A p53-like transcription factor, BbTFO1, contributes to virulence and oxidative and thermal stress tolerances in the insect pathogenic fungus, *Beauveria bassiana*

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Abstract

The p53-like transcription factor (TF) NDT80 plays a vital role in the regulation of pathogenic mechanisms and meiosis in certain fungi. However, the effects of NDT80 on entomopathogenic fungi are still unknown. In this paper, the NDT80 orthologue BbTFO1 was examined in *Beauveria bassiana*, a filamentous entomopathogenic fungus, to explore the role of an NDT80-like protein for fungal pest control potential. Disruption of BbTFO1 resulted in impaired resistance to oxidative stress (OS) in a growth assay under OS and a 50% minimum inhibitory concentration experiment. Intriguingly, the oxidation resistance changes were accompanied by transcriptional repression of the two key antioxidant enzyme genes cat2 and cat5. ΔBbTFO1 also displayed defective conidial germination, virulence and heat resistance. The specific supplementation of BbTFO1 reversed these phenotypic changes. As revealed by this work, BbTFO1 can affect the transcription of catalase genes and play vital roles in the maintenance of phenotypes associated with the biological control ability of *B. bassiana*.

Introduction

The p53-like transcription factor (TF) superfamily includes the NDT80/PhoG-like DNA-binding family (http://pfam.xfam.org/family/PF05224), which has only been discovered in unikont lineages [1]. NDT80-like proteins have different functions in different fungi, such as regulation of sexual development, meiosis, filamentation, virulence, drug resistance, programmed cell death and the response to nutrient stress [1, 2]. The NDT80 protein of *Saccharomyces cerevisiae* is considered to be the founding member of the NDT80 family within the p53-like superfamily [1] and a key regulator of sporulation and meiosis [3]. XprG, also known as an NDT80-like protein, positively regulates mycotoxin generation, carbon depletion-induced apoptosis, and extracellular protease expression in *Aspergillus nidulans* [4]. This protein is
similar to Neurospora crassa VIB-1, which plays a role in gene expression related to the positive regulation of extracellular protease generation as well as heterokaryon incompatibility-related apoptosis [2, 3, 5]. In Candida albicans, the roles of three NDT80-like proteins have been extensively studied. As regulators, CaNDT80 and CaRep1 control transporters (CDR1 and MDR1), which can eliminate drugs [6, 7]. Mutations in CaNDT80 and CaRON1 affect the induction of hyphal growth [7]. The CaNDT80 mutant also shows weakened virulence in a mouse model of infection [8]. NDT80 homologue mutations have certain impacts on secondary metabolism, including asexual or sexual spore pigmentation. N. crassa Δvib-1 mutants show pinkish conidia but not orange conidia (asexual spores); by contrast, mutants of XprG in A. fumigatus and A. nidulans possess pale conidia [3, 9]. Moreover, NDT80-like proteins are involved in fungal nutrition sensing. For example, suppressor of fusion (Suf), the Ndt80 homologue in Fusarium oxysporum, plays a vital role in regulating anastomosis depending on the nutrient [10]. As far as Trichoderma reesei is concerned, this protein also plays an important role in activating the N-acetylglucosamine (GlcNAc) gene cluster [2, 11]. C. albicans Rep1 and A. nidulans XprG are also required for GlcNAc catabolism [7].

The genus Beauveria, including the species B. bassiana, represents a strong insect pathogen with a wide range of hosts and has been prepared as a candidate mycoinsecticide with environmental friendliness [12]. As an important filamentous insect fungal pathogen, B. bassiana is the typical model for examining the pathogenic mechanism and growth of fungi together with their interactions with the host [13, 14]. Recently, some TFs in B. bassiana have been characterized. Typically, the BbMbp1 gene facilitates the transition of B. bassiana morphology with regard to saprophytic and pathogenic growth through a variety of genetic pathways [15]. In addition, in B. bassiana, the MADS-box TF Bbmcm1 plays a vital role in the regulation of the cell cycle, virulence and integrity [16]. Moreover, BbOhmm affects the homeostasis of reactive oxygen species (ROS) combined with the available oxygen to control BbSre1 transcriptional activity, thus regulating the homeostasis of mitochondrial iron, respiration-related genes and haem production to adapt to hypoxic conditions [14].

