce ganoderic acid biosynthesis through the calcineurin signal pathway, to upregulate its biosynthetic genes at the transcriptional level [17]. In Aspergillus nidulans, high levels of Mn\textsuperscript{2+} can induce an increase in intracellular Ca\textsuperscript{2+} levels, which leads to the nuclear accumulation of CrzA [18, 19]. These observations suggest that Ca\textsuperscript{2+} and Mn\textsuperscript{2+} have relevant impacts on the cellular physiology and metabolism of various organisms.

The ability to sense and respond to Mn\textsuperscript{2+} by the production of import and efflux systems to maintain Mn\textsuperscript{2+} homeostasis is critical for cells [20–23]. Such homeostasis factors include cell surface and intracellular Mn\textsuperscript{2+} transporters that collectively guide the metal through a designated trafficking pathway [20, 22]. PMR1, a P-type ATPase ion pump, is a transporter for both Ca\textsuperscript{2+} and Mn\textsuperscript{2+} and is also a homeostasis factor, associating with delivering both Mn\textsuperscript{2+} and Ca\textsuperscript{2+} to the secretory pathway [22, 24–26]. Cytosolic Mn\textsuperscript{2+} accumulates in yeast cells lacking the PMR1 transporter [22]. Although many studies have reported on Mn\textsuperscript{2+} homeostasis in various organisms, the detailed mechanisms are yet unclear. First, studies have focused mainly on yeast or bacterial species, and less work has been conducted with filamentous fungi. Second, the detailed roles of Mn\textsuperscript{2+} in the biological processes of filamentous fungi remain unclear and need further studies. Third, the conjunction between Mn\textsuperscript{2+} and calcium signaling in filamentous fungi is still not clear. Therefore, it is necessary to study the mechanism of Mn\textsuperscript{2+} stimulation in filamentous fungi.

In this study, the impact of Mn\textsuperscript{2+} on the growth and protein production of T. reesei Rut-C30 was investigated. The temporal dynamics of intracellular and extracellular Mn\textsuperscript{2+} were detected. Additionally, the function of Mn\textsuperscript{2+} transport proteins in T. reesei Rut-C30 was characterized. The conjunction between Mn\textsuperscript{2+} and Ca\textsuperscript{2+} was further investigated to elucidate how Mn\textsuperscript{2+} regulates the production of cellulase via calcium signaling in T. reesei Rut-C30. These results could be used for more efficient production of cellulase by T. reesei, and provide a new approach to understand the regulatory mechanisms that respond to environmental stimuli. This research may also offer the basis for the study of Mn\textsuperscript{2+}-induced signal transduction in other fungi.

**Results**

**Effects of the addition of Mn\textsuperscript{2+} on growth and cellulase production of T. reesei**

To determine how Mn\textsuperscript{2+} influences the hyphal growth, T. reesei Rut-C30 strains were cultured on MM (minimal medium) plates supplemented with different concentrations of Mn\textsuperscript{2+} (0, 1, 10, 20, and 40 mM final concentration) and 2% glucose as the sole carbon source. The mycelium length of T. reesei Rut-C30 after addition Mn\textsuperscript{2+} is shown in Fig. 1a. There was no significant difference in the hyphal growth with 1–10 mM Mn\textsuperscript{2+}. However, when the concentrations of Mn\textsuperscript{2+} increased to 20 mM, the strains grew more slowly and sparsely. As shown in Fig. 1b, treatment with 20 mM Mn\textsuperscript{2+} caused a 30% reduction in the colony diameter compared with that of the untreated strains. Moreover, after treatment with 40 mM Mn\textsuperscript{2+}, the treated strains showed a severe reduction in the colony diameter (53%).

To evaluate the effects of Mn\textsuperscript{2+} on cellulase production and total protein secretion, the same weight of precultured mycelia of T. reesei Rut-C30 was transferred to liquid MM containing 1% Avicel as the sole carbon source and different concentrations of Mn\textsuperscript{2+} (0, 1, 10, 20 and 40 mM). As shown in Fig. 1c (Additional file 8: Figure S7A), addition of Mn\textsuperscript{2+} at a final concentration of 10 mM significantly stimulated CMCase activity (representing endo-β-glucanase activity), with an increase of approximately 140% compared to the controls without addition of Mn\textsuperscript{2+}. However, 1, 20, and 40 mM of Mn\textsuperscript{2+} did not evidently affect CMCase activity. As shown in Fig. 1d (Additional file 8: Figure S7B) and e, the addition of Mn\textsuperscript{2+} at a final concentration of 10–40 mM significantly stimulated pNPCase activity (representing exo-β-glucanase activity) and increased total protein concentration after 1 day of fermentation, with an increase of approximately 327 and 55%, respectively.

The above results demonstrated that 10–40 mM Mn\textsuperscript{2+} could stimulate cellulase production and total protein secretion in T. reesei Rut-C30, and that 20–40 mM Mn\textsuperscript{2+} could delay hyphal growth. The optimal concentration of Mn\textsuperscript{2+} to enhance cellulase production was 10 mM, which was selected for further research in our study. CMCase or pNPCase activity was directly used to represent cellulase activity in our study.

To further determine the effects of Mn\textsuperscript{2+} supplementation on the synthesis of cellulases or total protein secretion, the expression levels of four main cellulase genes (cbh1 encoding cellobiohydrolase I, cbh2 encoding cellobiohydrolase II, egl1 encoding endoglucanase I, and egl2
encoding endoglucanase II), and a transcriptional regulator of cellulases, xyr1, were compared by quantitative reverse-transcription PCR (RT-qPCR) after induction for 24, 48, and 72 h in cultures with 0 or 10 mM Mn²⁺ supplementation. The primers used to detect transcriptional changes of these genes are listed in Additional file 1: Table S1. The transcriptional levels of four main cellulase genes significantly increased by almost 2- to 3.5-fold, after 24, 48, and 72 h of induction following supplementation with 10 mM Mn²⁺ (see Additional file 2: Figure S1A–D). In T. reesei, XYR1 is a global transcriptional activator of cellulose and hemicellulase genes [27]. In accordance with the transcription of cbh1, cbh2 and egl1, egl2, the expression level of xyr1 was also significantly stimulated after 72 h of induction (see Additional file 2: Figure S1E). These results were consistent with the upregulation of cellulase activity through the addition of 10 mM Mn²⁺. However, when compared to expression of other cellulase-related genes, the delayed upregulation of xyr1 expression implied that other putative regulators may participate in Mn²⁺ metabolism/regulation to directly induce cellulase gene expression, besides indirect induction through xyr1.

