A Mitogen-Activated Protein Kinase Tmk3 Participates in High Osmolarity Resistance, Cell Wall Integrity Maintenance and Cellulase Production Regulation in Trichoderma reesei

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Abstract

The mitogen-activated protein kinase (MAPK) pathways are important signal transduction pathways conserved in essentially all eukaryotes, but haven’t been subjected to functional studies in the most important cellulase-producing filamentous fungus Trichoderma reesei. Previous reports suggested the presence of three MAPKs in T. reesei: Tmk1, Tmk2, and Tmk3. By exploring the phenotypic features of T. reesei Δtmk3, we first showed elevated NaCl sensitivity and repressed transcription of genes involved in glycerol/trehalose biosynthesis under higher osmolarity, suggesting Tmk3 participates in high osmolarity resistance via derepression of genes involved in osmotic stabilizer biosynthesis. We also showed significant downregulation of genes encoding chitin synthases and a β-1,3-glucan synthase, decreased chitin content, ‘budded’ hyphal appearance typical to cell wall defective strains, and increased sensitivity to calcofluor white/Congo red in the tmk3 deficient strain, suggesting Tmk3 is involved in cell wall integrity maintenance in T. reesei. We further observed the decrease of cellulase transcription and production in T. reesei Δtmk3 during submerged cultivation, as well as the presence of MAPK phosphorylation sites on known transcription factors involved in cellulase regulation, suggesting Tmk3 is also involved in the regulation of cellulase production. Finally, the expression of cell wall integrity related genes, the expression of cellulase coding genes, cellulase production and biomass accumulation were compared between T. reesei Δtmk3 grown in solid state media and submerged media, showing a strong restoration effect in solid state media from defects resulted from tmk3 deletion. These results showed novel physiological processes that fungal Hog1-type MAPKs are involved in, and present the first experimental investigation of MAPK signaling pathways in T. reesei. Our observations on the restoration effect during solid state cultivation suggest that T. reesei is evolved to favor solid state growth, bringing up the proposal that the submerged condition normally used during investigations on fungal physiology might be misleading.

Introduction

The systematic risk inherent to a fossil fuel based economy has drawn worldwide attention, leading to the proposal of replacing these non-renewable energy sources with renewable energy sources including energy from renewable biomass. One such biomass is lignocellulose, which is abundantly available as agricultural or forestry wastes. Conversion of lignocellulose to achieve this ‘workhorse’ are therefore essential in both scientific and technological regards [2].

Cells sense their surrounding environments and react to external signals via signal transduction pathways. Among all known signal transduction pathways, the mitogen-activated protein kinase (MAPK) pathway is ubiquitous in almost all eukaryotic species and is one of the most well characterized pathways [3]. This pathway features a signal relay cascade in which three kinases are involved: the MAPK kinase kinase (MAPKKK), the MAPK kinase (MAPKK) and MAPK [4]. MAPK further phosphorylates downstream elements involved in the regulation of physiological activities. The most extensive research of these pathways was carried out in Saccharomyces cerevisiae, in which six MAPK pathways were identified [5]. In...
filamentous fungi, three major classes of MAPKs are present, respectively homologous to yeast Hog1p, Slt2p, and Fus3p. These MAPKs have been shown to function in a variety of physiological processes such as fruiting body development [6], polarized growth [7], biosynthesis [8], conidiation [9], pathogenicity [10], circadian rhythmicity [11], stress response [12,13], protein production [9,14] and cell wall integrity maintenance [15].

The HOG (high-osmolarity glycerol) pathway in *S. cerevisiae* is involved in combating high osmolarity by activating genes required for viability under hypertonic stress, which include genes in the glycerol synthesis pathway encoding glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol–3-phosphatase (*GPP2*) [16,17]. Governing this pathway are kinases in the Ste11p/Ssk2p/Ssk22p-Pbs2p-Hog1p [MAPKKK-MAPKK-MAPK] pathway [18]. In filamentous fungi, this function of the Hog1p pathway seems to be generally conserved. Hog1p homologues were shown to be involved in high osmolarity resistance in *Aspergillus nidulans* [19], *Magnaporthe grisea* [10], *Cryphonectria parasitica* [13], *Neurospora crassa* [20], *Trichoderma harzianum* [21], and *Aspergillus fumigatus* [22].

Investigations carried out on Slt2p homologues in filamentous fungi suggested the involvement of this MAPK in the maintenance of cell wall integrity in *A. fumigatus* [12,15], *Magnaporthe oryzae* [23], *Trichoderma viridae* [24], *Claviceps purpurea* and *Fusarium graminearum* [25], but not *Mycosphaerella graminicola* (*Mgslt2*) and *Botrytis cinerea* [26]. The involvement of a gene in cell wall integrity maintenance is generally examined by testing for hypersensitivity in the gene deletion strain to cell wall lysis enzymes or cell wall interfering compounds such as calcofluor white (CFW) and Congo red (CR). Interestingly, the deletion of *hog1* homologues in *T. harzianum* and *A. fumigatus* showed unchanged or even increased resistance to CFW and CR, suggesting the hypersensitivity to improved osmolarity is unrelated to weakening of the cell wall [21,27].