In our study, it is shown that the NDT80-like protein in B. bassiana plays an important role in oxidative stress (OS) resistance by impacting the expression of key catalase genes. Therefore, this protein was designated TFO1 (transcription factor regulating oxidation) in B. bassiana. This study was conducted to examine how BbTFO1 affects B. bassiana phenotypic changes related to its biological control ability through multi-phenotypic analyses of specific gene deletion or complementation strains combined with the parental WT. The results indicate that BbTFO1 plays an important role in regulating antioxidant effects, thermostolerance and pathogenesis in insect fungal pathogens.

Materials and methods

Phylogenetic analysis of BbTFO1

In NCBI databases, using the S. cerevisiae NDT80 sequence as a query, BLAST analysis based on NCBI was carried out to identify the genome from ARSEF 2860, the wild-type B. bassiana strain (WT), and some typical filamentous fungi. Next, each NDT80 homologous protein sequence found in the fungi was aligned to compare the structures and analyse the phylogenetics using MEGA7 (http://www.megasoftware.net).

Construction of BbTFO1 deletion and complementation mutants

BbTFO1 deletion together with a complementation mutant was created via Agrobacterium-mediated transformation [17]. The synthetic plasmids for homologous transformation were transformed by Agrobacterium tumefaciens and mobilized into B. bassiana according to a
previous procedure [18]. Using the paired primers (Table 1), PCR was carried out to clone \( BbTFO1 \)’ 5’ or 3’ coding/flanking fragment sequences based on the WT genome, with \( TaKaRa \) Taq (TaKaRa, Japan) used as the catalyst, followed by specific enzyme digestion and insertion to the target plasmid to form target gene deletion plasmids. \( BbTFO1 \) was knocked out in the WT by homogenously recombining the 5’ and 3’ fragments with a bar marker. The gene deletion mutant was initially screened by herbicide (200 \( \mu \text{g ml}^{-1} \) phosphinothricin) resistance [19].

For the reverse complement of the \( BbTFO1 \) deletion mutants, using Gateway BP Clonase™ II Enzyme Mix (Shanghai, China), primers (Table 1) were used to clone the entire \( BbTFO1 \) coding sequence based on the WT genome, followed by insertion into the p0380-sur-gateway for exchange for gateway fragments, and finally, p0380-sur-\( BbTFO1 \) was formed [20]. The plasmid was transformed in deletion via \( Agrobacterium \)-mediated transformation to form complementation strains of \( BbTFO1 \), and a putative complementary strain was selected by chlorimuron ethyl (10 \( \mu \text{g ml}^{-1} \)) tolerance. Quantitative real-time PCR (qPCR) and PCR were used to detect the expected deletion and complementation strains.

### Multi-phenotypic assays

Phenotypic assays were performed to detect the potential heterogeneities in the phenotype of \( \Delta BbTFO1 \) compared with controls (WT and \( \Delta BbTFO1/BbTFO1 \)). All assays were repeated three times.

An appropriate amount of conidial suspension (1 \( \mu \text{l}, 10^7 \) conidia \text{ml}^{-1}, identical concentration hereafter) harvested from SDAY (4% glucose, 1% yeast extract, 1% peptone plus 1.5% agar) plates was spotted onto media containing CPZ (3% sucrose, 0.1% K\textsubscript{2}HPO\textsubscript{4}, 0.3%}

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**Table 1. Paired primers used for gene cloning, deletion, complement and expression.**

