The Tudor Domain-Containing Protein BbTdp1 Contributes to Fungal Cell Development, the Cell Cycle, Virulence, and Transcriptional Regulation in the Insect Pathogenic Fungus Beauveria bassiana

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ABSTRACT Beauveria bassiana is an insect pathogenic fungus that serves as a model system for exploring the mechanisms of fungal development and host-pathogen interactions. Clinical and experimental studies have indicated that SND1 is closely correlated with the progression and invasiveness of common cancers as a potential oncogene, but this gene has rarely been studied in fungi. Here, we characterized the contributions of an SND1 ortholog (Tdp1) by constructing a BbTdp1 deletion strain and a complemented strain of B. bassiana. Compared with the wild-type (WT) strain, the ΔBbTdp1 mutant lost conidiation capacity (~87.7%) and blastospore (~96.3%) yields, increased sensitivity to chemical stress (4.4 to 54.3%) and heat shock (~44.2%), and decreased virulence following topical application (~24.7%) and hemocoel injection (~40.0%). Flow cytometry readings showed smaller sizes of both conidia and blastospores for ΔBbTdp1 mutants. Transcriptomic data revealed 4,094 differentially expressed genes (log2 ratio > 2 and q value of <0.05) between ΔBbTdp1 mutants and the WT strain, which accounted for 41.6% of the total genes, indicating that extreme fluctuation in the global gene expression pattern had occurred. Moreover, deletion of BbTdp1 led to an abnormal cell cycle with a longer S phase and shorter G2/M and G0/G1 phases of blastospores, and enzyme-linked immunosorbent assay confirmed that the level of phosphorylated cyclin-dependent kinase 1 (Cdk1) in the ΔBbTdp1 strain was ~31.5% lower than in the WT strain. In summary, our study is the first to report that BbTdp1 plays a vital role in regulating conidial and blastospore yields, fungal morphological changes, and pathogenicity in entomopathogenic fungi.

IMPORTANCE In this study, we used Beauveria bassiana as a biological model to report the role of BbTdp1 in entomopathogenic fungi. Our findings indicated that BbTdp1 contributed significantly to cell development, the cell cycle, and virulence in B. bassiana. In addition, deletion of BbTdp1 led to drastic fluctuations in the transcriptional profile. BbTdp1 can be developed as a novel target for B. bassiana development and pathogenicity, which also provides a framework for the study of Tdp1 in other fungi.

KEYWORDS entomopathogenic fungi, SND1, cell cycle, pathogenicity

As a widely used insect pathogenic fungus, Beauveria bassiana has become a novel model organism for the analysis of fungal development, pathogenesis, and interactions with hosts (1, 2). Transcription factors (TFs) are considered critical proteins involved in the pathogenicity of fungi (3). B. bassiana TFs are widely distributed and...
implicated in multiple biological processes (4, 5). For example, the MADS-box protein Mcm1 serves as the central TF in cell cycle regulation, and deletion of Mcm1 represses the expression of corresponding cellular event-involved genes, which further leads to abnormal cell development, morphology, and pathogenesis as well as a disordered cell cycle (6). BbMbp1, a component of the MluI cell cycle box-binding complex, has been shown to significantly contribute to fungal morphological changes, cell development, and virulence by regulating the transcription of the downstream targets BbCwp and BbImp (7). Furthermore, the Zn(II)2Cys6 TF Thm1 specifically functions in mediating heat and cell wall stress by altering membrane structure to participate in virulence (8).

As key upstream activators of the central developmental pathway, BrlA and AbaA are considered major controllers of conidia production, dimorphic transition, and insect pathogenicity (9); however, there are still many unreported TFs involved in the regulation of fungal pathogenicity. Research on these TFs will help improve the fungal pathogenic regulatory network and enhance fungal biocontrol potential.

