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Cytokinesis-required Cdc14 is a signaling hub of asexual development and multi-stress tolerance in *Beauveria bassiana*

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A dual-specificity, paralogue-free Cdc14 phosphatase was located in the nuclei of *Beauveria bassiana* (filamentous entomopathogen) and functionally characterized. Inactivation of *cdc14* caused defective cytokinesis due to multinucleate cells formed in *Δcdc14* and 89% decrease of blastospore production, followed by slower growth and a loss of ≥ 96% conidial yield under normal conditions. These defects coincided well with drastic down-regulation of 25 genes required for mitosis and conidiation. Moreover, *Δcdc14* became hypersensitive to oxidative, osmotic, and cell wall and mitosis perturbing stresses, and lost 41−70% of conidial thermotolerance, UV-B resistance and virulence, accompanied with transcriptional down-regualtion of various signaling factors and stress-responsive effectors and depressed phosphorylation signals of Hog1 and Slt2 in high-osmolarity glycerol and cell-wall integrity pathways. All changes were well restored by rescuing *cdc14*. Our findings indicate that Cdc14 vital for the fungal cytokinesis acts as a signaling hub in regulating not only asexual development but multi-stress responses and virulence.

Coordinating nuclear division with growth and cell cycle is vital for eukaryotic development[1]. Cdc14 is a key regulator of nuclear behavior in the family of dual-specificity phosphatases that dephosphorylate the residues of phosphotyrosine and phosphoserine/phosphothreonine[2]. This phosphatase is highly conserved in almost all eukaryotes and primarily involved in cell division[3] as has been elucidated in yeasts[4,5]. In budding yeast, for instance, Cdc14 can inactivate cyclin dependent kinases (CDKs) at the end of mitosis for cell entry into G1 phase because Cdc14 inactivation may result in overexpressed CDC28-CLB kinase, elongated mitotic spindles and separated chromosomes[6,7]. Moreover, Cdc14 may act as a hub of five phosphatases and 23 kinases[8], including mitosis-associated CDKs and mitogen-activated protein kinase (MAPK) cascades, which constitute the pathways of high-osmolarity glycerol (HOG), cell wall integrity (CWI), filamentous/invasive growth (FIG) and pheromone response (PR)[9,10]. Thus, Cdc14 is essential not only for cell cycle, checkpoint and metabolism but likely involved in multi-stress responses. In fact, Cdc14 orthologues take similar, but not always identical, parts in the regulation of cell division in some eukaryotes, such as human[11] and nematode[12]. A Cdc14-like phosphatase Clp1 (also known as Flp1) in fission yeast is required for cell entry into mitosis rather than exit and for septation rather than cyclin B destruction[2,6,13]. Cdc14A and Cdc14B, two Cdc14 paralogues in human[14], also function like yeast Cdc14[3]. Deletion of *cdc14p* from *Candida albicans* affected late cell-cycle events and morphogenesis, such as large cell aggregates, decreased invasion into solid substrate and impaired hyphal growth[15]. Knockdown expression of Cdc14 orthologues may result in defective sporulation in *Phytophthora*[1,16]. However, most of the previous studies have focused primarily on Cdc14 contribution to nuclear events but paid little attention to possible effects on multi-stress responses and other biological aspects, such as virulence, although Cdc14 may interact with other phosphatases and MAPKs in budding yeast[8]. The functions of Cdc14 orthologues in filamentous fungi remain poorly understood.

Fungal virulence and multi-stress tolerance are determinant to the biocontrol potential of filamentous entomopathogens against arthropod pests because their cells, such as unicellular conidia, are excellent active ingredients of mycoinsecticides and mycoacaricides[17]. Genetic engineering of fungal candidate strains for improved virulence[18] and stress tolerance[19] often requires the understanding of involved molecular mechanisms. Genomic analysis of *Beauveria bassiana*[20] has revealed the existence of a single Cdc14 in the fungal entomopathogen. This study sought to characterize *B. bassiana* Cdc14 by analyzing multiple phenotypes and transcriptional profiles of

its mutants under various stresses. We found that Cdc14 controlled not only cytokinesis but also *B. bassiana* conidiation, virulence and responses to a wide range of nutritional, chemical and environmental stresses by governing the expressions of many stress-responsive effectors and signaling factors, such as phosphatases, protein kinases and cascaded MAPKs.