| Primers          | Paired sequences (5’-3’)* | Purpose                          |
|------------------|--------------------------|---------------------------------|
| TFO1up-F/R       | AAAGAATTCCGAGCATCATCCGAGACAGTGG/AAAGGATCGGAGGAGGAGGAGGAA | Cloning 5’ \( BbTFO1 \)          |
| TFO1dn-F/R       | AAAGCTAGGGATCCAGGAGGAGGAGGAA/AAAGGATCGGAGGAGGAGGAA   | Cloning 3’ \( BbTFO1 \)          |
| TFO1fl-F/R       | GGGGACCAAGTGGTACCCACCAACAGGTGGATAG/ GGGGACCAGCTTGGTCCACCAACAGATGGAAGA | Cloning full-length \( BbTFO1 \) |
| pTFO1-F/R        | AAATCTAGGGATCCAGGAGGAGGAA/AAAGGATCGGAGGAGGAGGAA   | PCR detecting \( BbTFO1 \)        |
| qTFO1-F/R        | GAGGATCCGGGTGCTCATA/CACTGGACACTTGGGATA             | qPCR detecting \( BbTFO1 \)        |
| q18S-F/R         | TGGTTTCTAGGACCACGGGAA/CTTGGCAAAATGGTTCGCG         | qPCR detecting 18S rRNA           |
| qcat1-F/R        | CGGTCTGGAATCAACTGGGAA/GCTGGCGGTGTGGAGAGGAGGAGGAA | qPCR detecting \( Bbcat1 \)        |
| qcat2-F/R        | CCTCTAGGGATGGGGTTTGCTCCCTTCG/CCGTGTGCTGGTCCCTGGT | qPCR detecting \( Bbcat2 \)        |
| qcat3-F/R        | GAGGAGCCACAGAACGACAGAGA/TTGAGCAGAAGGGGCCGCAATT | qPCR detecting \( Bbcat3 \)        |
| qcat4-F/R        | CGGGCTGGGTGCTCTTGGCATA/CCTCTGGGCGGTCTGGGAA        | qPCR detecting \( Bbcat4 \)        |
| qcat5-F/R        | GCTGGGCCTGATGGTCTGCTTGGCT/TTGCTGGTAAACGGGGTCGTGG | qPCR detecting \( Bbcat5 \)        |
| qcat6-F/R        | TCAAGTCGGTTAGAGAAGGAC/TTGGCTGGCTCTCAATCGGGATG     | qPCR detecting \( Bbcat6 \)        |
| qSol1-F/R        | CGGCAGCTCCCACATCCACACCTTCGG/CGCCACAGGAGGTAGGATAG  | qPCR detecting \( BbSol1 \)        |
| qSol2-F/R        | CCAGTGGTTGTGGATTGACGAT/TCAGGGGCTCTTGGCAGTATGG     | qPCR detecting \( BbSol2 \)        |
| qSol3-F/R        | TCTCCGGGCAAGATATAGGGGCC/TTTGGGCGATCTGGCCTTGGCT    | qPCR detecting \( BbSol3 \)        |
| qSol4-F/R        | CGAGAATGGCTTCCGCTAGGGCCGAGGAG/CGCCGAGGAGGGCCAGG | qPCR detecting \( BbSol4 \)        |
| qSol5-F/R        | CGGCAGCTCCCACACACACACACCC/CCGCGTACCGGATCCACACACC | qPCR detecting \( BbSol5 \)        |
| qOhmm-F/R        | CAGCATATACAGAAGGACCAC/GATGCACATCACCACCTT          | qPCR detecting \( BbOhmm \)       |

* Underlined regions suggest the sites of restriction enzyme in the \( BbTFO1 \) deletion mutant (EcoRI/BamHI and XbaI/BglII) or gateway fragments exchanged for the targeted \( BbTFO1 \) complementation mutant.

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NaNO₃, 0.05% MgSO₄, 0.001% FeSO₄, 0.05% KCl combined with 1.5% agar), SDAY and 1/4 SDAY (containing 1/4 of every SDAY nutrient). The diameter of each colony was determined, and photos were taken at day 7 after incubation at temperatures of 25, 30 and 35˚C. Additionally, the cross-sectional size of the colony was determined to be the growth rate index of diverse plates.

For chemical stresses, an appropriate amount of conidium suspension (1 μl) was added to CPZ plates containing different chemical stresses, including oxidative (2 mM H₂O₂ along with 0.2 mM menadione), cell wall (30 μg ml⁻¹ Congo red) and osmotic (0.5 M NaCl) stressors. The colony diameters were measured and photographed as described earlier, and a CPZ plate without any chemical stress was used as the control.

To better assess how OS affected deletion mutant growth, an appropriate amount of conidium suspension (1 μl) was added to CPZ media containing gradient menadione doses (5–50 μM) and H₂O₂ (1–4 mM). The diameters of all colonies under OS and the control were measured to calculate the colony area after 7 days of incubation at 25˚C. A minimum inhibitory concentration (MIC₅₀) value of H₂O₂ and menadione that suppressed 50% colony growth was adopted as the OS resistance index in every strain.