Tudor domain-containing protein (Tdp1; a homologous protein of SND1) has been proven to be an evolutionarily conserved regulator composed of four staphylococcal nuclease (SNC) domains, providing endonuclease activity for SND1 and a tudor domain with the extraordinary ability to interact with nucleic acids and proteins (10). Originally, SND1 as a transcriptional coactivator was relevant for the regulation of gene expression-related processes as well as for the mediation of the dynamic balance of proteins and lipids (10, 11). SND1 has also been shown to participate in cell cycle regulation by mediating the G1/S phase transition (12, 13). Over the years, clinical and experimental studies have shown that, as a potential oncogene, SND1 expression is positively correlated with the progression and invasiveness of many common cancers, suggesting that SND1 may be a novel candidate gene for tumor therapy and an important indicator of prognosis (11, 14). Moreover, in Drosophila melanogaster, SND1 recognizes and binds to methyl-arginine/lysine residues to significantly participate in various epigenetic interactions, gene expression, and the regulation of various small RNAs (15). In Entamoeba histolytica, SND1 has been confirmed to be a multifunctional protein involved in transcription, stress responses, signaling, metabolism, and human pathogenicity (16, 17).

However, SND1/Tdp1 has rarely been studied in fungi. Hence, we used B. bassiana to illuminate the roles of Tdp1 through polymorphic phenotypic, pathogenicity, and transcriptional analyses of a BbTdp1 disruption mutant (ΔBbTdp1) compared to control strains, including the wild-type (WT) strain and a complemented strain (ΔBbTdp1/BbTdp1). Our results demonstrated that Tdp1 is required to maintain fungal cell growth, morphological changes, and the cell cycle and also participates in infection processes.

RESULTS

Identification of BbTdp1 in B. bassiana and generation of its mutant strains. BbTdp1 (locus tag, BBA_02313) encoded a putative 883-amino acid protein containing four SNC domains and one tudor domain in B. bassiana (Fig. S1A in the supplemental material). Phylogenetic analysis revealed that BbTdp1 had higher homology in fungi belonging to entomopathogens and phytopathogens than homology in animal pathogens and yeast fungi (Fig. S1B), but no orthology was observed in Saccharomyces cerevisiae. To explore the roles of BbTdp1 in B. bassiana, the ΔBbTdp1 and ΔBbTdp1/BbTdp1 strains were generated as described in Materials and Methods. The results were verified through PCR amplification and quantitative real-time PCR analysis (Fig. S1C and D).

Effect of BbTdp1 on colony growth and hypha extension. After culturing for 10 days, colonies on the medium of different nutrient sources showed obvious differences. For the enrichment medium (SDAY), the ΔBbTdp1 colony was ~24.7% larger than the WT colony, whereas the hyphal dry weight of the ΔBbTdp1 colony grown for 10 days was ~37.7% lighter than that of the WT. For the sterile medium (CZA), the colony areas of the ΔBbTdp1 strain displayed a decrease of ~71.8% compared with the WT, and, similarly, the hyphal biomass of the ΔBbTdp1 strain was ~49.0% lighter than that of the WT (Fig. 1A to C). In terms of hyphal micromorphology, ΔBbTdp1-mutant hyphae were thinner than those of the WT strain and ΔBbTdp1/BbTdp1 strains, and the...
stained ΔBbTdp1-mutant hyphae showed sparser septa than those of the WT under the fluorescence field (Fig. 1A). All the above data indicated abnormal colony growth and hyphae extension in the ΔBbTdp1 strain.

Roles of BbTdp1 in conidial development and quality. To investigate the effects of BbTdp1 ablation on conidial development and quality, we performed a multiphenotypic analysis. For the ΔBbTdp1 strain, conidiation was significantly lower than that of the WT strain from day 4 to day 7, and this gap reached ~87.7% on day 6 (Fig. 2A), which also could be confirmed by analysis of the spore balls (Fig. 2B). Confocal microscopy showed that the size of the ΔBbTdp1-mutant conidia was smaller than that of the WT conidia (Fig. 2B), which was in agreement with the flow cytometry (FCM) readings showing that conidial size decreased by ~28.6% in the ΔBbTdp1 strain compared to the WT strain (Fig. 2C).

The half-germination time (GT50; an indicator of germination ability) of the ΔBbTdp1 strain was ~39.3% more delayed than that of the WT (Fig. 2D), which meant that the ΔBbTdp1 strain required more time for conidial germination than WT (Fig. 2B). Conidial surface hydrophobicity, a cell surface characteristic, has become a vital factor for conidial infection of fungi against hosts (18). The test results showed that the conidial hydrophobicity of the ΔBbTdp1 strain (0.56 ± 0.02) was ~36.4% lower than that of the WT strain (0.88 ± 0.02) (Fig. 2E).