In filamentous fungi, yeast Fus3p homologues are involved in quite diverse processes, one of which is the production of glycoside hydrolases. Cellulase and chitinase production was upregulated in the *tmkA* (*a fus3* homologue) deletion strain of *T. virens* [9]. Chitinase production was improved in the *tmk1* (*a fus3* homologue) knockout strain of *Trichoderma atroviride* [14]. The expression levels of N-acetylglucosaminidase- and chitinase-encoding genes increased when *tmk1* (*a fus3* homologue) was disrupted in *T. virens* [28]. This phenomenon is of particular interest in cellulase-producing filamentous fungi like *Trichoderma* and *Aspergillus* species, as they are well-known industrial cellulase hyper-producing strains, out of which *T. reesei* is the most widely studied and used.

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**Figure 1. Growth of** *T. reesei* **parent strain and** *T. reesei Δtmk3* **on plates.**

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**Figure 2. Biomass accumulations in submerged media.** Panel A, biomass measured by ATP concentration in cultures; Panel B, biomass measured by total DNA content in cultures.
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*T. reesei* (syn. *Hypocrea jecorina*) was first isolated on the Solomon Islands during World War II, and has received considerable improvements over the last seventy years for industrial applications [29]. It has turned a paradigm for the investigations of cellulases, hemicellulases, and the molecular mechanisms underlying their synthesis and regulation [30]. However, knowledge on
the signal transduction cascades in this fungus is limited to a few aspects: 1) light-modulated cellulase production mediated by G proteins (Gna1 and Gna3), a PAS/LOV domain protein ENVOY and cAMP-dependent protein kinase A signaling [31–34]; 2) regulation of the sexual development process [35]; 3) cellulose- and cAMP-independent modulation of cellulase production mediated by Ras GTPase TrRas2 [36]. Relatively little is known in other well-known signal transduction pathways such as MAPK pathways, Ca\(^{2+}\)-signaling pathways and factors such as casein kinase II, germinal center kinases and protein kinase C.

In silico reconstruction of the MAPK signal transduction cascade in *T. reesei* identified three distinct pathways, in which three putative MAPKs are involved: the yeast Fus3p homologue Tmk1, Slt2p homologue Tmk2, and the Hog1p homologue Tmk3 [37]. None of these MAPKs have been studied in *T. reesei* yet. In this study, by characterizing the properties of the *T. reesei Δtmk3* deletion strain, we attempt to identify the role of Tmk3 in *T. reesei*, particularly in cell wall integrity maintenance and cellulase production that have not been identified for Hog1-type MAPKs in other fungal species. Further comparison between submerged and solid state growth leads to the finding of novel, interesting restoration effects during solid state cultivation. These studies are the first research done on MAPKs in *T. reesei*, leading up to further in-depth understanding of the regulatory mechanisms of this well-known industrial cellulase hyper-producing workhorse.

**Figure 3. Test of sensitivity to NaCl, CR and CFW.** Panel A, sensitivity to NaCl; Panel B, sensitivity to CR; Panel C, sensitivity to CFW. doi:10.1371/journal.pone.0072189.g003
Materials and Methods

Strain and chemicals

*T. reesei Aku70* strain, derived from the QM9414 uridine auxotrophic *pyr4*-negative strain TU-6 (ATCC MYA-256), was used as the high transformation efficiency parent strain for the gene deletion experiment, as is previously reported [38]. Uridine and sorbitol were purchased from Sanqun Biotech Co., Ltd. (Shanghai, China). Calcinhor white (CFW), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-β-D-cellobioside (pNPC), carboxymethylcellulose (CMC), cytoligase from *Helix pomatia* Ltd., Yucheng, Shandong, China. All other chemicals were used as the high transformation efficiency parent strain for the experiment. Amplification of *pyr4* auxotrophic strain, derived from the QM9414 uridine auxotrophic strain, was carried out using Kod FX high fidelity enzymes (TOYOBO CO. LTD. Osaka, Japan). Congo red was purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Wheat bran was kindly provided by Longlive Bio-Technology Co., Ltd., Yucheng, Shandong, China. All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Phylogenetic analysis

Phylogenetic tree construction and protein sequence comparison were carried out using the Clustal X2 software [39].

Construction of tmk3 deletion strain

Construction of *T. reesei Atmk3* was carried out essentially as previously described [38], using the *pyr4* selection marker which restores uridine biosynthesis capabilities in the *pyr4*-deficient parent strain. Amplification of *tmk3* from *T. reesei* genome was carried out using Kod FX high fidelity enzymes (TOYOBO CO. LTD. Osaka, Japan).

Southern blotting analysis

Southern blotting experiment was used to confirm whether *T. reesei Atmk3* was successfully constructed. Genomic DNA extracted from *T. reesei Aku70* or *Atmk3* strain was digested using HindIII prior to hybridization. Detection of probe-hybridized DNA fragment was carried out using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany).