Several conidial properties related to biocontrol potential were detected. The conidiation on SDAY was measured according to a related description [19]. Briefly, an appropriate amount of conidial suspension (100 μl) was uniformly added onto SDAY media, followed by incubation at 25˚C. Then, three rounded cultures were collected from every plate every day by a cork borer (6 mm diameter) from day 5 onwards. The conidia on rounded cultures were completely added to 0.02% Tween-80 (1 ml). Later, conidial suspension content was assessed, followed by conversion to conidia count in the plate culture per unit area. In germination broth (GB: 0.5% peptone and 2% sucrose in 0.02% Tween-80), the median germination time (GT₅₀, h) necessary to achieve 50% conidial germination under shaking at 110 rpm and 25/30˚C was evaluated to be the conidial viability index at different temperatures [21]. The yield of blastospores was quantified in nitrogen-limited broth (NLB: 0.4% NH₄NO₃, 4% glucose, 0.3% MgSO₄, 0.3% KH₂PO₄). Briefly, 40 μl conidial suspensions were added to 20 ml of NLB and cultured at 25˚C with shaking (110 rpm). The number of blastospores was counted after 3 days, and then the blastospore production was transformed into cell count per ml culture medium.

Conidial thermotolerance was evaluated using a previously described method [22]. Conidia tolerance to high temperature was defined as the median lethal time (LT₅₀, min) when conidia were treated with 0–120 min heat stress exposure at 45˚C. In this process, 100 μl samples were taken from each tube at 15 min intervals, and then 1 ml of GB was added. Afterwards, the sample was subjected to 24 h of shaking at 110 rpm and 25˚C, and a cytometer was used to evaluate the conidial germination rate microscopically.

**Fungal virulence**

*Galleria mellonella* larvae were used to assay fungal virulence according to immersion and injection approaches. The larvae were soaked in conidia suspension for 10 seconds and dry paper towels were used to remove excess liquid from the larvae. For intraheamocoel injection, 1 μl conidial suspension (5 × 10⁶ conidia ml⁻¹) was added to the larval haemocoel. The two control groups were exposed to sterile 0.02% Tween-80. Each experiment was carried out three times, with 30 larvae being adopted in every experiment. Moreover, the number of dead larvae was measured at intervals of half a day. After analysing the time-mortality trend, the LT₅₀ (d) for every strain was determined against every larval group. After curing under the optimal conditions for 6 days, the fungal growth on the surface of the mummified cadavers...
was photographed. During fungal virulence assessment, haemolymph samples were extracted from larvae that survived 72 h after injection, and hyphal bodies were formed in vivo, as seen by laser scanning confocal microscopy (LEICA DMi8, Germany).

**Transcript profile analysis of antioxidant-related genes**

The CAT family, SOD family and *Bbohnn* involved in OS responses were analysed by qPCR. In brief, 100 μl of conidial suspensions were inoculated into CPZ medium containing H₂O₂ (2 mM). After growing at 25°C for 5 days, an RNAiso Plus Kit (TaKaRa, Dalian, China) was utilized to extract total RNA from Δ*BbTFO1* and the WT strain. Then, a PrimeScript<sup>RT</sup> reagent kit (TaKaRa) was utilized for cDNA synthesis by reverse transcription. The resultant cDNA was then diluted at 10 ng ml<sup>-1</sup> and adopted as a template in qPCR by the use of paired primers (Table 1) [23]. For *B. bassiana*, 18S rRNA was applied as the internal standard of each transcript. The gene transcriptional level was determined and repeated three times. The relative transcription level of the antioxidant-related genes was calculated as the fold change of the mutant strain transcript relative to the WT strain transcript in stress culture.

**Statistical methods**

One-way ANOVA, as well as Tukey’s HSD test, were used to analyse phenotype heterogeneities in the WT compared with control strains.

**Results**

**BbTFO1 characteristics in and generation of BbTFO1 mutants**

Using the NDT80 sequence of *S. cerevisiae* as a query, the annotated *B. bassiana* ARSEF 2860 strain genome was searched against the NCBI database, generating the most highly related sequence of 502 amino acids (identity: 31%; E-value: 3e-05). Phylogenetic analysis (S1A Fig) showed that the BbTFO1 sequence had the closest relationship with NDT80 from *N. crassa* (XP 011394327) and *T. reesei* (XP 006961996) because they were located on the same branch. Similar to the transcription factor NDT80 of *S. cerevisiae*, BbTFO1 had an NDT80/PhoG-like DNA-binding domain (residues 131–318). Successful Δ*BbTFO1* deletion and complementation were verified through PCR and qPCR using paired primers to examine correct recombination events (S1B–S1D Fig). Additionally, *BbTFO1* transcription was not detected in the deletion strains, but similar transcription levels were detected in the WT and complementary strains (S1D Fig).