Stress response experiments were performed to measure the cellular sensitivity of the ΔBbTdp1-mutant strain and two control strains to osmolarity (NaCl), oxidation (H2O2), cell wall disturbance (Congo red [CR]), or fungicide (carbendazim [CAR]) chemical stress using the relative inhibition rate. Compared with the control strains, the ΔBbTdp1 strains showed more sensitivity to chemical stress. For the relative inhibition rate, the measurement results demonstrated that NaCl, H2O2, CR, and CAR were ~45.6%, ~54.3%, ~33.2%, and ~4.4% more inhibitive, respectively, to the ΔBbTdp1 strain on day 10 (Fig. 2F). Conidial thermostolerance was evaluated by the half-lethal time (LT50) after heat shock at 45°C. The LT50 of the ΔBbTdp1 strain (23.6 ± 0.7 min) was ~44.2% lower than that of the WT strain (42.3 ± 1.3 min), which meant that the ΔBbTdp1 strain was more sensitive to thermal stress (Fig. 2G).
Contribution of BbTdp1 to blastospore yield and size. After successful infection, *B. bassiana* survived in insect hemocoels as unicellular blastospores. We used nitrogen-limited broth (NLB) medium to simulate the insect hemocoels for producing blastospores, and the results showed that the blastospore yield of the ΔBbTdp1 strain was far below the yield observed in the WT strain. When cultivated for 3 days, the blastospore yield of the ΔBbTdp1 strain was \(96.3\%\) less than that of the WT strain (Fig. 3A).

Furthermore, from the FCM readings, the blastospore size of the ΔBbTdp1 strain was \(33.8\%\) smaller than that of the WT strain (Fig. 3B). In addition, confocal microscopy revealed a small blastospore size for ΔBbTdp1 mutants, and the blastospores also had an ellipsoidal shape, whereas the normal blastospores of the WT strain appeared fusiform (Fig. 3C).

Requirements of BbTdp1 for virulence. Topical application and hemocoel injection were used to distinguish the resistance of insect cuticle. Regardless of whether
infection occurred in vivo or in vitro, the larvae infected by the \( \Delta BbTdp1 \) mutant died significantly more slowly than larvae infected with the two control strains (Fig. 4A and B). For topical application, the LT\(_{50} \) was 7.3 ± 0.9 days for \( \Delta BbTdp1 \) mutants, which was ~24.7% longer than that observed in WT (5.5 ± 0.2 days) (Fig. 4C); moreover, for hemocoel injection, the LT\(_{50} \) of \( \Delta BbTdp1 \) mutants was 6.0 ± 0.5 days, which was ~40.0% longer than that of WT (3.6 ± 0.3 days) (Fig. 4D). At 3 days postinjection, the insect hemo-lymph was removed and observed. \( \Delta BbTdp1 \) strains produced a smaller quantity of in vivo blastospores and shorter mycelia than the control strains (Fig. 4E). When the insect cadavers were cultured to day 5, the hyphal growth of the larval surface infected by the \( \Delta BbTdp1 \) strains was significantly slower than that of the larvae killed by the control strains (Fig. 4E).

**Analysis of the BbTdp1 transcriptional profile.** High-throughput RNA sequencing was used to analyze the differences between the WT strain and \( \Delta BbTdp1 \) mutants. All the clean tags, which corresponded to a total of 9,852 genes obtained after filtering, were mapped to the genome of *B. bassiana* (19); among them, 9,305 genes were shared by both strains. The transcriptomic data indicated that the disruption of *BbTdp1* led to an altered expression pattern of 4,094 differentially expressed genes (DEGs; \( |\log_2 \) ratio\( | > 2 \) and \( q \) value of \( < 0.05 \)), with 3,499 downregulated and 595 upregulated genes in the \( \Delta BbTdp1 \)-mutant strain compared with the WT strain (Fig. 5A). The DEGs accounted for 41.6% of the total number of genes, which is a very large fluctuation in the transcriptional profile, suggesting that the deletion of *BbTdp1* caused a drastic fluctuation to strain function.