Variation in intracellular and extracellular Mn²⁺ concentration after the addition of Mn²⁺

Extracellular Mn²⁺ can significantly augment the intracellular Mn²⁺ content, and subsequently affect the physiology and metabolism of G. lucidum [17]. To investigate whether enhanced cellulase production is linked to intracellular Mn²⁺ in T. reesei Rut-C30, the intracellular and extracellular Mn²⁺ concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS) during cultivation. As illustrated in Fig. 2a, the levels of intracellular and extracellular Mn²⁺ were almost constant in the control sample without Mn²⁺ addition. On the contrary, upon Mn²⁺ addition (10 mM final concentration), the intracellular Mn²⁺ concentration initially markedly increased, reaching its maximum at 24 h and then declined gradually, while the extracellular Mn²⁺ concentration dropped initially, reaching its minimum at 24 h and then increased gradually, suggesting that Mn²⁺ was transported into cells initially in response to a higher extracellular Mn²⁺ concentration. Subsequently, intracellular Mn²⁺ gradually effused into the medium from 24 h. We hypothesized that a mechanism could pump Mn²⁺ in and out of the cell.
Jensen et al. [16] suggested that PHO84, a low-affinity transporter of Mn^{2+} from Saccharomyces cerevisiae, transports Mn^{2+} when cells are exposed to higher Mn^{2+} concentrations. In our study, Mn^{2+} efficiently transported into T. reesei Rut-C30 cells at an Mn^{2+} concentration of 10 mM (Fig. 2a). To identify the protein(s) responsible for transporting Mn^{2+} in T. reesei, we conducted a homology search with the protein sequence of PHO84 (GenBank: KZV08715.1) in the T. reesei genome from the JGI database (http://genome.jgi.doe.gov/Trire2/Trire2.home.html). Five high score hits were obtained with the following protein identity matches: TRE77552, 59.6%, TRE81389, 51.8%, TRE45852, 49.0%, TRE45868, 40.1%, and TRE106118, 35.2%.

We hypothesized that Mn^{2+} transport proteins would be highly expressed during Mn^{2+} addition. To assess which proteins encoded putative Mn^{2+} transport activity, transcriptional levels of tre77552, tre81389, tre45852, tre45868 and tre106118 were monitored by RT-qPCR with 10 mM Mn^{2+} compared to no Mn^{2+} addition at different induction times. As illustrated in Additional file 3: Figure S2, expression of these genes was significantly upregulated in the samples treated with 10 mM Mn^{2+}.

Based on their induction at higher Mn^{2+} concentration, all five genes might be responsible for Mn^{2+} transport of T. reesei Rut-C30.

To further investigate whether any of these five proteins displayed a function similar to that of PHO84, involved in transport of Mn^{2+} in S. cerevisiae, the five deletion mutants, Δ77552, Δ81389, Δ45852, Δ45868, and Δ106118, respectively, were collected to measure intracellular Mn^{2+} concentrations by ICP-MS compared to that in the parental strain. As shown in Fig. 2b, Δ77552 and Δ45868 showed a marked decrease in steady-state levels of cellular Mn^{2+} when compared with the Rut-C30 strain, in which a 70–80% decrease in metal accumulation was obtained. However, under the same conditions, Δ81389, Δ45852, or Δ106118 had an Mn^{2+} concentration similar to that in Rut-C30 strain (see Additional file 4: Figure S3). The tre77552 and tre45868 double mutant strain Δptho84-1/2 showed lower intracellular Mn^{2+} concentration than each single mutant (Fig. 2b). Δ77552 and Δ45868 were also complemented by transforming vectors ptpho84-1-rc and ptpho84-2-rc into them, respectively (see Additional file 5: Figure S4). Complementation strains (ptpho84-1-rc and ptpho84-2-rc) were obtained to demonstrate the restoration of Mn^{2+} transport with intracellular Mn^{2+} concentrations similar to...
those of the parent strain Rut-C30 (Fig. 2b). The results demonstrated that the intracellular Mn$^{2+}$ concentration can be increased via putative Mn$^{2+}$ transport proteins TRE77552 and TRE45868 in T. reesei Rut-C30.

As predicted by SMART (http://smart.embl-heidelberg.de/), both TRE77552 and TRE45868 are membrane proteins with an 11- and 9-transmembrane domain topology, respectively. To visualize the location of TRE77552 and TRE45868, we constructed two chimeric proteins, RFP-TRE77552 and RFP-TRE45868, by fusing red fluorescence protein to their N-terminus (see Additional file 6: Figure S5A). The two chimeric proteins were overexpressed via cbh1 promoter, which allowed us to confirm that TRE77552 and TRE45868 are located at the mycelial surface. Additional file 6: Figure S5B shows the in vivo epifluorescence analysis of the two chimeric protein (RFP-TRE77552 and RFP-TRE45868) transformants, rfp-tpho84-1 and rfp-tpho84-2, respectively, depicting strong and stable fluorescent signal at the mycelial surface, as expected for plasma membrane proteins. However, overexpression of genes encoding membrane bound proteins by a strong promoter might cause some uncertainty.

Based on these findings, we considered that both tre77552 and tre45868 encode Mn$^{2+}$ transports with Mn$^{2+}$ transport function located at the plasma membrane, and named the two genes as tpho84-1 and tpho84-2, respectively. Intracellular Mn$^{2+}$ can be transported into T. reesei Rut-C30 cells via TPHO84-1 and TPHO84-2. It is presently unclear whether tre81389, tre45852, and tre106118 are inactive in the transport of Mn$^{2+}$.

Role of TPHO84-1 and TPHO84-2 in cellulase production

Δ77552 (Δtpho84-1) and Δ45868 (Δtpho84-2), and their complementation strains tpho84-1-rc and tpho84-2-rc were used to determine the effect of TPHO84-1 and TPHO84-2 in mediating the growth and cellulase production in T. reesei Rut-C30. To determine the effect of TPHO84-1 and TPHO84-2 on mediating growth, Rut-C30, Δtpho84-1, Δtpho84-2, tpho84-1-rc, tpho84-2-rc and Δtpho84-1/2 strains were cultured on minimal medium plates adding 0 or 10 mM Mn$^{2+}$ and 2% glucose as the sole carbon source. The mycelium morphology is shown in Fig. 3a. There was no significant effect on the hyphal growth of Rut-C30, Δtpho84-1, Δtpho84-2, tpho84-1-rc and tpho84-2-rc strains at 0 or 10 mM Mn$^{2+}$. However, the hyphal growth of the Δtpho84-1/2 transformant was slightly repressed, compared with that of the parent strain at both 0 and 10 mM Mn$^{2+}$ (Fig. 3b).

To further test the effect of Mn$^{2+}$ transport proteins on cellulase production, we investigated the effect of Rut-C30, Δtpho84-1, Δtpho84-2, tpho84-1-rc, tpho84-2-rc, and Δtpho84-1/2 strains on cellulase production after supplementation of 0 or 10 mM Mn$^{2+}$. There was no obvious difference in CMCase (see Additional file 7: Figure S6A) and pNPCase (see Additional file 7: Figure S6B) activities between these six strains without Mn$^{2+}$ addition. However, Δtpho84-1 and Δtpho84-2 strains showed approximately 30–40% reduction in CMCase activity, compared with the parental strain Rut-C30 with 10 mM Mn$^{2+}$ addition. Furthermore, Δtpho84-1/2 strain had lower CMCase activity than each single mutant, at approximately 50% reduction compared with Rut-C30 (Fig. 3c and Additional file 8: Figure S8A). Similarly, Δtpho84-1 and Δtpho84-2 strains showed approximately 25 to 30% reduction in the pNPCase activity, compared with the parental strain Rut-C30, with 10 mM Mn$^{2+}$ addition. Furthermore, the Δtpho84-1/2 strain showed lower pNPCase activity than each single mutant, and approximately 40% reduction compared with Rut-C30 (Fig. 3d and Additional file 8: Figure S8B). The cellulase production capabilities of tpho84-1-rc and tpho84-2-rc were complemented at 10 mM Mn$^{2+}$ supplementation compared to parental strains Δtpho84-1 and Δtpho84-2, respectively, to a level similar to that of the original strain Rut-C30 (Fig. 3c, d and Additional file 8: Figure S8A, B).