**Contribution of BbTFO1 to chemical stress tolerance**

The growth of deletion, complementation, and WT strains in different nutrient and chemical stress media is shown in Fig 1. The colony size of deletion strains was similar to that of control strains, and there was no significant difference with those in the rich medium SDAY, nutrient-deficient medium 1/4 SDAY, or minimal medium CPZ (Tukey’s HSD, *P* > 0.05) (Fig 1). Similar results were also found in CPZ medium containing NaCl and Congo red (Tukey’s HSD, *P* > 0.05). Nevertheless, Δ*BbTFO1* showed markedly higher sensitivity to OS than the control strains (Tukey’s HSD, *P* < 0.05). The above findings indicate that BbTFO1 plays an essential role in maintaining OS tolerance but not in responding to other nutritional and chemical stresses.
Transcriptional changes in antioxidant-related genes in ΔBbTFO1

The colony size of deletion strains decreased by 41% and 63% compared with the control in CPZ medium containing menadione and H$_2$O$_2$, respectively (Fig 2A). However, the colony size of the deletion strain was not significantly different from that of the control in CPZ medium (Fig 2A). ΔBbTFO1 showed markedly high sensitivity to OS compared with controls. According to the MIC$_{50}$ (Fig 2B), the deletion mutant also displayed high sensitivity to the oxidants H$_2$O$_2$ (19%) and menadione (23%). To explain the reduced tolerance of ΔBbTFO1 to oxidative stress, the transcriptional levels of some antioxidant-related genes were analysed by qPCR. Among them, four of six CAT family genes and Bbohmm showed transcriptional down-regulation compared with WT. Significantly, two key catalases, cat2 and cat5, were transcriptionally repressed by 68% and 54%, respectively (Fig 2C), in response to OS in ΔBbTFO1 [23]. However, three of five SOD family genes showed transcriptional upregulation compared with WT. These results suggest that ΔBbTFO1 sensitivity to OS is accompanied by the transcriptional downregulation of these two key catalases.

Role of BbTFO1 in conidiation and conidial quality

For conidiation, the ΔBbTFO1 strain was decreased by 44%, 22% and 21% on days 5, 6, and 7 (Fig 3A), respectively, which was significantly different from the controls (Tukey’s HSD, $P < 0.05$). However, final ΔBbTFO1 conidiation was similar to that of the control strain (Tukey’s HSD, $P > 0.05$). These results showed that the deletion of BbTFO1 affected conidiation growth but not conidiation ability. Moreover, the production of blastospores at 25˚C showed no significant difference in ΔBbTFO1 compared with the corresponding controls (Tukey’s HSD, $P > 0.05$, Fig 3B).

Despite having no impact on conidiation capacity and the production of blastospores, deletion of BbTFO1 exerts a marked influence on conidial germination and thermotolerance. The ΔBbTFO1 mutant showed GT$_{50}$ values of 2.1 h and 2.5 h longer than those of the WT at 25˚C.
and 35˚C, respectively (Fig 3C). The colony sizes of ΔBbTFO1 decreased by 26% and 65% compared with WT at 30˚C and 35˚C, respectively (Fig 3D). Moreover, heat stress resistance of ΔBbTFO1 at 45˚C decreased significantly compared with that of the WT strain, and its LT50 decreased by 67% (Fig 3E). These data revealed the role of BbTFO1 in conidial germination and its contribution to thermotolerance but not in the production of blastospores.

**Contribution of BbTFO1 to insect virulence**

For normal infection via the cuticular penetration method, time-mortality trends and means (± SD) LT50 of G. mellonella larvae following conidial suspension immersion are shown in Fig 4A and 4B. On the ninth day after immersion, the mortality of the WT and complementation strain was close to 100%, while that of ΔbTFO1 was approximately 80% (Fig 4A). The mean (± SD) LT50 values of the WT and complementation strains against larvae were 6.0 ± 0.2 and 5.9 ± 0.2 days via normal cuticle penetration (Fig 4B), respectively. The mean (± SD) LT50 values of the ΔbTFO1 mutant against larvae were extended until 7.3 ± 0.3 days (Fig 4B). For intrahemocoel injection, the mortality of the WT and complementation strains were close to

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Fig 2. Disruptions to BbTFO1 affect OS resistance and antioxidant-related enzyme gene expression in B. bassiana. (A) Colony size was measured on the seventh day of culture at 25˚C after inoculation. (B) MIC50 estimates for two oxidants (H2O2 and menadione) to inhibit 50% fungal growth in diverse strains on CPZ at 25˚C. (C) Relative antioxidant-related gene transcript levels of ΔBbTFO1 strains compared with WT cultured for 5 days at 25˚C on CPZ containing H2O2 (2 mM). The bar marked with an asterisk in each group shows a significant difference (Tukey’s HSD, P < 0.05). Error bars: SD of 3 repeated assays. 