Functional category (FunCat) analysis revealed that the downregulated DEGs (Fig. 5B; Table S2) were enriched in the following categories: (i) protein with binding function or cofactor requirement (structural or analytic), including genes involved in RNA binding; (ii) transcription, including genes related to rRNA synthesis, rRNA processing, tRNA processing, and tRNA modification; (iii) protein synthesis, including massive numbers of genes associated with translation, ribosomal proteins, and ribosome biogenesis (e.g., ribosomal proteins, eukaryotic translation initiation factors, tRNA
synthetases, helicases, and WD domain-containing proteins); and (iv) metabolism, including many genes associated with nucleotide degradation and metabolism. The upregulated DEGs in the \( \Delta BbTdp1 \)-mutant strain were enriched only in secondary metabolism within the metabolism category; these genes were members of the cytochrome P450 family or encoded major facilitator superfamily transporters and ABC multidrug transporters (Fig. 5B; Table S3).

For Gene Ontology (GO) classification, the total DEGs were divided into three primary categories, including molecular function, biological process, and cellular component (Fig. 5C). At the level of molecular function, most DEGs were mapped into the terms of catalytic activity, binding, transcription regulator activity, and transporter activity, and at the biological process level, there were more DEGs belonging to metabolic process and cellular process (Fig. 5C). Moreover, DEGs associated with cell, membrane, and organelle were classified at the level of cellular component (Fig. 5C). Based on the analysis of the above GO classification, the deletion of \( BbTdp1 \) may lead to abnormalities of enzyme catalytic activity, metabolic processes, and cellular components. In terms of KEGG pathway analysis, both downregulated DEGs and upregulated DEGs were almost sorted into nine pathways covering global and overview maps, carbohydrate metabolism, transport and catabolism, signal transduction, translation, amino acid metabolism, lipid metabolism, folding, sorting, and degradation, and metabolism of cofactors and vitamins (Fig. 5D).

From the analysis of the transcriptome, knockout of \( BbTdp1 \) caused the suppression of a large proportion of genes involved in the metabolism of carbohydrates, amino acids, and lipids, catalytic activity, protein synthesis and transport, and signal transduction, which suggested global regulation by \( BbTdp1 \) in \( B. bassiana \).
Ablation of BbTdp1 alters the cell cycle. Fluorescence-activated cell sorter (FACS) analysis revealed that in terms of the blastospore cell cycle, D BbTdp1 mutants had a significantly (~65.6%) longer S phase, an ~54.4% shorter G2/M phase, and an ~12.6% shorter G0/G1 phase than the control strains, suggesting that more D BbTdp1-mutant cells were blocked in the S phase than the other phases and they failed to enter the G2 phase (Fig. 6A).

To further verify the role of BbTdp1 in the cell cycle, we used enzyme-linked immunosorbent assay (ELISA) technology to detect the levels of phosphorylated and unphosphorylated cyclin-dependent kinase 1 (Cdk1), which are necessary to maintain the normal cell cycle in B. bassiana (20). The results showed that the concentration of...
phosphorylated Cdk1 in the ΔBbTdp1 strain was ~31.5% lower than in WT, while the concentration of nonphosphorylated Cdk1 showed no significant difference among the three strains (Fig. 6B).

**DISCUSSION**

Similar to other SND1 orthologs (e.g., TSN, Tudor-SN, and p100), BbTdp1 contains four SNc domains and one tudor domain. As a transcription coactivator, SND1 is considered to be involved not only in the regulation of gene expression, such as RNA splicing, interferon-stability, and editing, but also in the control of protein and lipid homeostasis (10). In human cells, mature functional microRNAs (miRNAs) are degraded by SND1, which promotes the progression of the cell cycle, indicating that targeting SND1 nuclease activity could inhibit cell proliferation (13). Clinically, SND1 is considered a marker of malignant cancer because it is overexpressed in a variety of primary tumors and invasive cancer cells (14, 21). Here, BbTdp1 was characterized to be involved in the mediation of cell development, the cell cycle, and virulence and causes various alterations in the transcription patterns of corresponding cellular event-associated genes in *B. bassiana*.

In nature, *B. bassiana* mainly exists in the form of aerial conidia whose yield and quality are extremely important for fungal pathogenicity (1, 22). Lower conidiation in ΔBbTdp1-mutant strains than in WT strains may be associated with the downregulation of the transcriptional activity of related genes (Table 1). FluG proteins are required for the synthesis of the extracellular conidiation-inducing factors, and the FluG-null mutant showed profuse aerial growth but no conidiation (23). The conidiation process is also controlled by a central developmental pathway composed of the BrlA, AbaA, and WetA (Table 1) regulatory factors in *B. bassiana* (9). As a C2H2-zinc-finger-type TF, BrlA controls the progression and completion of conidiation (9, 24). AbaA proteins with a TEA/ATTS DNA-binding motif are considered dimorphic transition switches with BrlA proteins (9), and WetA has a conserved ESC/WetA-related domain that contributes to conidiation and secondary metabolism (25). In addition, delayed germination and