Results

Features of *cdc14* and deduced protein in *B. bassiana*. The coding sequence of *cdc14* amplified from the wild-type strain *B. bassiana* ARSEF 2860 (Bb2860 or WT herein) is 1962 bp long, encoding a protein of 642 amino acids (molecular weight: 71.51 kDa; isoelectric point: 8.97). The deduced Cdc14 is characteristic of a highly conserved signature motif typical for the superfamily of protein tyrosine phosphatases and four CDK consensus phosphorylation sites (S/TPXK/R), i.e., $S_{43}PRK_{46}$, $T_{414}RIR_{417}$, $S_{534}PMR_{537}$ and $S_{599}PLR_{602}$. There are two to six similar sites in other fungal Cdc14 orthologues but none of them exists in *Saccharomyces cerevisiae*[15]. The deduced protein has no paralogue found in *B. bassiana* and shares 40−100% sequence identity with the fungal/yeast orthologues in NCBI database (Fig. S1A).

As a result of quantitative real-time PCR (qRT-PCR) analysis, the transcript level of *cdc14* in Bb2860 was much higher during conidiation than during hyphal growth under normal conditions (Fig. S1B) and greatly elevated by different chemical stresses but less affected by heat shock (Fig. S1C).

A transformant expressing the fusion protein Cdc14::eGFP in Bb2860 was created to show intracellular location of Cdc14. As a result, green fluorescence was emitted from the nuclei of hyphal cells grown in Sabouraud dextrose broth (SDB) for 2 days at 25°C and the expressed Cdc14 was well stained with the nuclear stain DAPI (Fig. 1A). This confirmed the nuclear location of Cdc14 in *B. bassiana*.

Disruption of *cdc14* caused defects in cytokinesis and asexual development. The disruption and complementation of *cdc14* in Bb2860 were verified via PCR and Southern blotting analyses (Fig. S1D) with paired primers (Fig. S1E). Hyphal cells gained by shaking 10^6 conidia/ml SDB for 3 days at 25°C were stained with both DAPI and calcofluor white. In three batches of > 300 stained cells, Δ*cdc14* showed three or more nuclei at the mean (± SD) percentage of 13.3 ± 0.9 whereas only one or two nuclei were consistently present in two control strains (Fig. 1B). Strikingly, 18 genes involved in cytokinesis and cell division (Table S1) were all down-regulated by 72−87% in the SDB culture of Δ*cdc14* versus WT but their transcripts in Δ*cdc14*/*cdc14* were well restored to normal WT levels (Fig. 1C). These indicated that cytokinesis was defective in Δ*cdc14* due to the drastic down-regulation of those genes.

As a result of abnormal cytokinesis in Δ*cdc14*, its blastospore yield was reduced by 89% in the SDB culture compared to the mean yield of 4.58 (± 0.17) × 10^7 blastospores/ml from two control strains (Fig. 2A). After 7-day growth at 25°C on nutrition-rich SDAY (Sabouraud dextrose agar plus 1% yeast extract), Δ*cdc14* colonies were 16% smaller than those (~7 cm^2 each) of the control strains ($F_{2,6} = 81.8$, $P < 0.0001$). Conidial yields measured from the cultures during days 4−7 were reduced by ≥ 96% in Δ*cdc14* (Fig. 2B). Microscopic examination of colony samples revealed that, during the incubation, Δ*cdc14* failed to form zig-zag conidiophore clusters and spore balls typical for *B. bassiana* as observed in the control strains (Fig. S2). Eight genes essential for hyphal development and conidiation (Table S1) were assessed for their transcript levels in 3-day SDAY cultures via qRT-PCR. All of them except *flbA* were down-regulated by 24−94% in Δ*cdc14* versus the control strains (Fig. 2C), a possible cause of the severe defect of its conidiation. All the data indicated that Cdc14 was indispensable for both cytokinesis and asexual development of *B. bassiana*.

Disruption of cdc14 reduced *B. bassiana* adaptation to environment and host. During 8-day incubation at 25°C on minimal CZA (Czapek agar) and eight CZA-derived media, Δ*cdc14* grew significantly slower than the control strains ($P < 0.01$ in F tests). The final colony size of the disruptant was reduced by 46−58% on the mere carbon source of sucrose, glucose, galactose, acetate or glycerol and 35−45% on the mere nitrogen source of NO_3^-, NO_2^- or NH_4^+, and more affected by the starvation of carbon than nitrogen (Fig. 3A).