Additionally, RT-qPCR was performed to determine the transcription levels of the important cellulase genes cbh1 and egl1 in the T. reesei Rut-C30, Δtpho84-1, Δtpho84-2, tpho84-1-rc, tpho84-2-rc, and Δtpho84-1/2 mutants. The primers used to detect transcriptional levels of these genes are listed in Additional file 1: Table S1. In agreement with the CMCase activity levels, no obvious difference in transcription levels of cbh1 and egl1 between above six strains was observed without Mn$^{2+}$ addition (data not shown). However, the deletion strains Δtpho84-1, Δtpho84-2, and Δtpho84-1/2 showed a marked reduction in cellulase transcription compared with the parental strain Rut-C30 upon 10 mM Mn$^{2+}$ addition at all time points examined (Fig. 3e, f). Similarly, the expression levels of the cbh1 and egl1 genes of tpho84-1-rc and tpho84-2-rc were complemented under Mn$^{2+}$ supplementation, to a level similar to that of the original strain Rut-C30.

These results indicate that TPHO84-1 and TPHO84-2 participate in inducing cellulase production of T. reesei Rut-C30 only under Mn$^{2+}$ addition, which is in accordance with its function as Mn$^{2+}$ transport protein.

Increase in cytosolic Ca$^{2+}$ level and calcium signaling after Mn$^{2+}$ addition

Next, we investigated how Mn$^{2+}$ addition can upregulate cellulase gene expression via Mn$^{2+}$ transport proteins TPHO84-1 and TPHO84-2. Using Fluo-3/AM fluorescent dye, a dye that only emits green fluorescence after crossing the cell membrane and binding with Ca$^{2+}$ [28], whose intensity represents relative amounts of free
Fig. 3 TPHO84-1 and TPHO84-2 regulate cellulase production of *T. reesei* Rut-C30 with Mn$^{2+}$ supplementation. **a** The hyphal growth of *T. reesei* Rut-C30 and its derivative mutant strains under 0 or 10 mM MnCl$_2$. **b** The measurement of colony diameter. The CMCase activity (c) and pNPCase activity (d) of *T. reesei* Rut-C30 and its derivative mutant strains were examined after culture in medium containing 0 or 10 mM MnCl$_2$. The transcriptional levels of *cbh1* (e) and *egl1* (f) in the parental strain Rut-C30 and its derivative mutant strains were analyzed after cultured in medium containing 0 or 10 mM MnCl$_2$. Values are the mean ± SD of the results from three independent experiments. Asterisks indicate significant differences (*p* < 0.05, Student’s *t* test).
intracellular Ca\textsuperscript{2+} [29], we found that the cytosolic Ca\textsuperscript{2+} concentration was increased after Mn\textsuperscript{2+} addition. As shown in Fig. 4A, a stronger green fluorescence intensity was observed in the Rut-C30 cells under 10 mM Mn\textsuperscript{2+} supplement on the second day than that observed with the control (no Mn\textsuperscript{2+} supplement), demonstrating that Mn\textsuperscript{2+} leads to an increase in the level of cytosolic Ca\textsuperscript{2+}. The fluorescence level emitted by the Ca\textsuperscript{2+}-activated fluorochrome reached a 2.23-fold increase under 10 mM Mn\textsuperscript{2+} addition compared to that in the control (Fig. 4B). These results indicate that Mn\textsuperscript{2+} induced an increase in the concentration level of cytosolic Ca\textsuperscript{2+}. A similar phenomenon was reported in S. cerevisiae [30].

We measured the level of cytosolic Ca\textsuperscript{2+} in the \textit{\textgreek{d}tpho84-1}, \textit{\textdaggerpho84-2}, and \textit{\textdaggerpho84-1/2} strains with Mn\textsuperscript{2+} addition. As illustrated in Fig. 4A, B, the significant increase in intracellular Ca\textsuperscript{2+} induced by Mn\textsuperscript{2+}, observed in parental strain Rut-C30, was absent in the \textit{\textdaggerpho84-1}, \textit{\textdaggerpho84-2}, and \textit{\textdaggerpho84-1/2} strains. The augmentation of cytosolic Ca\textsuperscript{2+} levels induced by Mn\textsuperscript{2+} was blocked in the \textit{\textdaggerpho84-1}, \textit{\textdaggerpho84-2}, and \textit{\textdaggerpho84-1/2} strains. These results suggest that rising cytosolic Ca\textsuperscript{2+} levels depend on Mn\textsuperscript{2+} transport into Rut-C30 cells via TPHO84-1 and TPHO84-2. This also indicates that Ca\textsuperscript{2+} influx in cells is closely associated with Mn\textsuperscript{2+} homeostasis.

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**Fig. 4** Cytosolic Ca\textsuperscript{2+} levels and calcium signaling increase after Mn\textsuperscript{2+} addition. A The analysis of cytosolic Ca\textsuperscript{2+} levels via a Ca\textsuperscript{2+} fluorescent probe Fluo-3/AM. The \textit{T. reesei} Rut-C30 and its derivative mutant strains were cultured in liquid minimal medium for 48–60 h with 0 or 10 mM final concentration of MnCl\textsubscript{2} (0 or 10 Mn, respectively). For detection, 50 \textmu M Fluo-3/AM was used, and the intensity was monitored using Automatic Inverted Fluorescence Microscopy. Green fluorescence represents the free cytosolic Ca\textsuperscript{2+}. DIC, differential interference contrast. B Comparative fluorescence ratio analysis of Mn\textsuperscript{2+} influence on cytosolic Ca\textsuperscript{2+} levels. The y-axis represents the Ca\textsuperscript{2+} fluorescence ratio measured by CLSM and the x-axis the different strains tested. The effects of Mn\textsuperscript{2+} on the expression levels of certain calcium signaling-related genes \textit{cam} (C), \textit{cna1} (D) and \textit{crz1} (E) in \textit{T. reesei} Rut-C30. 0 Mn, no MnCl\textsubscript{2} was added to the medium; 10 Mn, final concentration of 10 mM MnCl\textsubscript{2}. Values are the mean ± SD of the results from three independent experiments. Asterisks indicate significant differences from untreated strains (*p < 0.05, **p < 0.01, Student’s t test). Different letters indicate significant differences between the columns (p < 0.05, according to Duncan’s multiple-range test).
Previous studies have demonstrated that the calcium signal transduction pathway can upregulate cellulase gene expression [13]. We investigated whether the increased levels of cytosolic Ca\(^{2+}\), induced by Mn\(^{2+}\), can trigger calcium signal transduction pathways in *T. reesei*. To test our hypothesis, RT-qPCR was carried out to analyze the transcriptional levels of calcium signaling-related genes, including calmodulin (*cam*, GenBank: ACZ26150.1), calcineurin (*cna1*, GenBank: EGR49476.1) [31], and calcineurin-responsive zinc finger transcription factor 1, *crz1* [13], under Mn\(^{2+}\) addition. The primers used to detect transcription of these genes are listed in Additional file 1: Table S1. As shown in Fig. 4C–E, similar to the increase in the contents of cytosolic Ca\(^{2+}\), expression of these genes was significantly upregulated with Mn\(^{2+}\) addition. These results suggested that Mn\(^{2+}\) can increase the concentration of cytosolic Ca\(^{2+}\), thus stimulating the calcium signal transduction pathway to induce cellulase production in *T. reesei* Rut-C30.