**FIG 6** Examination of fungal cell cycle. (A) Cell cycle (G0/G1, S, and G2/M phases) of blastospores indicated by unduplicated (1C), duplicated (2C), and intermediate DNA content profiles according to FACS analysis. (B) The concentration of phosphorylated and nonphosphorylated Cdk1 protein (P-Cdk1 and NP-Cdk1, respectively) in each strain measured by ELISA. Error bars represent SDs of three replications (*, \( P < 0.05 \)). (C) The knockout of BbTdp1 destroyed the balance ratio between phosphorylated and unphosphorylated Cdk1, causing alterations of the cell cycle of ΔBbTdp1-mutant fungal cells.
relatively small cell size were observed for the $\Delta BbTdp1$ strain, which may be the reason for the decreased virulence. The positive correlation of pathogenicity with spore size and germination speed has been confirmed to be highly significant (26). In *Mucor circinelloides*, strains with larger spores were more virulent, which may be associated with the germination ability of spores (27). When the pathogenicity of different *Mucorales* species to *Galleria mellonella* was investigated, a positive correlation was found between spore size and virulence for the same species (28).

In the insect hemocoel, entomopathogenic fungi are present in the form of unicellular blastospores and fight against the host immune system through cell proliferation (29, 30). When blastospores were cultured under simulated conditions, the yield of blastospores was dramatically reduced in the $\Delta BbTdp1$ mutant, indicating that $BbTdp1$ contributes to blastospore proliferation. In previous studies of oncogenes, SND1 was found to be highly expressed during the stage of malignant proliferation and aggressiveness of many common human cancer cells (10). In colon cancer, SND1 overexpression significantly accelerated cell proliferation in the exponential growth phase (31). In hepatocellular carcinoma, SND1 has been shown to be involved in controlling cell growth and proliferation (32). Furthermore, compared with the WT strain, the $\Delta BbTdp1$-mutant strain displayed a longer S phase and shorter G2/M and G0/G1 phases of blastospores, demonstrating that the disruption of $BbTdp1$ induced cell cycle arrest during the S phase and delayed entry into the G2/M phase (Fig. 6C). In human tumor cells, Cdk1 promoted the phosphorylation of ribose-phosphate diphosphokinase 1 (PRPS1) at S103, and the loss of phosphorylation at S103 altered the cell cycle, which was similar to the alteration of the cell cycle observed in $\Delta BbTdp1$ mutants (33). In *Schizosaccharomyces pombe*, the Ser/Thr protein kinase Cdk1 is an important factor in the cell cycle pathway that controls the G2/M phase transition by mediating the