Modeling analyses of relative growth trends over the gradients of stressful chemicals added to 1/4 SDAY for 6-day incubation at 25°C generated effective concentration (EC_{50}) of each inhibiting 50% colony growth. Compared to the control strains, Δ*cdc14* became most sensitive to Congo red and SDS, followed by H_2O_2, carbendazim, menadione and NaCl in order (Fig. 3B−D).

A sensitive concentration was used to assess the effects of NaCl, H_2O_2, menadione, Congo red and SDS on conidial germination. Based on residual viability (germination), Δ*cdc14* was 24−69% less tolerant to the stressful chemicals (Fig. 3E). Conidial thermotolerance and UV-B resistance were reduced by 41% and 50% in Δ*cdc14*, as indicated by lowered LT_{50} and LD_{50} (Fig. 3F) respectively. Under a uniform spray of 10^7 conidia/ml suspension, the LT_{50} of Δ*cdc14* against *Spodoptera litura* second-instar larvae was 3-day longer than those (~4.3 days) from the control strains (Fig. 3G).

All the data indicated that Cdc14 acted as a positive regulator of *B. bassiana* virulence and responses to nutritional, oxidative, hyperosmotic, cell wall disturbing, fungicidal, thermal and UV-B stresses and contributed greatly to the fungal biocontrol potential.

Disruption of *cdc14* altered transcriptional profiles of stress-responsive genes and phosphorylaiton levels of CWI and HOG pathways. Up to 61 stress-responsive genes were assessed for their transcript levels in the 3-day cultures of 1/4 SDAY supplemented with menadione, Congo red, NaCl and carbendazim via qRT-PCR with paired primers (Table S1), including those involved in various signal transduction pathways (STP), such as protein kinases (pkA, pkC and Snf1), Ras GTPases (Ras1 and Ras2), and all MAPK cascades. Under each chemical stress, all effector and/or SIP genes were transcribed at similar levels in two control strains, as those shown for cytokinesis (Fig. 1C) and conidiation (Fig. 2C).

In contrast, many of the examined genes were down-regulated in Δ*cdc14* versus WT. Transcript levels of 11 STP genes were lowered by 30−96% under oxidative stress (Fig. 4A), including *ras1/2*, *pkC*, *snf1*, *mkk5* and the full cascades of HOG (*ste11*, *pbs2* and *hog1*) and CWI (*bck1*, *mkk1* and *slt2*). Also, three catalases (Cat2/4/5), two peroxidases (Pod1/3) and three superoxide dismutases (SODs) (Sod2−4) were transcriptionally down-regualted by 32−99% under the same stress. Cell wall disturbance by Congo red resulted in 43−98% reductions of all the STP gene transcripts except those of *snf1*, *ste11*, *pbs2* and *mkk5* (Fig. 4B), accompanied with 20−97% transcriptional down-regulation of nine chitin synthases (Chs1/2/5−10), two cell wall biosynthesis proteins (Mhp1 and Ssd1) and five chitinases (Chn1/4/6/7/10). Under osmotic stress, 13 STP and four osmosensitive genes were down-regulated by 31−95% and 74−94% respectively (Fig. 4C). Inclusion of carbendazim in the culture also down-regulated all the STP genes except *sskB* and *mpk1* (Fig. 4D). Notably, the STP genes repressed by Congo red, NaCl and carbendazim were involved in almost all the MAPK cascades while Msn2, an important transcription factor with multiple stress-responsive elements, was highly responsive to all the chemical cues except menadione.