**Mn\(^{2+}\) induces cellulase production via cytosolic Ca\(^{2+}\)**

To investigate the assumption that Mn\(^{2+}\) induces cytosolic Ca\(^{2+}\) improvement and cellulase production via Ca\(^{2+}\) channels, we used LaCl\(_3\), a plasma membrane Ca\(^{2+}\) channel blocker to prevent influx of external Ca\(^{2+}\) [32]. Figure 5A, B shows that the Fluo-3/AM fluorescence intensity of mycelia remarkably reduced almost 60% with LaCl\(_3\) compared with no LaCl\(_3\) addition, under 10 mM Mn\(^{2+}\). The increased content of cytosolic Ca\(^{2+}\) induced by Mn\(^{2+}\) could be effectively attenuated by adding LaCl\(_3\) to *T. reesei* Rut-C30. Meanwhile, the increased expression of calcium signaling-related genes, *cam*, *cna1*, and *crz1*, which are induced by 10 mM Mn\(^{2+}\), was also effectively prevented by adding LaCl\(_3\) (data not shown).

Ca\(^{2+}\) participates in regulating cellulase production in *T. reesei* [13]. To investigate whether a cellulase increase, induced by Mn\(^{2+}\), was blocked by LaCl\(_3\), we analyzed CMCase and pNPCase activities and transcription of key cellulase genes *cbh1* and *egll*. As shown in Fig. 5C, D (Additional file 8: Figure S9A, B), under Mn\(^{2+}\) supplementation and with LaCl\(_3\), the CMCase and pNPCase activities in *T. reesei* Rut-C30 decreased by 50% and 49%, respectively, compared with no LaCl\(_3\) addition. Meanwhile, the expression levels of *cbh1* and *egll* were reduced by 60% at 72 h (Fig. 5E, F). However, there was no obvious change to CMCase activities and transcriptional levels of *cbh1* and *egll* with or without LaCl\(_3\) when Mn\(^{2+}\) was not added (Fig. 5C–F, Additional file 8: Figure S9A, B). These results showed that LaCl\(_3\) could significantly decrease the Mn\(^{2+}\)-induced high expression levels of key cellulase genes in *T. reesei* Rut-C30.

These data indicated that Mn\(^{2+}\) induced cytosolic Ca\(^{2+}\) increase via the Ca\(^{2+}\) channel. When a Ca\(^{2+}\) channel blocker LaCl\(_3\) was added, the increase in cytosolic Ca\(^{2+}\) concentration and cellulase production induced by Mn\(^{2+}\) were effectively attenuated.

To investigate whether a cellulase increase, induced by Mn\(^{2+}\), is associated with calcium signal transduction, we constructed a crz1 deletion mutant Δcrz1 as Chen et al. [13] to block the calcium signal transduction pathway. As shown in Fig. 6a, b (Additional file 8: Figure S10A, B), the remarkable increase of CMCase and pNPCase activities induced by Mn\(^{2+}\), observed in parental strain Rut-C30, was effectively attenuated by deleting *crz1*. Similarly, the transcriptional levels of *cbh1* and *egll* were markedly reduced in the Δcrz1 mutant at all time points examined (Fig. 6c, d).

Taken together, these data indicated that Mn\(^{2+}\) induces cellulase improvement via calcium signal transduction in *T. reesei* Rut-C30. The improvement of cellulase production induced by Mn\(^{2+}\) is effectively prevented in a *crz1* mutant, blocking the calcium signal transduction pathway.

**TPMR1 functions in conjunction with channels of Mn\(^{2+}\) and Ca\(^{2+}\)**

Mn\(^{2+}\)/Ca\(^{2+}\) homeostasis exists in other fungi [33–36]. PMR1 plays an important role in Mn\(^{2+}\)/Ca\(^{2+}\) homeostasis, and may act as a Ca\(^{2+}\)/Mn\(^{2+}\) exchanger to balance Mn\(^{2+}\) via Ca\(^{2+}\) [17]. To clarify how Mn\(^{2+}\) increased cytosolic Ca\(^{2+}\) concentration, using *Neurospora crassa* PMR1 (GenBank: CAB65296.1) as the query, we searched for a PMR1 homolog (TRE119592) in the *T. reesei* genome, named as TPMR1.
We constructed a tpmr1 deletion mutant Δtpmr1. To investigate whether TPMR1 is responsible for pumping in Ca²⁺ upon 10 mM Mn²⁺ addition in T. reesei, we measured the level of intracellular Ca²⁺ in the Δtpmr1 strain with or without Mn²⁺ addition. As shown in Fig. 7A and B, the increase in intracellular Ca²⁺ induced by Mn²⁺ in Rut-C30 was absent in the Δtpmr1 strain. The augmentation of Ca²⁺ levels induced by Mn²⁺ was blocked in

(See figure on next page.)

Fig. 6 Influence of CRZ1 on Mn²⁺-induced cellulase production. The CMCase activity (a) and pNPCase activity (b) of T. reesei Rut-C30 and Δcrz1 strains supplemented with 0 or 10 mM MnCl₂. The expression levels of cbh1 (c) and egl1 (d) in T. reesei Rut-C30 and Δcrz1 strains supplemented with 0 or 10 mM MnCl₂. Values are the mean ± SD of the results from three independent experiments. Asterisks indicate significant differences (*p < 0.05, Student’s t test)

Fig. 7 Influence of TPMR1 on Mn²⁺-induced cytosolic Ca²⁺ burst and cellulase production. A Fluorescence analysis of the influence of TPMR1 on the cytosolic Ca²⁺ burst induced by Mn²⁺. The T. reesei Rut-C30 and Δtpmr1 strains were cultured in liquid minimal medium for 48–60 h with 0 or 10 mM MnCl₂ (0 or 10 Mn, respectively). For detection, 50 µM Fluo-3/AM was used, and the intensity was monitored using Automatic Inverted Fluorescence Microscopy. Green fluorescence represents the free cytosolic Ca²⁺. DIC, differential interference contrast. B Comparative fluorescence ratio analysis of TPMR1 influence on cytosolic Ca²⁺ burst induced by Mn²⁺. The y-axis represents the Ca²⁺ fluorescence ratio measured by CLSM and the x-axis the different treatments. The CMCase activity (C) and pNPCase activity (D) of T. reesei Rut-C30 and Δtpmr1 strains were examined after culture in medium containing 0 or 10 mM MnCl₂. The expression levels of cbh1 (E) and egl1 (F) in T. reesei Rut-C30 and Δtpmr1 strains were analyzed after culture in medium containing 0 or 10 mM MnCl₂. Values are the mean ± SD of the results from three independent experiments. Asterisks indicate significant differences from untreated strains (*p < 0.05, **p < 0.01, Student’s t test). Different letters indicate significant differences between the columns (p < 0.05, according to Duncan’s multiple-range test)
the Δtpmr1 strain. These results suggested that TPMR1 is responsible for the increase of cytosolic Ca\(^{2+}\) under Mn\(^{2+}\) addition in T. reesei Rut-C30.