Submerged and solid state growth

Minimal media solution containing 0.5% NH₄SO₄, 0.06% MgSO₄, 1.5% KH₂PO₄, 0.08% CaCl₂, 0.00005% FeSO₄·7H₂O, 0.00016% MnSO₄·H₂O, 0.00014% ZnSO₄·7H₂O and 0.00002% CoCl₂ was first prepared. Spores of *T. reesei Aku70* or *Atmk3* strain were harvested after 6 days of growth, and counted using a hemacytometer. For submerged growth, approximately 10⁵ spores were inoculated in submerged media containing 2 g avicel, 2 g wheat bran and 100 ml minimal media solutions. The media for growth of *T. reesei Aku70* contain 0.1% uridine. The growth of 100 ml submerged cultures took place in 500 ml flasks at 30°C in a rotary shaker (Model SKY-1112B, Shanghai Sukun Ltd., Shanghai, China) rotated at 200 rpm. Solid state media contain 6 ml minimal media solution, 2 g avicel and 2 g wheat bran. Approximately 10⁶ spores were inoculated to glass plates containing 10 g solid state media. The plates were subsequently incubated in an incubator (Model MJX-250, Ningbo Jiangnan Instrument Factory, Ningbo, China) at 30°C without shaking.

Phenotypic analysis

Approximately 10⁵ conidiospores of *T. reesei Aku70* or *Atmk3* strain were inoculated on minimal media (MM) plates containing glucose, starch, sucrose, lactose or glycerol, as well as PDA plates. The plates were incubated in an incubator (Model MJX-250, Ningbo Jiangnan Instrument Factory, Ningbo, China) at 30°C for 4 days. Double-layer avicel plates were prepared by first casting a MM agarose bottom layer containing no carbon sources, and a second MM agarose top layer containing 1% avicel. Approximately 10⁶ conidiospores of *T. reesei Aku70* or *Atmk3* strain were inoculated on the plates, which were incubated at 30°C for 4 days prior to examination.

Images of *T. reesei Aku70* or *Atmk3* hyphae were taken with a bright field microscope (Nikon eclipse E100, 400 fold magnification) from cultures grown in submerged minimal media containing glucose as the carbon source.

NaCl, CFW and CR sensitivity assays

Approximately 10⁵ spores of *T. reesei Aku70* or *Atmk3* strain were inoculated on PDA plates containing various concentrations of NaCl, CFW and CR for sensitivity tests on these chemicals. These plates were subsequently incubated in an incubator (Model MJX-250, Ningbo Jiangnan Instrument Factory, Ningbo, China) at 30°C for 3 days. The dosages of NaCl used in this assay are respectively 0, 0.3, 0.5, 0.7, 0.9, 1.1 M. The dosages of CFW used in this assay are respectively 0, 10, 20, 30, 40 mg/L. The dosages of CR used in this assay are respectively 0, 75, 125, 150, 175, 200 mg/L. The diameters of colonies on CR used in this assay are respectively 0, 10, 20, 30, 40 mg/L. The diameters of colonies on CR- and CFW-containing plates were measured for comparison between *T. reesei Aku70* and *Atmk3*. Three individual replicates of each experiment were performed.

Biochemical assays

The ATP level was assayed using the Checklite 250 plus ATP kit (Kikkoman Biochemifa Company, Minato-ku, Japan). The concentration of ATP indicates the average ATP concentration in cultures. Five individual replicates were carried out for ATP level analysis in solid state cultures. Three individual replicates were carried out for ATP level analysis in submerged cultures.

Figure 4. Response of transcriptional levels of glycerol and trehalose biosynthesis genes to elevated osmolarity. Y-axis indicates transcriptional abundance ratio between strains grown under 0.15 M NaCl and 0 M NaCl. Trire2_76620, Trire2_2574, glycerol-3-phosphate dehydrogenase-coding genes; Trire2_75295, Trire2_77602, phosphate dehydrogenase-coding genes; Trire2_75295, Trire2_77602, glycerol-3-phosphate synthase-coding genes.
The DNA content of a T. reesei Δku70 or Δtmk3 culture was also used as a measure of biomass. To measure the DNA content, solid state and submerged cultures were first prepared as described in this work. For submerged cultures, 1 ml of the culture was drawn for DNA content determination. For solid state cultures, 25 ml of distilled water was used to suspend the cultures, and 1 ml of suspended culture was drawn for DNA content determination. The cultures were subsequently diluted 5-fold in 15 ml centrifuge tubes, and were subjected to a brief centrifugation to remove supernatant. One milliliter of 10% trichloroacetic acid (TCA) was then added to the tube, thoroughly mixed, and incubated on ice for 3 minutes. The tube was boiled in a water bath (Shanghai Jinghong Laboratory Instrument Co., Ltd., Shanghai, China) for 30 minutes, followed by centrifugation at 10,000 rpm for 10 minutes. Absorbance at 260 nm in the supernatant was subsequently measured using a UV-visible spectrophotometer (Model 2802, UNICO, Dayton, NJ, US). The total DNA content in the cultures was calculated from $A_{260}$. Three individual replicates were carried out for each assay.