### TABLE 1 Associated genes identified from the BbTdp1-mediated transcriptional profile

| Gene category/name                   | Locus tag | Annotation                        | log$_2$ ratio | Q-value       |
|--------------------------------------|-----------|-----------------------------------|---------------|---------------|
| Conidial development                 | FluG      | BBA_04942 FluG protein            | −8.6          | 9.65e-81      |
|                                      | BrlA      | BBA_07544 zinc finger protein      | −4.7          | 0             |
|                                      | AbaA      | BBA_00300 FoabaA-like protein      | −6.2          | 0             |
|                                      | WetA      | BBA_06126 hypothetical protein     | −5.4          | 0             |
| Blastospore proliferation            | Cdk1      | BBA_02861 Cyclin-dependent protein kinase | −2.9          | 0             |
|                                      | Wee1      | BBA_08543 mitosis inhibitor protein kinase | −1.0          | 2.94e-740     |
| Cell wall integrity                  | Hyd1      | BBA_03015 class I hydrophobin      | −5.9          | 0             |
|                                      | Pmt1      | BBA_07762 mannosyltransferase      | −2.9          | 0             |
|                                      | Pmt2      | BBA_07508 mannosyltransferase      | −2.4          | 0             |
|                                      | Pmt4      | BBA_00053 O-mannosyl-transferase   | −2.4          | 0             |
|                                      | Ktr1      | BBA_07003 $\alpha$-1,2-mannosyltransferase | −1.8          | 2.07e-192     |
|                                      | Ktr4      | BBA_00842 $\alpha$-1,2-mannosyltransferase | −3.5          | 0             |
|                                      | Kre2      | BBA_08768 glycolipid 2-alpha-mannosyltransferase | −3.0          | 0             |
| Insect epidermal degradation          | CDEP-1    | BBA_00443 cuticle-degrading protease | −0.9          | 0             |
|                                      | Chi2      | BBA_02381 endochitinase Chi2       | −2.9          | 0             |
| Superoxide dismutation                | Sod1      | BBA_02311 Cu/Zn superoxide dismutase | −3.1          | 0             |
|                                      | Sod2      | BBA_09706 cytosol Mn-superoxide dismutase | −0.8          | 1.65e-247     |
|                                      | Sod3      | BBA_09382 mitochondrial manganese superoxide dismutase | −1.8          | 0             |
|                                      | Sod4      | BBA_04317 superoxide dismutase     | −1.4          | 7.52e-141     |
|                                      | Sod5      | BBA_01984 superoxide dismutase     | −1.9          | 0             |
| Secondary metabolism                 | Ops1      | BBA_08179 polyketide synthase      | −1.2          | 6.11e-17      |
|                                      | BalS      | BBA_02630 bassianolide nonribosomal peptide synthetase | −4.4          | 2.23e-7       |
phosphorylation of Tyr15 (34, 35). The knockout of BbTdp1 destroyed the balance ratio between phosphorylated and unphosphorylated Cdk1, causing alterations in the cell cycle of the ΔBbTdp1 strain (Fig. 6C). In B. bassiana, the balance of Cdk1 dephosphorylation and phosphorylation are coordinated by the phosphatase cell division cycle 25 (Cdc25) and the kinase Wee1 (20). The ΔWee1 mutant displays smaller blastospores, a delayed cell cycle, and reduced virulence, confirming that the change in virulence had a linear relationship with blastospore size (20), and these changes were consistent with the changes in blastospores in the ΔBbTdp1-mutant strain. Hence, the defects in virulence in the ΔBbTdp1-mutant strain were partly attributable to blastospore alterations, such as slow proliferation and aberrant cell cycle and size.

BbTdp1 is indispensable for fungal virulence, which is reflected not only in cell development but also in the cell infection process. First, the conidia of ΔBbTdp1-mutant cells had lower hydrophobicity and a more fragile cell wall than those of WT cells, and they were therefore sensitive to cell wall interference agents. Hydrophobin (Table 1) causes decreased spore hydrophobicity and altered surface carbohydrate epitopes, expression of such as slow proliferation and aberrant cell cycle and size.

B. bassiana enhances virulence due to the contribution of conidial germination and cuticle penetration (38). In Isaria fumosorosea, the disruption of Chi2 led to an apparent delay in insect lethality, suggesting that chitinase is required for fungal penetration of the insect body wall and is a crucial virulence factor (39). Third, ΔBbTdp1 strains exhibited sensitivity to oxidative stress, which may be attributed to the downregulated transcription of superoxide dismutase (SOD; Table 1), which is involved in scavenging intracellular reactive oxygen species (ROS) in vivo (40). In B. bassiana, all five SODs have been reported to be vital for antioxidation and virulence, and among them, BbSod2/3 (i.e., cytosol manganese superoxide dismutase and mitochondrial manganese superoxide dismutase, respectively) dominates the total SOD activity (40, 41). Fourth, BbTdp1 contributed to virulence by mediating the transcription of genes associated with secondary metabolism (e.g., oosporein and bassianolide). Oosporein, a secondary metabolite synthesized by the polyketide synthase cluster (PKS) oosporein synthase, facilitates hyphal infection by inhibiting host immunoreaction responses, and when the BbPKS9 gene is interrupted, oosporein production is lost and virulence is reduced (42). Another secondary metabolic toxin bassianolide, which is synthesized by the nonribosomal peptide synthetase BbBsls, has been shown to significantly contribute to virulence in B. bassiana (43).

MATERIALS AND METHODS

Strains, media, and growth conditions. All strains of B. bassiana ARSEF 2860 were maintained in sand tubes stored at −80°C and cultured at 25°C in SDAY enrichment medium (4% glucose, 1% peptone, 1% yeast extract, and 1.5% agar). Fungal stress responses were assayed in sterile CZA medium (3% sucrose, 0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄, and 0.001% FeSO₄) with different chemical stresses (20).