To further investigate the function of TPMR1 in conjunction with Mn\(^{2+}\) and Ca\(^{2+}\) channels, the intracellular and extracellular Mn\(^{2+}\) concentrations in the Δtpmr1 strain with or without Mn\(^{2+}\) addition were measured by ICP-MS during cultivation. As illustrated in Additional file 9: Figure S12, upon Mn\(^{2+}\) addition (10 mM final concentration), the intracellular Mn\(^{2+}\) concentration initially markedly increased, reaching and maintaining a high level after 24 h. The extracellular Mn\(^{2+}\) concentration dropped initially, reaching its minimum from 24 to 72 h. The intracellular and extracellular Mn\(^{2+}\) concentrations of the Δtpmr1 strain (Additional file 9: Figure S12) are quite different from that of Rut-C30 (Fig. 2a), whose intracellular Mn\(^{2+}\) gradually effused into the medium. These results suggested that TPMR1 is responsible for pumping Mn\(^{2+}\) out of the cell.

To further test the effect of TPMR1 on cellulase production under Mn\(^{2+}\) addition, we compared the cellulase production in parental strain Rut-C30 and Δtpmr1 mutant. Upon the addition of Mn\(^{2+}\), the Δtpmr1 strain caused about 26 and 51% reductions in the CMCase and pNPCase activities, respectively, compared with that of Rut-C30 (Fig. 7C, D and Additional file 8: Figure S11A, B). Moreover, the transcriptional levels of cbh1 and egl1 observed are in agreement with the CMCase and pNPCase activity levels, which were markedly reduced in the Δtpmr1 mutant at all time points examined (Fig. 7E, F). The increase in cellulase production induced by Mn\(^{2+}\) was remarkably weakened in the Δtpmr1 deletion mutant. These results demonstrated that TPMR1 functions in conjunction with channels of Mn\(^{2+}\) and Ca\(^{2+}\) in T. reesei Rut-C30.

**Discussion**

Metal ions are important in regulating cellular metabolism. For example, Ca\(^{2+}\) plays an important role in the regulation of cellulase or hemicellulase in T. reesei [13–15]. Paraszkiewicz et al. [37] reported Cd\(^{2+}\), Zn\(^{2+}\), and Pb\(^{2+}\) as environmental stress factors, which increased the biosynthesis of fungal emulsifier in *Curvularia lunata*. Addition of Ca\(^{2+}\), Na\(^{+}\), and Mn\(^{2+}\) enhanced ganoderic acid production in *Ganoderma lucidum* liquid cultures, through induction of the calcineurin signal pathway [16, 22, 43, 44]. PHO84, a family of phosphate/proton symporters [45], can transport Mn\(^{2+}\) under excess Mn\(^{2+}\) stress [16, 25], indicating that PHO84 is a low-affinity transporter of Mn\(^{2+}\), contributing to Mn\(^{2+}\) accumulation only when cells are exposed to higher Mn\(^{2+}\) concentrations. *T. reesei* might contain both high- and low-affinity transporters of Mn\(^{2+}\), similar to what has been described for the metal transport systems for iron, copper, and zinc [46–50]. A BLAST search of the genome sequence of *T. reesei* identified a gene (*tre79644*) similar to SMF1, and this gene was named *tsmf1*. There was no significant difference on Mn\(^{2+}\) transport and cellulase production under 10 mM Mn\(^{2+}\) surplus using a *tsmf1* deletion strain (data not shown). The alternative PHO84 homologs identified as PHO84-1 and PHO84-2 are similar to PHO84. We proved that extracellular Mn\(^{2+}\) is transported into Rut-C30 cells via PHO84-1 and PHO84-2, which are annotated as putative low-affinity transports of Mn\(^{2+}\) responding to higher Mn\(^{2+}\) concentration. Meanwhile, there were no significant differences between the cytosolic Ca\(^{2+}\) concentrations for the *T. reesei* Rut-C30, Δtpho84-1, Δtpho84-2, and Δtpho84-1/2 mutants (see Additional file 10: Figure S13).

Calcium is widely used as a second messenger in prokaryotic and eukaryotic cells. It is known that a cytosolic Ca\(^{2+}\) burst and further induced calcium signaling regulate cellular responses when exposed to different external stimuli [51, 52]. For instance, Na\(^{+}\) induction enhances cytosolic Ca\(^{2+}\) to induce the ganoderic acid biosynthesis through calcineurin signal pathway, to upregulate its biosynthetic genes at the transcriptional level in *Ganoderma lucidum* [29]. High temperatures are known to trigger the generation of cytosolic Ca\(^{2+}\) in plants [53]. Alkaline pH triggers an immediate calcium burst in *Candida albicans* [54]. Our results indicated that Mn\(^{2+}\) induces a significantly increased cytosolic Ca\(^{2+}\) level in *T. reesei* Rut-C30 (Fig. 4A, B). Additionally, in our experiments, inhibition of cytosolic Ca\(^{2+}\) level by LaCl\(_3\) effectively attenuated the cellulase increase induced by Mn\(^{2+}\) (Fig. 5C–E). The results showed that 10 mM Mn\(^{2+}\) stress led to a cytosolic Ca\(^{2+}\) burst in *T. reesei* Rut-C30. An earlier study demonstrated that Ca\(^{2+}\) (/calmodulin)–calcineurin–CRZ1 signaling could induce cellulase production at the transcriptional level by Ca\(^{2+}\)
In our study, Mn$^{2+}$ could also activate the expression of Ca$^{2+}$ (calmodulin)–calcineurin–CRZ1 signaling-related genes in T. reesei Rut-C30 (Fig. 4C–E). Furthermore, CRZ1 participated in regulating cellulase production in Mn$^{2+}$-induced strains (Fig. 6a–d). The results presented here indicate that the regulation of cellulase gene expression and production by Mn$^{2+}$ are dependent on cytosolic Ca$^{2+}$ burst and further induce calcium signaling.

PMR1, encoding the Ca$^{2+}$/Mn$^{2+}$ P-type ATPase, is required to either scavenge trace amounts of Mn$^{2+}$ and Ca$^{2+}$ from the medium or maintain sufficient levels of Mn$^{2+}$ and Ca$^{2+}$ in an intracellular compartment [22, 24–26, 33]. Bowman et al. [33] reported that a pmr1 deletion strain accumulates 80% lesser Ca$^{2+}$ than the wild type. Under higher levels of intracellular Mn$^{2+}$, extracellular Ca$^{2+}$ might transport into the cells through PMR1 to increase intracellular Ca$^{2+}$ and then trigger calcium signaling to regulate cellular responses [35]. Our work found that the increase in Ca$^{2+}$ levels induced by Mn$^{2+}$ treatment was blocked in the tpmr1 deletion strain (Fig. 7A, B) and that the improvement of cellulase production induced by Mn$^{2+}$ was remarkably weakened in the tpmr1 deletion mutant. These results demonstrated that TPMR1 functions as a channel of Mn$^{2+}$ and Ca$^{2+}$ in T. reesei Rut-C30. Cytosolic Ca$^{2+}$ concentration was enhanced through TPMR1 under Mn$^{2+}$ addition, thus inducing calcium signaling to upregulate cellulase genes. Additionally, PMR1 provides a major route for cellular sequestration of Mn$^{2+}$ by pumping excess Mn$^{2+}$ into the Golgi, from where the metal may exit the cell via the secretory pathway vesicles that merge with the cell surface and release the Mn$^{2+}$ contents back into the extracellular environment [20, 22, 24, 34]. These data correlate well with our data indicating that intracellular Mn$^{2+}$ gradually effused to the medium from 24 h (Fig. 2a). However, in the Δtpmr1 mutant, the intracellular Mn$^{2+}$ concentration maintained its high level after 24 h, and the extracellular Mn$^{2+}$ concentration reached its minimum from 24 to 72 h. These results implied that TPMR1

![Fig. 8](image.png)
participates in pumping excess Mn\(^{2+}\) into the Golgi, and then releases it extracellularly, meanwhile accumulating cytosolic Ca\(^{2+}\) in T. reesei Rut-C30. However, the detailed role of TPMR1 in T. reesei needs further research.