Protein concentration was determined using the Lowry method [40]. Filter paperase activity (FPA) was assayed following previously published protocols [41]. Cellobiohydrolase, endoglucanase, β-glucosidase and β-xylosidase activities were respectively assayed by abilities to hydrolyze pNPC, CMC, pNPG and pNPX following published protocols [42]. Six replicates were performed to determine extracellular protein concentrations, βNPCase, CMCase, βNPGase and βNPXase activities in solid state cultures. Three replicates were performed to determine extracellular

Table 1. Diameters of T. reesei parent strain and T. reesei Δtmk3 colonies on CR- and CFW-containing PDA plates.

| CR concentration (mg/L) | T. reesei parent strain | Percentage of reduction (versus no CR addition) | T. reesei Δtmk3 | Percentage of reduction (versus no CR addition) |
|-------------------------|-------------------------|-----------------------------------------------|-----------------|-----------------------------------------------|
| 0                       | 48.3±0.6                | 0.0%                                          | 32.0±0.0        | 0.0%                                          |
| 75                      | 33.0±0.0                | 31.7%                                         | 18.5±0.7        | 42.2%                                         |
| 125                     | 28.7±0.6                | 40.7%                                         | 0.0±0.0         | 100.0%                                        |
| 150                     | 25.3±0.6                | 47.6%                                         | 0.0±0.0         | 100.0%                                        |
| 175                     | 22.0±1.0                | 54.5%                                         | 0.0±0.0         | 100.0%                                        |
| 200                     | 18.0±1.0                | 62.8%                                         | 0.0±0.0         | 100.0%                                        |

| CFW concentration (mg/L) | T. reesei parent strain | Percentage of reduction (versus no CFW addition) | T. reesei Δtmk3 | Percentage of reduction (versus no CFW addition) |
|--------------------------|-------------------------|-----------------------------------------------|-----------------|-----------------------------------------------|
| 0                        | 50.3±1.5                | 0.0%                                          | 42.0±0.0        | 0.0%                                          |
| 10                       | 47.3±0.6                | 6.0%                                          | 32.5±0.7        | 22.6%                                         |
| 20                       | 24.7±0.6                | 51.0%                                         | 16.5±0.7        | 60.7%                                         |
| 30                       | 22.3±0.6                | 55.6%                                         | 0.0±0.0         | 100.0%                                        |
| 40                       | 14.3±1.2                | 71.5%                                         | 0.0±0.0         | 100.0%                                        |

The DNA content of a T. reesei Δku70 or Δtmk3 culture was also used as a measure of biomass. To measure the DNA content, solid state and submerged cultures were first prepared as described in this work. For submerged cultures, 1 ml of the culture was drawn from flasks for DNA content determination. For solid state cultures, 25 ml of distilled water was used to suspend the cultures, and 1 ml of suspended culture was drawn for DNA content determination. The cultures were subsequently diluted 5-fold in 15 ml centrifuge tubes, and were subjected to a brief centrifugation to remove supernatant. One milliliter of 10% trichloroacetic acid (TCA) was then added to the tube, thoroughly mixed, and incubated on ice for 3 minutes. The tube was boiled in a water bath (Shanghai Jinghong Laboratory Instrument Co., Ltd., Shanghai, China) for 30 minutes, followed by centrifugation at 10,000 rpm for 10 minutes. Absorbance at 260 nm in the supernatant was subsequently measured using a UV-visible spectrophotometer (Model 2802, UNICO, Dayton, NJ, US). The total DNA content in the cultures was calculated from $A_{260}$. Three individual replicates were carried out for each assay.

Protein concentration was determined using the Lowry method [40]. Filter paperase activity (FPA) was assayed following previously published protocols [41]. Cellobiohydrolase, endoglucanase, β-glucosidase and β-xylosidase activities were respectively assayed by abilities to hydrolyze pNPC, CMC, pNPG and pNPX following published protocols [42]. Six replicates were performed to determine extracellular protein concentrations, βNPCase, CMCase, βNPGase and βNPXase activities in solid state cultures. Three replicates were performed to determine extracellular

Figure 5. Microscopic images of T. reesei parent strain and T. reesei Δtmk3 hyphae. Panel A–B, T. reesei parent strain; Panel C–D, T. reesei Δtmk3. Bar, 20 μm. doi:10.1371/journal.pone.0072189.g005

Figure 6. Transcriptional changes of chitin synthase- and β-1,3-glucan synthase-coding genes. Y-axis indicates transcriptional abundance ratio between T. reesei Δtmk3/T. reesei parent strain. Trire2_112271, Trire2_58188, Trire2_55341, Trire2_51492, Trire2_124228, Trire2_122172, Trire2_71563, Trire2_67600, chitin synthase-coding genes; fks, β-1,3-glucan synthase-coding genes. doi:10.1371/journal.pone.0072189.g006
protein concentrations, \( \beta \)NPCase, CMCase, \( \beta \)NPase and \( \beta \)NPase activities in submerged cultures.