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100% on the fifth day after injection (Fig 4C); the mean (± SD) LT$_{50}$ values of the WT and supplementation were 3.6 ± 0.2 and 3.4 ± 0.1 days, respectively; and the mean LT$_{50}$ of ΔBbTFO1 strains against the larvae increased to 4.0 ± 0.1 days (Fig 4D). After 6 days of preservation under the optimal conditions, the fungal growth on larvae infected with the control strain was greater than that on the larvae killed by ΔBbTFO1 (Fig 4E).

To probe the potential reason for the reduced virulence by cuticle-bypassing infection with ΔBbTFO1, haemolymph samples obtained from larvae that survived 72 h after injection were analysed. As a result, there were rich hyphal bodies in the control strains but rare hyphal bodies within the haemolymph samples from the larvae following injection with ΔBbTFO1 conidia (Fig 4F).

**Discussion**

Compared with the controls, the ΔBbTFO1 strains showed significant defects in tolerance to OS, together with certain typical defects in phenotype related to the biological control potential of fungi, such as virulence and conidial quality. Typically, two catalase genes were markedly
downregulated in the deletion mutants. The complicated phenotypic changes caused by the deletion of *BbTFO1* are discussed below.

First, *B. bassiana* was subjected to biotic and abiotic OS in the field [24], which negatively affects the insect host infection capacity of fungi [25, 26]. Recently, more than 24 antioxidant enzymes showing dispersion within diverse families have been analysed for *B. bassiana* [27]. Some SODs in entomopathogenic fungi have been demonstrated to be crucial factors of fungal biocontrol potential [28]. Five members of the SOD family characterized in *B. bassiana* showed that Sod2 and Sod3 played a dominant role in fungal SOD activity [28, 29]. Each type of peroxide can be decomposed by members of the peroxidase (POD) family, including larger hydrogen peroxide, H$_2$O$_2$ and xenobiotics. KatG1, a bifunctional catalase-peroxidase, has been
suggested to display POD and CAT activities, which play a vital role in chemical OS tolerance in *Metarhizium acridum* [24]. Both the GRX-GLR and TRX-TRR families have been examined to analyse their impacts on the antioxidation of *B. bassiana* [30, 31]. Some strong antioxidant genes have been adopted for entomopathogenic fungal genetic engineering for the sake of enhancing biological control abilities [32]. Overexpression of cytoplasmic manganese core superoxide dismutase (BbSod2) in *B. bassiana* significantly increased antioxidant capacity, UV-B virulence and tolerance [33].

In addition, catalase family members decompose H₂O₂. Knockout mutants of catalases have shown discrete phenotypes in susceptibility to oxidative, heat, and UV-B stress in *B. bassiana* [34]. Among them, *cat5*, *cat1* and *cat4* are influential virulence factors, while *cat1* and *cat4* show equal importance to the regulation of UV-B resistance of conidia, with *cat2* ranking second. In addition, *cat1* is the most important regulator of conidial heat tolerance. It is believed that the CAT family affects fungal virulence by changing other virulence-related phenotypes of *B. bassiana* [23, 27]. Prior studies have shown that total catalase activity decreased by 89% and 56% in Δ*cat2* and Δ*cat5*, respectively, while this decrease was only 9–12% in additional *B. bassiana* catalase knockout mutants [23]. *cat2* and *cat5* exert viral parts in *B. bassiana* antioxidation of catalase family [23]. Moreover, *BbTFO1* deletion led to four of six CAT family genes showing transcriptional downregulation compared with the WT. In particular, the two key catalases *cat2* and *cat5* were transcriptionally repressed by 68% and 54%, respectively. In Δ*BbTFO1*, the defect of colony growth under OS may be due to the repression of catalase gene expression. Therefore, it was speculated that *BbTFO1* can contribute to fungal antioxidation by affecting the expression of catalase genes. Beyond expectation, the absence of *BbTFO1* did not increase the sensitivity to cell wall stressors in *B. bassiana*, while for additional fungi, such as *C. albicans*, *NDT80* deletion resulted in enhanced sensitivity to Congo red and SDS [2]. The lack of *NDT80* resulted in a denser biofilm relative to WT and might initiate the *TEC1*-dependent compensatory response, mainly through the *TEC1*-ROBI pathway [35].