In ELISA experiments with available phospho-antibodies, the phosphorylation levels of Hog1 and Slt2 as hallmarks of the HOG and CWI pathways of Δ*cdc14* were significantly lowered by 26−52% (Fig. 4E) and 38−93% (Fig. 4F) under all the chemical stresses respectively. In contrast, their phosphorylation levels did not differ

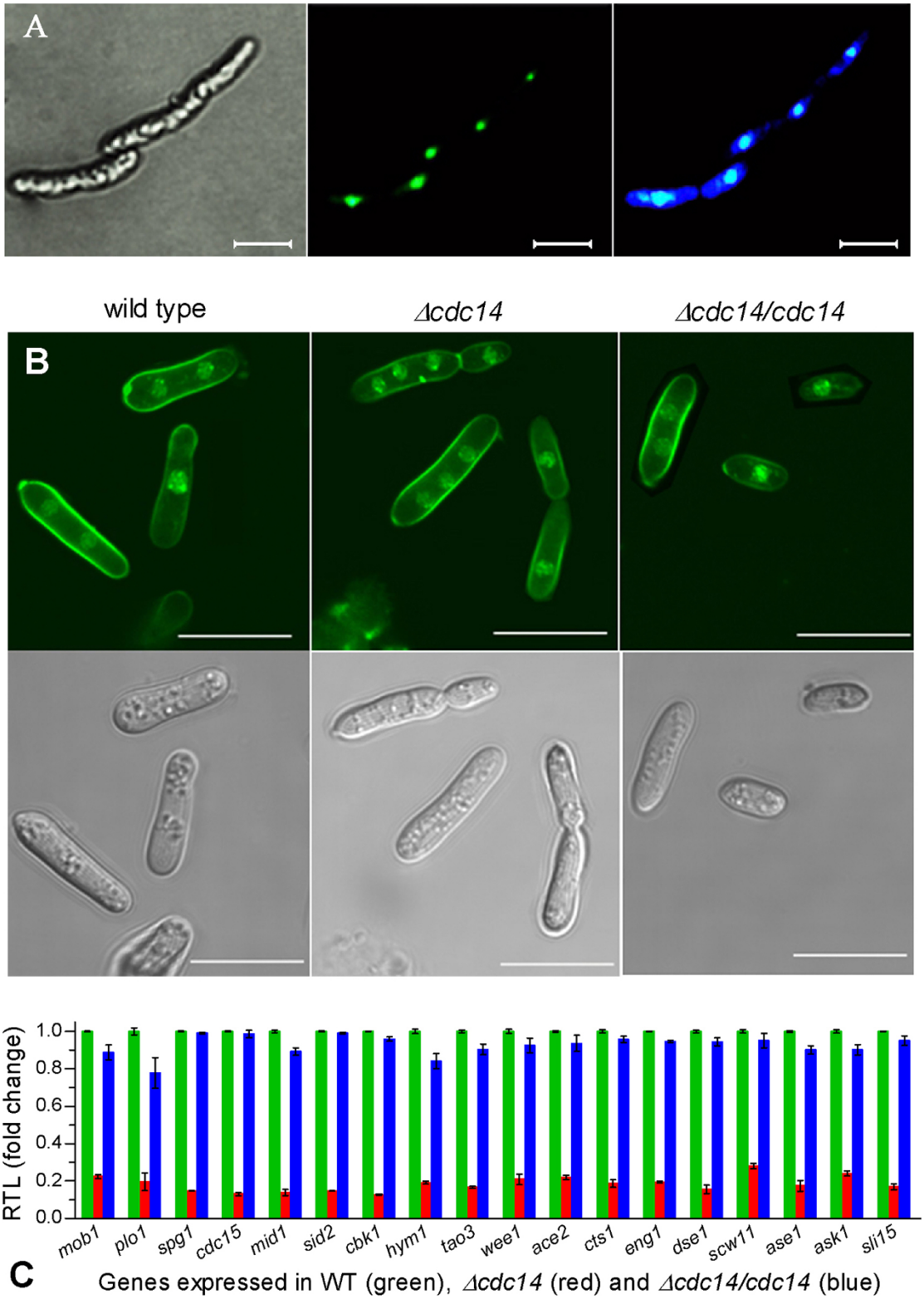

Figure 1 | **Disruption of *cdc14* caused cytokinesis defect in *B. bassiana*.** (A) Microscopic images of transgenic hyphal cells expressing the fused protein eGFP::Cdc14 in SDB at 25°C. Left: bright image of the cells stained with DAPI. Middle: fluorescent image of the expressed eGFP in nuclei at the excitation/emission wavelengths of 488/507 nm. Right: fluorescent image of the DAPI-stained Cdc14 in nuclei at the excitation/emission wavelengths of 358/360 nm. (B) Bright (upper) and fluorescent (lower) images of hyphal cells stained with both DAPI and calcofluor white (a stain specific to cell wall). Note that cytokinesis was normal (with one or two nuclei per cell) in wild type but abnormal (with three or more nuclei per cell) in Δ*cdc14*. Scale bars: 10 μm. (C) Relative transcript levels (RTL) of 18 cytokinesis-associated genes in Δ*cdc14* and Δ*cdc14/cdc14* versus wild type grown for 3 days in SDB at 25°C. Error bars: SD from three cDNA samples assessed via qRT-PCR with paired primers (Table S1).