**Chitin content assay**

Chitin contents of *T. reesei\( D_{ku70} \) and *T. reesei\( D_{tmk3} \) were assayed similarly to previous reports [43,44]. Approximately \( 10^6 \) spores of *T. reesei\( D_{ku70} \) or \( D_{tmk3} \) strain were inoculated in submerged media containing 2\% glucose. The mycelia were harvested after 3 days of growth, and subsequently dried by heating at 105\°C for 4 hours in an oven (Model DHG-9030, Shanghai Jinghong Laboratory Instrument Co. Ltd., Shanghai, China) for the determination of dry cell weight (DCW). Sixty mg of mycelia (14 mg DCW) of both *T. reesei\( D_{ku70} \) or \( D_{tmk3} \) strain were heated at 80\°C for 90 minutes in 1 ml of 6\% KOH using a water bath (Shanghai Jinghong Laboratory Instrument Co. Ltd., Shanghai, China), followed by addition of 0.1 ml glacial acetic acid. The treated mycelia were further centrifuged in a microcentrifuge (Centrifuge 5415R, Eppendorf, Hamburg, Germany) at 13,000 rpm for 10 minutes. The pellet was suspended in 0.5 ml phosphate buffer (pH 6.3) and digested using 0.1 U chitinase by incubation at 37\°C for 1 hour. The reaction system was centrifuged again at 13,000 rpm for 10 minutes. Zero point two five mg of cytohelicase was subsequently added to 500 \( \mu \)l supernant, followed by incubation at 37\°C for 1 hour. The

![Figure 7. Production of extracellular proteins, cellulases and hemicellulase in submerged media.](image)

Panel A, Extracellular protein levels; Panel B, FPA levels; Panel C, \( \beta \)NPCase activities; Panel D, CMCase activities; Panel E, \( \beta \)NPase activities; Panel F, \( \beta \)NPase activities.  

**Figure 7. Production of extracellular proteins, cellulases and hemicellulase in submerged media.** Panel A, Extracellular protein levels; Panel B, FPA levels; Panel C, \( \beta \)NPCase activities; Panel D, CMCase activities; Panel E, \( \beta \)NPase activities; Panel F, \( \beta \)NPase activities.

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amount of released N-acetylglucosamine was assayed according to previous reported procedures [44].

Real-time PCR reactions and data manipulation

Total RNA was extracted from *T. reesei Aku70* or *Atmk3* strain growing in solid state media and submerged media containing 2% avicel and 2% wheat bran for the examination of expression of cellulase-, hemicellulase-, chitin synthase- and β-1,3-glucan synthase-coding genes, as well as from *T. reesei Aku70* or *Atmk3* strain growing in submerged media containing 2% glucose and 0 or 0.15 M NaCl for the examination of expression of glycerol-3-phosphate dehydrogenase- and α,α-trehalose-6-phosphate synthase-coding genes. cDNA was synthesized using PrimeScript RT reagent kit with gDNA erase (Perfect Real Time) from Takara Bio Inc. (Shiga, Japan).

Real-time PCR reactions were carried out on a LightCycler 480II Real-Time PCR system (Roche Applied Science, Mannheim, Germany) using SYBR Premix EX Taq™ II (Takara Bio Inc., Shiga, Japan) as the dye. Three individual biological replicates and three individual technical replicates for each biological sample (a total of 9 replicates for each reaction) were carried out. The relative abundance of genes was calculated using the $2^{-\Delta\Delta Ct}$ method as previously described [45].

Results and Discussion

tmk3 encodes a Hog1-type MAPK in *T. reesei*

Phylogenetic analysis of Tmk3 from *T. reesei* and other previously characterized MAPKs showed Tmk3 apparently cluster with Hog1-type MAPKs from other species (Fig. S1), leading to the suggestion that *tmk3* likely encodes a Hog1 type MAPK. Sequence comparison between Tmk3 from *T. reesei* and Hog1 from *S. cerevisiae* showed a sequence identity of 66%, further supporting this suggestion (Fig. S2). It can therefore be concluded that Tmk3 is homologous to Hog1-type MAPKs, which function in high osmolarity resistance in *S. cerevisiae* and other filamentous fungi [3].

Construction and growth patterns of *T. reesei Atmk3*

The *tmk3* deletion strain was constructed via homologous recombination using *T. reesei Aku70* as the parent strain (Fig. S3), in which the non-homologous end joining pathway was defective [38]. Examination of the growth of parent and *Atmk3* strains on plates lead to several interesting findings: 1) The growth of *T. reesei Atmk3* on each minimal media plate containing a tested carbon source (glucose, starch, lactose, sucrose, glycerol) is significantly worse than the parent strain; 2) Only slightly slower growth was observed when *T. reesei Atmk3* was grown on complete media (PDA plates); 3) a significantly larger transparent zone was observed around the colony of *T. reesei Atmk3* in comparison to the parent strain, when the two strains were grown on double layer plates containing avicel (Fig. 1).

The differences between the performance of *T. reesei Atmk3* on minimal and complete media reflect damaged biosynthesis of certain critical compounds in the *tmk3*-deficient strain. The availability of these compounds in complete media partially...
compensates for the defects. Our results cannot specifically suggest which pathways are damaged, which remains to be elucidated in further studies.