Second, according to a previous report in *C. albicans* [2], *NDT80* deletion mutants also showed sensitivity to heat stress in *B. bassiana*. Heat stress could promote cellular ROS generation, thereby damaging certain biomolecules, such as DNA, lipids or proteins [33, 36]. Antioxidant enzymes can scavenge diverse intracellular ROS [27, 37]. Typically, SOD can decompose superoxide anions and produce H₂O₂ and oxygen [38], while H₂O₂ can be additionally decomposed to oxygen and water via catalase [39]. In the present work, 4 catalase gene expression levels, especially *cat2* and *cat5*, in Δ*BbTFO1* were repressed, resulting in a decrease in fungal ability to decompose H₂O₂, which naturally caused the reduced tolerance of Δ*BbTFO1* conidia to high temperature. In addition, *BbTFO1* knockout mutants were demonstrated to suffer defects in the development of conidiation and conidial germination. Similarly, the XprG mutant was slightly slower to initiate conidiation development in *A. nidulans* [4]. Conidial quality is also considered an essential virulence factor of *B. bassiana* [40]. In this study, defects in conidial quality may be one of the reasons for the decrease in virulence.

Finally, *BbTFO1* mutants showed reduced virulence to hosts in *B. bassiana* by immersion and injection, as observed in *NDT80* mutants of *C. albicans*. The virulence loss of *NDT80* mutants in *C. albicans* may be associated with the critical part of *NDT80* in the control of hyphal growth and stress resistance [2, 8]. An *NDT80* homologue in *F. oxysporum* (Suf, suppressor of fusion) is associated with horizontal transport of virulence gene-carrying small chromosomes under conditions with limited nutrients [10]. For *B. bassiana*, the host immune response will produce cytotoxic molecules such as ROS when it infects insects [41]. Host phagocytes also generate reactive oxygen intermediates (ROIs), which are toxic to a variety of microorganisms [42]. Based on this experiment, it was believed that the disruption of *BbTFO1* leads to a reduction in the transcription levels of four catalases and complex phenotypic
changes, which are responsible for the reduction in fungal virulence. Catalase is considered a potential virulence factor in pathogenic fungi [23]. Overexpression of one catalase led to increased virulence of *B. bassiana* [43]. Catalase deletion within *A. fumigatus* mycelium and conidia leads to a significant decrease in virulence to mice [44]. In *Magnaporthe grisea*, the absence of *cat2* enhances fungal sensitivity to OS and significantly decreases virulence to barley [45]. An NDT80-like protein was further examined in *A. fumigatus*, a pathogenic fungus in animals, showing that XprG makes no difference in the virulence of mice [9]. The deletion of *BbTFO1* leads to defects in antioxidant capacity and germination defects, indicating that *BbTFO1* is a critical virulence factor of *B. bassiana*.

**Conclusion**

Our results confirmed the previously reported functional diversity of NDT80-like proteins in different fungi [7]. In addition, *BbTFO1* plays a crucial role in antioxidant activity, thermotolerance, conidiation, conidial quality and virulence in *B. bassiana*. According to transcript analysis, *BbTFO1* can positively affect the expression of two key catalases to influence the antioxidant capacity of fungi, following the impact of the *B. bassiana* biological control potential.

**Supporting information**

S1 Fig. Bioinformatics description of *BbTFO1* and generation of its mutant strains. (A) Phylogenetic tree of *B. bassiana* NDT80 homologues and additional fungi. The NCBI accession codes for all proteins, together with the sequence identities in *B. bassiana*, are provided in brackets after the fungal names. (B) Sketch map of *BbTFO1* deletion strategy. (C) *BbTFO1* deletion identification (lanes 1–3) by PCR. Lane M: DNA marker (bp). Lane CK: blank control. Lane 1: WT. Lane 2: Δ*BbTFO1*. Lane 3: Δ*BbTFO1*/BbTFO1. (D) Identification of *BbTFO1* deletion by qPCR.

**Author Contributions**

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**Methodology:** Juan-Juan Wang.

**Software:** Juan-Juan Wang, Wen Cheng.

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**Writing – review & editing:** Lei Qiu.

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