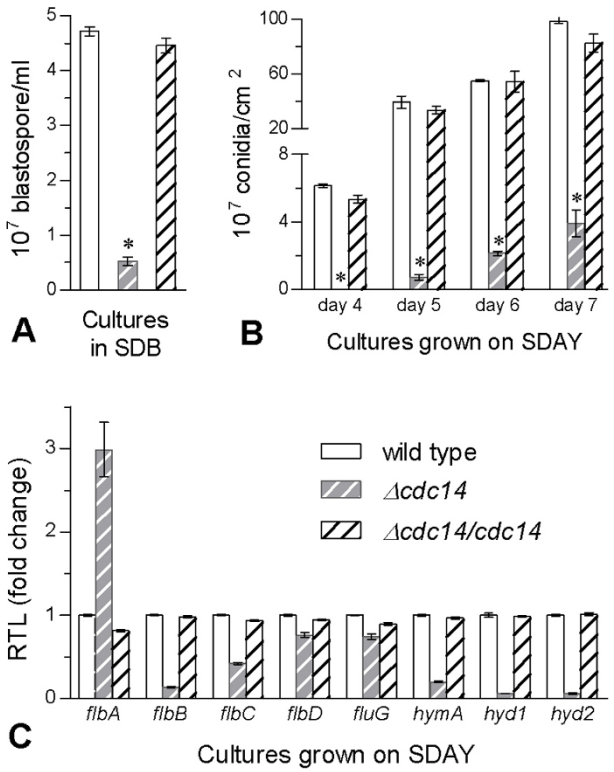

Figure 2 | Disruption of *cdc14* in *B. bassiana* caused severe defects in the production of blastospores and conidia. (A) Blastospore yields in liquid SDB cultures initiated with 10^6 conidia/ml and shaken for 3 days at 25°C. (B) Conidial yields over the period of incubation on SDAY plates at 25°C. The asterisked bar in each group differs significantly from those unmarked (Tukey's HSD, $P < 0.05$). (C) Relative transcript levels (RTL) of eight conidiation-associated genes in 3-day SDAY cultures at 25°C based on qRT-PCR with paired primers (Table S1). Error bars: SD from three replicates.

significantly among the tested strains under normal culture conditions ($P \geq 0.58$ in F tests) and were restored in the complemented mutant under the chemical stresses.

All the data indicated that Cdc14 was a core phosphatase to activate the expressions of various STP and phenotype-related effector genes under the chemical stresses of different types.

Discussion

As a dual-specificity phosphatase located in the nuclei of *B. bassiana*, Cdc14 has proved to play a crucial role in cytokinesis, a key step of cell cycle well known in eukaryotes. The nuclear behavior of Cdc14 exerted profound effects not only on asexual development but on virulence and multi-stress tolerances of *B. bassiana*, thereby contributing greatly to the fungal biocontrol potential. All phenotypic and transcriptional events altered by Cdc14 inactivation are discussed below.

First of all, Cdc14 is required for the cytokinesis and asexual development of *B. bassiana* because cytokinesis was abnormal in Δ*cdc14* (Fig. 1B). The hypersensitivity of Δ*cdc14* cells to the mitosis inhibitor carbendazim (Fig. 3C) implicates that the defective cytokinesis could result from abnormal mitosis, as was evidenced with 13% multinucleate cells formed in our Δ*cdc14* and reported previously from fission yeast[13,21]. The abnormal nuclear behavior in Δ*cdc14* is well supported by the drastic downregulation of 18 genes (Fig. 1C), such as *sli15*, *ask1*, *fin1* and *ase1* involved in the stabilization and extension of anaphase spindle[22–24], *ace2*, *cts1*, *eng1*, *dse1* and *scw11* associated with cell wall hydrolysis[25–29], and *cdc15* required for septum formation[21].