The larger transparent zone around the colony on avicel-containing double layer plate is an indication of improved avicelase production in *T. reesei* Dtmk3. The term ‘avicelase’ here refers to the avicel-hydrolyzing enzymatic activities, which are primarily determined by cellobiohydrolase activities for two reasons: 1) cellobiohydrolase is the most abundant enzyme in secreted *T. reesei* cellulases [46]; 2) avicel has a highly crystalized cellulose structure and lacks the amorphous regions favored by endoglucanases. Although the contribution of other major cellulases cannot be excluded, the improved avicelase production in *T. reesei* Dtmk3 can be interpreted primarily as the increase of cellobiohydrolase secretion.

Growth of *T. reesei* parent and Dtmk3 strains was also compared during submerged cultivation. The levels of ATP production and DNA content were used as measures of biomass accumulation. As is shown in Fig. 2, growth is significantly hampered in the tmk3-deficient strain, suggesting the important role Tmk3 plays in vegetative growth.

Figure 10. Production of extracellular proteins, cellulases and hemicellulase in solid state media. Panel A, Extracellular protein levels; panel B, FPA levels; Panel C, pNPCase activities; Panel D, CMCase activities; Panel E, pNPase activities; Panel F, pNPXase activities.

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Participation of Tmk3 in high osmolarity resistance via derepression of glycerol and trehalose synthesis genes

We examined the tolerance of T. reesei parent and Δtmk3 strains to elevated osmotic pressure by growing them on plates containing various concentrations of NaCl. It appears that T. reesei Δtmk3 colonies ceased to develop at the NaCl level of 0.5 M, while the parent strain can still grow in the presence of 0.9 M NaCl (Fig. 3A). The apparent higher sensitivity to NaCl for the tmk3 deletion strain is an indication of hampered tolerance to high osmolarity, which is indicative of a Hog1-like function for Tmk3.

It was reported that glycerol and trehalose are involved in high osmolarity resistance by improving intracellular osmolarity in S. cerevisiae [16,47]. In particular, gpd1 that encodes a glycerol-3-phosphate dehydrogenase and functions in glycerol biosynthesis was upregulated during exposure to high osmolarity [16]. In T. reesei, we found two glycerol-3-phosphate dehydrogenase-coding genes functioning in glycerol biosynthesis (Trire2_76620 and Trire2_2574) and five α,α-trehalose-6-phosphate synthase-coding genes (Trire2_73295, Trire2_77602, Trire2_121491, Trire2_73134 and Trire2_48707) functioning in trehalose biosynthesis. Unlike in S. cerevisiae, the upregulation of these genes in response to improved osmolarity is not apparent in T. reesei parent strain (Fig. 4). In T. reesei Δtmk3, however, the expression levels of these genes are downregulated when exposed to 0.15 M NaCl (Fig. 4). These results suggest the Hog1-homologous Tmk3 functions in high osmolarity resistance in T. reesei, similarly to S. cerevisiae. The mechanism of this function, however, appears different: Hog1p of S. cerevisiae functions in stimulation of genes in the osmotic stabilizer biosynthesis; Tmk3 of T. reesei functions in derepression of genes in the osmotic stabilizer biosynthesis.

Involvement of Tmk3 in cell wall structure integrity maintenance

The integrity of T. reesei parent and Δtmk3 strains’ cell wall was investigated by testing of sensitivity to cell wall interfering substances CFW and CR. On CFW- and CR-containing plates, T. reesei Δtmk3 colonies are significantly smaller than those of the parent strain (Fig. 3B, 3C). T. reesei Δtmk3 could not grow on plates containing 125 mg/L CR or 30 mg/L CFW, while the parent strain was able to tolerate at least 200 mg/L CR and 40 mg/L CFW. Analysis of colony diameters showed T. reesei Δtmk3 is affected by CFW and CR more severely than T. reesei parent strain (Table 1), further suggesting the deletion of tmk3 leads to hypersensitivity to CFW and CR and reduction of cell wall integrity.

Comparison of the hyphal phenotype of T. reesei parent and Δtmk3 strains further supported the presence of weakened cell wall in the tmk3 knockout strain. T. reesei parent strain hyphae have a smooth cell wall structure (Fig. 5A, 5B), while T. reesei Δtmk3 hyphae apparently adopt a ‘budded’ appearance, in agreement with having compromised cell wall structure (Fig. 5C, 5D), although other possible defects such as impaired polarity maintenance cannot be excluded [48,49].