Analysis of structure and phylogenetics. The BbTdp1 gene (NCBI accession code EJP68311.1) from the genome of B. bassiana was explored via BLAST within the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (19). The identified BbTdp1 protein was structurally compared with other queried representative entomopathogens, phytopathogens, animal pathogens, and yeast by means of the simple modular architecture research tool (SMART) database at http://smart.embl-heidelberg.de. Phylogenetic analysis was completed by using the neighbor-joining method in MEGA 7 software (http://www.megasoftware.net) (44).

Generation of BbTdp1 disruption and complemented strains. Disruption and complemented strains were constructed according to our previously described method (45–47). Recombination events were verified via PCR and quantitative real-time PCR, and primers used to build the knockout and complemented strains are included in Table S1 in the supplemental material.

Measurement of colonies and hyphae. For observations of nutritional requirements, colonies of ΔBbTdp1 mutants and two control strains were started by spotting 1 μl of a 10⁷ conidia ml⁻¹ suspension
Roles of BbTdp1 in B. bassiana

(3) the same concentration below unless specified) on the centers of SDAY and CZA plates (48), and, meanwhile, aliquots (100 µl) of conidial suspensions were spread on SDAY and CZA plates with cellophane overlaid evenly for the determination of biomass (hyphal dry weight).

To assess hyphal septation and morphology, hyphae were harvested from SDB (agar-free SDAY) cultures at 25°C for 48 h and stained with calcofluor white (Sigma) at ambient temperatures for 15 min. A laser scanning confocal microscope (Leica DMi8, Germany) was used to observe the stained hyphae under the bright/fluorescence fields (wavelength, excitation = 335 nm and emission = 433 ± 5 nm) (6).

Assessment of conidial development and quality. Aliquots (200 µl) of a 10⁷ conidial ml⁻¹ suspension were spread on SDAY plates with cellophane overlaid evenly. Three culture slices (5-mm diameter) were drilled daily from each plate using a borer and were crushed for suspension in 1 ml of 0.02% Tween 80; the plugs were counted using a hemocytometer at 1-day intervals from day 4, and the counts were calculated as the number of conidia per cm² to assess the conidiation capacity (45). The microbial adhesion to hydrocarbons method (50) was used as the control for the two treatments. All boxes were incubated at 25°C on a 12-h/12-h photoperiod; in addition, deaths were recorded every 12 h (44). As an indicator for assessing larval mortality, the LT₅₀ is a death trend index obtained by probabilistic analysis. When the insects were infected for 3 days, the morphology of fungi in the host was observed under a confocal microscope. The dead larvae after death.

Conidial harvested from 6-day-old SDAY plates were suspended in phosphate-buffered saline (PBS) solution (pH 7.4) for cell size analysis measured by FCM readings (49) using FACS analysis of 1 × 10⁶ cells per sample in an FC500 MCL flow cytometer (Beckman Coulter, CytoFLEX LX, USA).

Aliquots (100 µl) of conidial suspensions were inoculated into 900 µl of germination broth (GB; 2% sucrose, 0.05% peptone, and 0.02% Tween 80). The half-germination time (GT₅₀) was used to measure the conidial germination capacity (45). The microbial adhesion to hydrocarbons method (50) was used to assess the index of the surface hydrophobicity for conidia cultured on SDAY plates after 6 days.

Moreover, a laser scanning confocal microscope was used to provide intuitive visualizations of the bright field for conidial development. Moderate conidial suspension, conidiferous structures (spore balls) from 6-day SDAY plates, and the conidial suspension after 12 h of germination were observed under a confocal microscope.

One-microliter aliquots of conidial suspensions were pointed to the CZA plates with gradients of NaCl (30 mg ml⁻¹), H₂O₂ (2 mM), CR (30 µg ml⁻¹), and CAR (0.2 µg ml⁻¹) as chemical stressors. Colony diameters were determined on day 10, and the relative inhibitory rate was calculated using the formula (Sc − St)/Sc × 100% (i.e., Sc is the area of the colonies grown on CZA plates, and St is the area of the colonies grown on CZA plates with treatments) (51). Conidial thermostolerance was examined by exposing conidial samples to 45°C heat stress followed by a modeling analysis of relative mortality rates using LT₁₀ estimates of each strain (52).