We found that 10 mM Mn\(^{2+}\) could also stimulate cellulase production and increase total protein secretion from the T. reesei wild-type strain QM6a and the mutant strain Qm9414 (data not shown). However, the results from Rut-C30 may not be identical to those of the wild-type strain, because each strain likely has its own unique regulatory mechanism.

**Conclusions**

In summary, the putative mechanism of the extracellular Mn\(^{2+}\)-induced stimulation of cellulase production was characterized in T. reesei Rut-C30 (Fig. 8). Mn\(^{2+}\) induces a significantly increased cytosolic Ca\(^{2+}\) level and triggers Ca\(^{2+}\)-CRZ1 signaling to induce cellulase production at the transcription level. Moreover, we identified two Mn\(^{2+}\) transport proteins in T. reesei Rut-C30, and named TPHO84-1 and TPHO84-2. Furthermore, TPMR1 acts as a link between channels in Mn\(^{2+}\) and Ca\(^{2+}\) homeostasis in T. reesei. This study provides a successful approach to produce a higher yield of cellulase and to develop industrially applicable T. reesei strains, which is important for biofuel production from lignocellulosics. This study also provides a molecular basis for understanding the regulatory mechanism of divalent metal ions on the cellular metabolism of fungi.

**Methods**

**Strains and growth conditions**

*Escherichia coli* DH5α was used for plasmid amplification. *Agrobacterium tumefaciens* strain AGL-1 was used as a T-DNA donor for fungal transformation [55]. *T. reesei* Rut-C30 (ATCC 56765) was used throughout the study and as the host for genetic transformation. Luria–Broth (LB) was used for culture of *E. coli* and *A. tumefaciens*. Mandels’ medium [56] was used for the general fungal culture. All strains were maintained on potato dextrose agar (PDA) plates at 28 °C. The fungal strains constructed in this study are summarized in Table 1. All strains were cultured in the dark.

| Strains | Relevant features | Source |
|---------|------------------|--------|
| Rut-C30 | Parent strain   | ATCC   |
| Δtpho84-1(Δ77552) | Rut-C30-deleted tpho84-1 gene | This study |
| Δtpho84-2(Δ45868) | Rut-C30-deleted tpho84-2 gene | This study |
| Δtpho84-1/2 | Rut-C30-deleted tpho84-1 and tpho84-2 gene | This study |
| Δ81389 | Rut-C30-deleted tre81389 gene | This study |
| Δ45852 | Rut-C30-deleted tre45852 gene | This study |
| Δ106118 | Rut-C30-deleted tre106118 gene | This study |
| rfp(tpho84-1 | Rut-C30-harboring vector prfp-tpho84-1 | This study |
| rfp(tpho84-2 | Rut-C30-harboring vector prfp-tpho84-2 | This study |
| tpho84-1-rc | Rut-C30-harboring vector ptpho84-1-rc | This study |
| tpho84-2-rc | Rut-C30-harboring vector ptpho84-2-rc | This study |
| Δcrz1 | Rut-C30-deleted crz1 gene | This study |
| Δtpmr1 | Rut-C30-deleted tpmr1 gene | This study |

**Fungal growth, enzymatic activity, protein concentration, and biomass assays**

For fungal hyphal growth assays, conidia were collected and diluted to 10\(^7\) ml\(^{-1}\) in sterile water. An equal volume of the solution (2 μl) was inoculated onto the center of the MM plates as described above, and was grown for 3–5 days at 28 °C.

For enzymatic activity, protein concentration, and biomass assays, 1 ml of culture liquid was collected and subjected to 0.45-μm filtration. The culture supernatants were subjected to cellulase activity and protein concentration analysis. The mycelia were subjected to biomass measurement. Fungal CMCase and pNPCase activities were measured according to the method described by Wang et al. [57]. Protein concentrations were determined using the Bradford Protein Assay Kit (Generay, Shanghai, China). Biomass concentration was indirectly measured by calculating the amount of total intracellular proteins, with some modification [58]. Briefly, harvested mycelia using 0.85% NaCl, then transferred to 100 ml MM containing 1% (w/v) Avicel (PH-101, Sigma-Aldrich) with the addition of MnCl\(_2\) to final concentrations of 1, 10, 20, and 40 mM. Strains were induced for 1–5 days before being subjected to testing for enzymatic activity, protein concentration, or induced for 24, 48, or 72 h before being subjected to RNA extraction and RT-qPCR analyses, respectively. To assess the effect of plasma membrane Ca\(^{2+}\) channels on the regulation of cellulase production of T. reesei Rut-C30, 5 mM (final concentration) LaCl\(_3\) (Aladdin, Shanghai, China) was added after 1 day of culture in MM.
were suspended in 1 ml 1 M NaOH in a reaction tube and the mixture was incubated for 2 h and frequently vortexed. Total protein was collected via centrifugation at 14,000×g at 4 °C for 10 min. Total protein concentration was determined by the Modified Lowry Protein Assay Kit (Sangon Biotech, Shanghai, China). The final protein content was furthermore corrected using a set of substrate controls where no inoculum was added to the medium. The biomass dry weight was then calculated assuming an average content of 0.32 g intracellular protein per gram of dry cell mass.

**RNA isolation and quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR)**

The levels of gene-specific mRNA were assessed using RT-qPCR, according to our previous study, with some modification [59]. In brief, the total RNA of 50 mg fresh weight cells was extracted using a FastRNA Pro Red Kit (MPbio, Irvine, CA, USA), according to the manufacturer’s instructions. Synthesis of cDNA from total RNA was performed using the PrimeScript RT Reagent Kit with gDNA eraser (TaKaRa, Japan) as per the manufacturer’s instructions. For RT-qPCR, the TransStart TipTop Green qPCR SuperMix (TransGen, Shanghai, China) was used with 200 nM of forward and reverse primers (see Additional file 1: Table S1). Gene transcription was analyzed using SYBR green assays. Transcription levels of target genes were normalized to that of the sar1 gene of target genes were normalized to that of the

**Determination of extracellular and intracellular Mn2+ concentration**

Extracellular and intracellular Mn2+ concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS), as described for *Ganoderma lucidum* by Xu [17]. Five milliliter of culture liquid was collected and subjected to filtration. The culture supernatant and mycelium were subjected to extracellular and intracellular Mn2+ concentrations, respectively. The supernatant of the cultures was filtered through 0.22-μm membranes and then diluted with 1% HNO3 for measuring the extracellular Mn2+ concentration. Mycelia were washed in distilled water to remove any nonspecifically bound Mn2+, and digested with 1 ml 68% HNO3. The mixture was collected via centrifugation at 12,000×g for 5 min. The supernatant was then filtered through a 0.22-μm membrane and diluted with 1% HNO3 for measuring intracellular Mn2+ concentration. The final intracellular Mn2+ concentration was shown as micromole per gram biomass.