The fungal cell wall is primarily composed of proteins and polysaccharides including chitin, β-1,3-glucan and β-1,6-glucan [50]. It has been previously reported that chitin synthase and β-1,3-glucan synthase are involved in the synthesis of chitin and β-1,3-glucan in filamentous fungi [51,52]. Nine chitin synthase coding genes (Trire2_112271, Trire2_58188, Trire2_55341, Trire2_51492, Trire2_124228, Trire2_122172, Trire2_71563, Trire2_67600) and one β-1,3-glucan synthase coding gene (Trire2_78176, fks) are present in the genome of T. reesei. The transcriptional abundance of these genes in T. reesei parent and Δtmk3 strains was investigated with real-time PCR. The transcription of all these genes except for Trire2_67600 was detected, and all the detected genes except for Trire2_55341 are significantly (2–8 folds) downregulated in T. reesei Δtmk3 (Fig. 6), suggesting Tmk3 participates in the cell wall integrity pathway by regulating the synthesis of chitin and β-1,3-glucan. These transcriptional responses were further supported by the observation of higher chitin content in T. reesei parent strain (4.19±0.07 percent DCW) than in T. reesei Δtmk3 strain (3.48±0.07 percent DCW).

Our results reported in this study clearly suggest the involvement of Tmk3 in the maintenance of cell wall integrity in T. reesei. Interestingly, instead of Hog1-type MAPKs, Slt2-type MAPKs were reportedly involved in cell wall integrity maintenance (as is summarized in the introduction section). The unaltered or even increased resistance to CR and CFW in hog1 deletion strains of T. harzianum and A. fumigatus further suggested Hog1 is not involved in cell wall integrity pathways in these two filamentous fungi [21,27]. The ‘budded’ appearance of T. reesei Δtmk3 hyphae in minimal media is similar to that of A. fumigatus Δmpk4 (Mpka is a Slt2 homologue in A. fumigatus) [15], while this phenomenon was only apparent in hog1 deletion strains of M. grisea and A. nidulans when exposed in high salt media due to their incapability to cope with high osmotic pressure [10,19]. All these observations lead to the conclusion that Tmk3, unlike Hog1-type MAPKs in other fungi, functions in the cell wall integrity pathway similarly to Slt2-type MAPKs. This also explains the discrepancy of growth performance in standard low salt media between T. reesei Δtmk3 and other filamentous fungi in which hog1 homologues were inactivated [10,13,21]: Hog1 in other filamentous fungi only responds to high-salt environments, so they grow normally in low salt media; Tmk3 is involved in cell wall integrity maintenance, and there is a growth defect for T. reesei Δtmk3 even under low osmolarity.

Responses of cellulase and hemicellulase production to tmk3 deletion

The comparison of cellulase production from T. reesei parent and Δtmk3 strains under submerged cultivation conditions showed significantly decreased production of FPA, extracellular protein, pNPCase activity that measures celllobiohydrolase activity, CMCase activity that measures endoglucanase activity, pNPase activity that measures β-glucosidase activity, and pNPase activity that measures β-xylanase activity in T. reesei Δtmk3 (Fig. 7, D). Transcriptional analysis showed significant and strong downregulation of cbh1, cbh2, egl1, egl2, bg1 and bxl1, respectively coding CBHI, celllobiohydrolase II (CBHII), endoglucanase I (EGI), endoglucanase II (EGII), β-glucosidase I (BG1) and β-xylanase I (BXL1) (Fig. 8).

The decrease in cellulase and hemicellulase production in T. reesei Δtmk3 cannot be solely explained by the reduced biomass accumulation, because the transcriptional levels of cellulose- and hemicellulase-coding genes are clearly downregulated. These observations lead to the suggestion that Tmk3 is involved in cellulase production and/or induction. In T. reesei, several transcription factors were shown to regulate cellulase production, including the activator Xyr1 [53], repressor Cre1 [54], repressor ACE1 [55] and activator ACEII [56]. Using a phosphorylation prediction software KinasePhos [57], we were able to identify MAPK phosphorylation sites on all these transcription factors (Table 2). Previous reports have suggested that the phosphorylation of some of these transcription factors is the prerequisite for their activities [58,59]. It is therefore reasonable to propose that Tmk3 may regulate cellulase production by phosphorylating, and therefore activating, transcription factors. This proposal, however, should not be interpreted as the activation of ALL these
transcription factors by Tmk3, as two more MAPKs (Tmk1, Tmk2) are present in T. reesei, and the phosphorylation specificity of the transcription factors has not been identified.

Restored growth and cellulase production in solid state media

One common problem on the studies of T. reesei physiology is that the way T. reesei is cultured in these investigations does not resemble their natural habitats. In nature, T. reesei grows on solid lignocellulosic particles, instead of in submerged media which are normally used for T. reesei cultivation during physiological studies. There are considerable differences between these two environments: the level of encountered moisture varies significantly; the exposure to oxygen is different; and in the natural environment, secreted proteins do not diffuse like during submerged cultivation. We therefore compared the performance of T. reesei Tmk3 in submerged media and solid state media, in order to further identify the role of Tmk3 in T. reesei.