Examination of blastospores and the cell cycle. To examine blastospore production, 100-µl aliquots of conidial suspensions were inoculated into 50 ml of nitrogen-limited broth (NLB; 4% glucose, 0.4% NH₄NO₃, 0.3% KH₂PO₄, and 0.3% MgSO₄) and incubated at 25°C and 160 rpm for 4 days. During this period, blastospores were calculated under the microscope every 24 h from day 3. After 4 days, blastospores obtained from NB were suspended in PBS solution (pH 7.4), and blastospores were observed using confocal microscopy and the size was further determined using FCM. The cell cycles were analyzed by FACS for DNA concentration readings according to our previously described method (20). The G₀/G₁, S, and G₂/M cell cycle phases were determined based on unduplicated (1C), duplicated (2C), and intermediate DNA concentrations (20).

Enzyme-linked immunosorbent assay. Protein samples were extracted from the NLB cultures of fungal strains shaken at 25°C for 4 days and were suspended in 1-ml aliquots of PBS (pH 7.4) supplemented with 10 µl of protease inhibitors and phosphatase inhibitors (Mei5 Biotech, Beijing, China) on an ice bath for 10 min. After centrifuging the mixture for 10 min at 4°C and 10,000 × g, the supernatant was then centrifuged for another 10 min and used for ELISA. Levels of phosphorylated and nonphosphorylated Cdk1 (cyclin-dependent kinase 1) were detected with P-Cdk1 and NP-Cdk1 kits (Meimian Biotech, Jiangsu, China). The absorbance values of the treated ELISA plates were detected using a microplate reader (Thermo Fisher Scientific Inc.).

Fungal virulence bioassays. Fungal virulence tests were performed with third-instar G. mellonella larvae. Three groups of 30 larvae were soaked in 30 ml of conidial suspensions for 10 s for in vitro infection or injected with 1 µl of 5 × 10⁶ conidia ml⁻¹ suspensions for in vivo infection, after which they were immediately transferred into breathable boxes for normal growth. An equal dose of 0.02% Tween 80 was used as the control for the two treatments. All boxes were incubated at 25°C on a 12-h/12-h photoperiod; in addition, deaths were recorded every 12 h (44). As an indicator for assessing larval mortality, the LT₀ is a death trend index obtained by probabilistic analysis. When the insects were infected for 3 days, the morphology of fungi in the host was observed under a confocal microscope. The dead larvae carcasses were collected and cultured in a saturated humidity chamber to observe the conditions of larvae after death.

Sequencing of the BbTdp1-mediated transcriptome (RNA sequencing). To explore the role of BbTdp1, we performed comparative transcriptional analysis of the WT and ΔBbTdp1 strains to further understand the effects of BbTdp1 on the overall transcriptional pattern. The WT and ΔBbTdp1 strains were cultured for 6 days on SDAY plates; subsequently, total RNA was extracted. After purifying the RNA samples, six libraries (i.e., three libraries for the WT strain and three for the ΔBbTdp1-mutant strain) were constructed and sequenced on the BGISEQ-500 platform at BGI (Shenzhen, China).

After filtering the raw reads, the clean reads were queried against the B. bassiana genomic database (19). Cufflinks was used to quantify the identified genes based on the expected number of fragments per kilobase of exon per million mapped fragments (53). Then, the DEGs between WT and ΔBbTdp1 strains were filtered with the Cuffdiff method (54). Significant DEGs met the following conditions: q value of < 0.05 and a [log (ratio)] > 2.

To functionally classify the DEGs, we used FungiFun2 (https://elbe.hki-jena.de/fungifun2) to determine gene functional enrichment (55). The functional categories were significantly enriched when the
adjusted P value was <0.05 (56, 57). In addition, Gene Ontology and signal pathway classification were performed for describing the function of DEGs more intuitively with the help of GO (https://geneontology.org/) (58) and KEGG (https://www.kegg.jp/) (59).

**Statistical analysis.** All experiments comparing the ΔBbtDp1 strain and the two control strains, including phenotypic observations, measurements, and virulence, were performed in triplicate and were subjected to one-factor (strain) analysis of variance. In addition, Tukey’s honestly significant difference (HSD) test was applied to differentiate the means of each phenotype.

**Data availability.** The sequence data have been deposited in the NCBI Gene Expression Omnibus (accession no. GSE179456).

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**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.01 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.01 MB.

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