**Construction of plasmids and strains**

To construct a tpho84-1 deletion mutant, the upstream (−1 to −762 bp) and downstream (+1917 to +2681 bp) fragments of *tpho84-1* were generated from the genome of *T. reesei* Rut-C30 using KOD-Plus-Neo (TOYOBIO, Japan). All primers are indicated in Additional file 1: Table S1. First, the upstream fragment was ligated into the *PacI* - and *XbaI*-linearized LML2.0 [61] using the ClonExpressTM II One Step Cloning Kit (Vazyme, Nanjing, China) to form pFtpho84-1. Subsequently, the downstream fragment was inserted into Swal-linearized pFtpho84-1 to form the binary vector pDtpho84-1 (see Additional file 5: Figure S4A) for the knockout of *tpho84-1* in Rut-C30 using *Agrobacterium*-mediated transformation [59]. Strains were selected using hygromycin B and cefotaxime on Mandels’ medium. Then, the marker was excised using the method described by Zhang [61]. The putative *tpho84-1* disruption mutants (∆*tpho84-1*/*Δ77552) generated by double crossover were verified by diagnostic PCR using the primers *tpho84-1*-CF and *tpho84-1*-OR and *tpho84-1*-CR and *tpho84-1*-OF and *tpho84-1*-OR (see Additional file 5: Figure S4E).

Similarly, the 706-bp upstream and 710-bp downstream regions of *tpho84-2* were amplified and then inserted into LML2.0 as described above to generate the binary vector pDtpho84-2 (see Additional file 5: Figure S4B). After transformation and selection, the putative *tpho84-2* deletion mutant (∆*tpho84-2*/*Δ45868) was verified by diagnostic PCR using the primers *tpho84-2*-CF and *tpho84-2*-CR, and *tpho84-2*-OF and *tpho84-2*-OR (see Additional file 5: Figure S4E). The tre81389, tre45852, tre106118, crz1, and *tpmr1* disruption mutants (∆*tpho84-1*, ∆*tpho84-2*, ∆*tpho84-3*, ∆*tpho84-4*, and ∆*tpho84-5*) were constructed similarly (see Additional file 11: Figure S14).

To construct a *tpho84-1*-1/2 deletion mutant, the pDtpho84-2 cassette was transformed into ∆*tpho84-1* strain using *Agrobacterium*-mediated transformation. The *tpho84-1*-1/2 double deletion mutant (∆*tpho84-1*/2) was verified by diagnostic PCR.

For *tpho84-1* and *tpho84-2* re-complementation, 3916- and 4087-bp DNA fragments containing total *tpho84-1* and *tpho84-2* expression cassette were amplified from the Rut-C30 genome using *tpho84-1*-1/2 and *tpho84-2*-1/2 primers and then inserted into Swal-linearized LML2.0 to generate vector pFtpho84-1-1/2 and pFtpho84-2-1/2, respectively. *P tpho84-1*-1/2 and *p tpho84-2*-1/2 were then transferred to the *tpho84-1* and *tpho84-2* deletion strain by *Agrobacterium*-mediated transformation (see Additional file 5: Figure S4C, D). *tpho84-1* and *tpho84-2* re-complementation strains (∆*tpho84-1*-1/2 and ∆*tpho84-2*-1/2) were selected and verified by PCR using *tpho84-1*-1/2 and *tpho84-2*-1/2, and
tpho84-2-OF and tpho84-2-OR (see Additional file 5: Figure S4F).

For the construction of N-terminal RFP-tagged translational fusion of tpho84-1 under the control of the cbh1 promoter, the upstream (−1 to −1038 bp) and downstream (+1 to +1084 bp) fragments of tpho84-1 were generated from the genome of T. reesei Rut-C30 using KOD-Plus-Neo (TOYOBO, Japan). The promoter of cbh1 (P_{cbh1}) was obtained by PCR from the genome of T. reesei Rut-C30. Red fluorescent protein (rfp) was obtained by PCR using the plasmid pDsRed2-N1 (Clontech) as the template. First, the upstream fragment was ligated into the PacI- and XbaI-linearized LML2.0, using the ClonExpressTM II One Step Cloning Kit (Vazyme, Nanjing, China) to form pFrpRut-84-1. Subsequently, the P_{cbh1}, rfp, and downstream fragments were inserted into Swal-linearized FrpRut-84-1 to form the binary vector pRFPTPHO84-1 for subcellular location of tpho84-1 using Agrobacterium-mediated transformation. Subsequently, the marker was excised following the method of Zhang [61]. The putative rfp-tpho84-1 mutants (rfp-tpho84-1) generated by double crossover were verified by diagnostic PCR using the primers rfp-tpho84-1-CF and rfp-tpho84-1-CR (see Additional file 12: Figure S15).

Similarly, the 997-bp upstream and 797-bp downstream regions of tpho84-2 were amplified and then inserted into LML2.0 as described above to generate the binary vector pRFPTPHO84-2 (see Additional file 6: Figure S5A). After transformation and selection, the putative rfp-tpho84-2 mutants (rfp-tpho84-2) was verified by diagnostic PCR using the primers rfp-tpho84-2-CF and rfp-tpho84-2-CR (see Additional file 12: Figure S15).

The genome sequence of T. reesei is available at the US Department of Energy (DOE) Joint Genome Institute (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

**Fluorescence microscopy**

To localize RFP-TPHO84-1/2 fusion proteins using microscopy, the rfp-tpho84-1 and rfp-tpho84-2 strains were inoculated into Mandels’ medium and grown for 48–60 h. The mycelia were then observed using an S Plan Fluar ELWD 100×, 1.3 numerical aperture (NA) objective on a Laser Scanning Confocal Microscope (A1R, Nikon, Japan) comprising a Texas Red filter (500–620 nm band-pass excitation filter and emission filter of 670 nm). Images were processed using the NIS elements software (Nikon).

**Free cytosolic Ca2+ labeling and detection**

Fluo-3/AM (Sigma) was used as a Ca2+-specific probe to assess the level of cytoplasmic Ca2+ in T. reesei Rut-C30 according to the manufacturer’s protocol. Fluo-3/AM (50 μM final concentration) was loaded into 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 an S Plan Fluor ELWD 20×, 0.5 numerical aperture (NA) objective and a digital sight camera on an Eclipse Ti inverted microscope system (Ti-E, Nikon, Japan), comprising an FITC filter (420–490 nm band-pass excitation filter, and emission filter of 535 nm). The intensity of green fluorescence was quantified using NIS-Elements F package software. To eliminate the contribution of background fluorescence, cells without Fluo-3/AM labeling were also imaged under identical conditions.

**Statistical analysis**

All experimental data shown in this paper were carried out at least three times with identical or similar results. For every experiment, three biological replicates were performed with three technical replicates. The error bars indicate the standard deviation (SD) from the mean of triplicates. Student’s t test was used to compare two samples. Duncan’s multiple-range test was used for multiple comparisons. p < 0.05 was considered to be significant.

**Additional files**

Additional file 1: Table S1. Primers used in this study.

**Additional file 2: Figure S1.** Influence of Mn2+ on the transcriptional levels of cellulase-encoding genes in T. reesei Rut-C30. A–E The effects of Mn2+ on the transcriptional levels of cbh1 (A), cbh2 (B), xyl1 (C), eg1 (D) and eg2 (E). 0 mM, no Mn2+ was added to the medium; 10 mM, final concentration of 10 mM. *p < 0.05* compared with the control.

**Additional file 3: Figure S2.** Influence of Mn2+ addition on the transcriptional levels of putative Mn2+ transport-encoding genes. A–E The effect of Mn2+ on the transcriptional levels of tre77552 (A), tre11389 (B), tre5852 (C), tre45868 (D), and tre1661 (E). 0 mM, no Mn2+ was added to the medium; 10 mM, final concentration of 10 mM. *p < 0.05* compared with the control.