A significant improvement in ATP and DNA contents, as measures of biomass accumulation, could be observed when T. reesei Tmk3 was grown on solid state media (Fig. 9), when compared to submerged cultures (Fig. 2). However, in comparison with submerged cultivation, chitin synthase and β-1,3-glucan synthase transcription showed a similar magnitude of downregulation during solid state cultivation following tmk3 deletion, except for Trire2_55341 (Fig. 6). Therefore, the improved ATP and DNA contents, as measures of biomass production, during solid state cultivation for T. reesei Tmk3 can unlikely be attributed to recovered cell wall integrity. It has been known for a long time that cell walls serve as the skeletons of fungal cells, and that fungal protoplasts die due to inflow of water unless osmolarity in the growth medium is adjusted [60]. One proposed explanation to this improvement of cellulase production is that the weaker cell wall in T. reesei Tmk3 allows easier protein permeation and therefore may benefit secretion and/or release of cell bound enzyme or protein. This beneficial effect could not be offset by the slight downregulation of cbh1 and bgf1 transcription and decreased biomass accumulation, and therefore leads to the improvement of overall cellobiohydrolase and β-glucosidase production.

Providing an explanation for the restoration of transcription for all the cellulase-coding genes during solid state cultivation is difficult before investigations could provide further evidence for the involvement of an additional regulatory mechanism. However, these results do suggest the interplay between Tmk3 mediated and other cellulase/hemicellulase regulatory mechanisms. Indeed, cellulase/hemicellulase production is regulated by a complicated regulatory network that is governed by many signals (such as light, carbon source etc), as well as extracellular and cytoplasmic factors [34,36]. It is therefore not a surprise for Tmk3 mediated regulatory mechanism and other regulatory mechanisms to affect each other.

Our studies of T. reesei Tmk3 grown during solid state cultivation showed a clear restorative effect in comparison to submerged cultivation. The molecular mechanisms underlying these effects vary on different aspects of cellular physiology, but the unified final restorative effect suggests T. reesei physiology is evolved to favor solid state cultivation. Although secreted proteins or metabolites can be produced more efficiently from filamentous fungi during submerged cultivation, therefore benefiting industrial application, the submerged condition might actually alter fungal growth and metabolism, and care needs to be taken when assumptions are made that growth under certain submerged growth conditions is ‘optimum’ or ‘physiological’, particularly during mechanistic studies on fungal physiology.

Conclusions

Results reported herein lead to the suggestion of the physiological roles in high osmolarity resistance, cell wall integrity maintenance and cellulase production of a Hog1-type MAPK Tmk3 in T. reesei. Although sequence comparison suggests Tmk3 is homologous to Hog1p from S. cerevisiae, their functions appear to vary significantly. While Hog1p from S. cerevisiae and Tmk3 from T. reesei are both involved in the resistance to high osmolarity, the mechanism of this function differs: induction of osmotic stabilizer resistance genes in S. cerevisiae and derepression of these genes in T. reesei. Further investigations showed the function of Tmk3 in more novel aspects. Our phenotypic analysis, chemical sensitivity studies and transcriptional profiling all suggested Tmk3 functions in the cell wall integrity signaling pathway similarly to Slt2-type MAPKs, a role never identified for Hog1-type MAPK in filamentous fungi before. We further observed that deletion of tmk3 leads to an apparent decrease in cellulase transcription, and suggested the involvement of Tmk3 in cellulase production regulation by phosphorylating transcription factors.

When grown in submerged media, biomass accumulation and cellulase production of T. reesei were significantly reduced upon tmk3 deletion. The degree of reduction was much smaller after transferring to solid-state media. The mechanism of the restoration of growth likely differs from the mechanism of the restoration of cellulase production, but the similarity between the effects suggests T. reesei is evolved to favor solid state cultivation.

In conclusion, our results show the participation of Tmk3 in high osmolarity resistance and two novel aspects: cell wall integrity and cellulase production regulation. The restorative effect identified during solid cultivation, particularly regulation of cellulase production, is worthy of further investigations for the
identification of other regulatory pathways involved in celluose production.

Supporting Information

Figure S1 Phylogenetic analysis of MAPKs from filamentous fungi. The phylogenetic tree was constructed by the neighbour-joining method. Bootstrap values are shown at each node, and are calculated from 1000 trees. Bar, evolutionary distance of 0.1.

Figure S2 Sequence alignment of Tmk3 from T. reesei and Hog1 from Saccharomyces cerevisiae. Trtmk3, Tmk3 from T. reesei; Schog1, Hog1 from S. cerevisiae.

Figure S3 Southern blotting analysis of T. reesei parent and Atmk3 strains. M, DNA molecular size marker; Atmk3, T. reesei Atmk3; parent, T. reesei parent strain. Indicated by arrows are the predicted sizes of DNA fragments hybridized with the probe.

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Author Contributions

Conceived and designed the experiments: MW XF. Analyzed the data: MW JY XF. Contributed reagents/materials/analysis tools: XF. Wrote the paper: MW XF.

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Function of a Hog1-Type MAPK (Tmk3) in T. reesei

Figure S2 Sequence alignment of Tmk3 from T. reesei and Hog1 from Saccharomyces cerevisiae. Trtmk3, Tmk3 from T. reesei; Schog1, Hog1 from S. cerevisiae. (TIF)

Figure S3 Southern blotting analysis of T. reesei parent and Atmk3 strains. M, DNA molecular size marker; Atmk3, T. reesei Atmk3; parent, T. reesei parent strain. Indicated by arrows are the predicted sizes of DNA fragments hybridized with the probe. (TIF)
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