**Additional file 4: Figure S3.** Concentrations of Mn2+ in T. reesei Rut-C30 and its derivative mutants. The concentrations of intracellular Mn2+ of T. reesei Rut-C30 and its derivative mutant strains were examined after cultured in medium containing 10 mM MnCl2. Values are the means ± SD of the results from three independent experiments. *p < 0.05* compared with the control.

**Additional file 5: Figure S4.** Construction and verification of Δtpho84-1, Δtpho84-2, tpho84-1-cr, tpho84-2-cr mutants. (A) Schematic representation of the tpho84-1 locus from Rut-C30 and Δtpho84-1 mutant. The binding sites of primers on the genome of Rut-C30 and Δtpho84-1, and the expected sizes of the products in PCR verification are given. The region from +1 to +1916 bp relative to the translation start site of tpho84-1 (grey box) was replaced with the hygromycin resistance expression cassette (hygromycin, black box). (B) Schematic representation of the tpho84-2 locus from Rut-C30 and Δtpho84-2 mutant. The binding sites of primers
on the genome of Rut-C30 and Δphto84-2 and the expected sizes of the PCR products are given. The region from +1 to +2087 bp relative to the translation start site of phto84-2 (grey box) was replaced with the hygromycin resistance expression cassette (hygromycin, black box). (C) Schematic representation of the P-tpho84-1-tpho84-1-T-tpho84-1 cassette in phto84-1-c strains. The primer pairs indicated were used in the verification of the expression cassette. P-tpho84-1, the possible promoter of tpho84-1; T-tpho84-1, the possible terminator of tpho84-1. (D) Schematic representation of the P-tpho84-2-tpho84-2-T-tpho84-2 cassette in phto84-2-c strains. The primer pairs indicated were used in the verification of the expression cassette. P-tpho84-2, the possible promoter of tpho84-2; T-tpho84-2, the possible terminator of tpho84-2. (E) PCR verification of the Δphto84-1 and Δphto84-2 mutants. Lane M, DNA molecular mass maker. PCR amplification results of the Dpho84-1-F were obtained using tpho84-1-CF with D70-4 and Dpho84-1-R were obtained using HG3.6 with tpho84-1-1-CR (see Additional file 1: Table S1). PCR amplification results of the Dpho84-2-F were obtained using tpho84-2-2-CF with D70-4 and Dpho84-2-R were obtained using HG3.6 with tpho84-2-2-CR. (F) PCR verification of the tpho84-1-c and tpho84-2-c strains. Lane M, DNA molecular mass maker. PCR amplification results of the rfp-tpho84-1-c were obtained using rfp-tpho84-1-CF with rfp-tpho84-1-1-CR and rfp-tpho84-2-c were obtained using rfp-tpho84-2-CF with rfp-tpho84-2-2-CR. The genome of the Δphto84-1 and Δphto84-2 mutants were used as a negative control.

Additional file 6: Figure S5. Subcellular localization of TPHO84-1 and TPHO84-2. Schematic representation of the plasmids used for expression of RFP-TPHO84-1 and RFP-TPHO84-2. RFP was fused to the N-terminal of TPHO84-1 and TPHO84-2 and expressed under the control of the cbh1 promoter. B. In vivo epifluorescence analysis of rfp-tpho84-1 and rfp-tpho84-2 transformants. The transformants were cultured in liquid Mandels’ medium for 48–60 h and observed using Laser Scanning Confocal Microscopy.

Additional file 7: Figure S6. Cellulase activities of T. reesei Rut-C30 and its derivative mutant strains. The activities were examined after cultured in medium containing 0 mM Mn2+ with D70-4 and Dpho84-1-R were obtained using HG3.6 with Dpho84-1-1-CR. Values are the means ± SD of the results from three independent experiments.

Additional file 8. Data for corresponding cellulase activity per biomass. The CMCase/biomass activity and pNPCase/biomass activity of T. reesei strains were examined. For every experiment, three biological replicates were performed with three technical replicates each. Values are the means ± SD of the results from three independent experiments. The authors declare that they have no competing interests.

Additional file 9: Figure S12. Concentrations of Mn2+ in Δtphm1 strain. The concentrations of extracellular and intracellular Mn2+ of the Δtphm1 strain was analyzed after culturing in minimal medium containing 0 or 10 mM MnC12, filled circle, intracellular Mn2+ concentration after adding 10 mM MnCl2, blank circle, extracellular Mn2+ concentration after adding 10 mM MnCl2 filled circle, intracellular Mn2+ concentration without MnCl2 supplementation; blank square, extracellular Mn2+ concentration of control without MnCl2 supplementation. Values are the means ± SD of the results from three independent experiments.

Additional file 10: Figure S13. Cytosolic Ca2+ levels increase after Ca2+ addition. A. The analysis of cytosolic Ca2+ levels via a Ca2+ fluorescent probe Fluo-3/AM. The T. reesei Rut-C30 and its derivative mutant strains were cultured in liquid minimal medium (initial 5.4 mM Ca2+) for 48–60 h with extra 0 or 10 mM CaCl2 supplementation (low Ca or high Ca, respectively). For detection, 50 μM Fluo-3/AM was used, and the intensity was monitored using Automatic Inverted Fluorescence Microscopy. Green fluorescence represents the free cytosolic Ca2+; DIC, differential interference contrast. B. Comparative fluorescence ratio analysis of Ca2+ influence on cytosolic Ca2+ levels. The y-axis represents the Ca2+ fluorescence ratio measured by CSM, and the x-axis represents the different strains tested.

Additional file 11: Figure S14. Verification of Δtphm1, Δtphm2, Δtphm1Δtphm2, Δtphm1Δtphm2Δc2a1, and Δtphm1Δtphm2Δdphm1 mutants. Lane M, DNA molecular mass maker. PCR amplification results of the F were obtained using t81389-, t45852-, t106118-, tcrz1-, and tpmr1-CF, respectively, with D70-4 and R were obtained using HG3.6 with t81389-, t45852-, t106118-, tcrz1-, and tpmr1-1-CR, respectively. PCR amplification results of the F were obtained using rfp-tpho84-1-CF with D70-4 and Dpho84-1-R were obtained using HG3.6 with rfp-tpho84-1-CR. (Additional file 1: Table S1). PCR amplification results of the rfp-tpho84-2-F were obtained using rfp-tpho84-2-CF with D70-4 and rfp-tpho84-2-R were obtained using HG3.6 and rfp-tpho84-2-CR.

Abbreviations

crz1: calcineurin-responsive zinc finger transcription factor 1; CMCase: endo-β-glucanase activity; pNPCase: endo-β-glucanase activity; qRT-PCR: quantitative reverse-transcription PCR; ICP-MS: inductively coupled plasma mass spectrometry; NRAMP: Natural Resistance-Associated Macrophage Protein.

Authors’ contributions

WW initiated, designed, and coordinated the study and reviewed the manuscript. YC planned and carried out experiments and measurements, and interpreted experimental data. DW and WW supported the research funding. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and in Additional files 1 and 2].

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

This research was supported by the National Natural Science Foundation of China [No. C010302-5150066], and the Fundamental Research Funds for the Central Universities [No. 222201714053].

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 16 November 2017 Accepted: 18 February 2018

Published online: 01 March 2018

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