Accompanied with the abnormal cytokinesis, Δ*cdc14* became defective in hyphal growth and much worse in the formation of blastospores and conidia (Fig. 2). Its defective growth could be attributed to less efficient use of carbon and nitrogen sources (Fig. 3A). The severe conidiation defect is consistent with those caused by knockdown *cdc14* expression in two oomycetes[1,16] and likely attributable to the repressed expression of *hymA* required for conidiophore development[30], *hyd1/2* essential for hydrophobin biosynthesis and spore coat rodlet layer assembly during conidiation[31], and *flbB−D* as transcriptional factors required for morphogenesis and asexual development[32–35]. The exceptional up-regulation of *flbA* might contribute to *brlA* activation for the premature initiation of development[36] but its effect on *brlA* could be shaded by the repressed *flbB−D* and *fluG*, another *brlA* activator[37].

Moreover, *B. bassiana* Cdc14 may act as a hub of cellular signaling network in fungal response to various stresses. The fact that Δ*cdc14* was hypersensitive to oxidative, osmotic, and cell wall and mitosis perturbing agents (Fig. 3B−E) confirmed for the first time that Cdc14 is a powerful, positive regulator of multi-stress responses in filamentous fungi. The responses of our Δ*cdc14* to the stresses of different types are well in agreement with increased sensitivity of yeast Cdc14 mutants to both cell wall and mitosis disturbance[8,38] but reverse to the decreased osmosensitivity in the yeast mutants[8]. We found that many of the examined effector genes were down-regulated in Δ*cdc14* under the chemical stresses of different types (Fig. 4A−D). These effector genes could lower the activities of antioxidant and osmotolerant enzymes/proteins and impaire cell wall composition, thus increasing Δ*cdc14* sensitivity to each of the stressful chemicals. More importantly, all cascaded kinases in the CWI and HOG pathways of Δ*cdc14* were transcriptionally down-regulated by menadione and even more by NaCl, Congo red and carbendazim. With available phospho-antibodies, we confirmed that the phosphorylation levels of both Hog1 and Slt2 in Δ*cdc14* were significantly lowered by all the chemical stresses. Taking together, Cdc14 is a core phosphatase in the signaling network of *B. bassiana* as shown in budding yeast[8]. Thus, we propose that the positive regulation of multi-stress responses by Cdc14 is likely achieved by its interplay with multiple STP components because cellular multi-stress responses are well controlled by the pathways of HOG[39], CWI[40], Ras GTPases[41] and pkA/pkC[42]. However, we could not find more commercial phospho-antibodies to detect phosphorylation signal changes in other MAPK pathways.

Finally, Cdc14 contributes greatly to the biocontrol potential of *B. bassiana* against arthropod pests. Conidial virulence, thermotolerance and UV-B resistance determinant to the fungal biocontrol potential were greatly decreased by the *cdc14* disruption (Fig. 3). Previously, cellular antioxidant capability and cell wall integrity were linearly correlated with either UV-B resistance or virulence of *B. bassiana*[41,43]. In this study, Δ*cdc14* lost large parts of conidial tolerances to both oxidative and cell wall perturbing stresses, an interpretation for the decreased UV-B resistance and virulence. Moreover, Cat2, a single thermosensitive member in the catalase family of *B. bassiana*[44], was transcirptionally down-regulated in Δ*cdc14* under oxidative stress, partially interpreting the deceased thermotolerance. Therefore, Cdc14 is also a positive regulator of the fungal biocontrol potential.

Methods

Microbial strains and culture conditions. The wild-type strain Bb2860 was used as a recipient of target gene manipulation and cultivated in SDAY (4% glucose, 1% peptone and 1.5% agar plus 1% yeast extract) at 25°C and 12 : 12 h (light : dark cycle). CZA (3% sucrose, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$ and 0.001% $FeSO_4$ plus 1.5% agar) and 1/4 SDAY (SDAY nutrients diluted to 1/4) were used in phenotypic assays of fungal mutants. *Escherichia coli* DH5α from Invitrogen (Shanghai, China) grown in Luria-Bertani medium at 37°C was used for plasmid propagation. *Agrobacterium tumefaciens* AGL-1 cultivated at 28°C in YEB medium[45] was used as a T-DNA donor for fungal transformation.

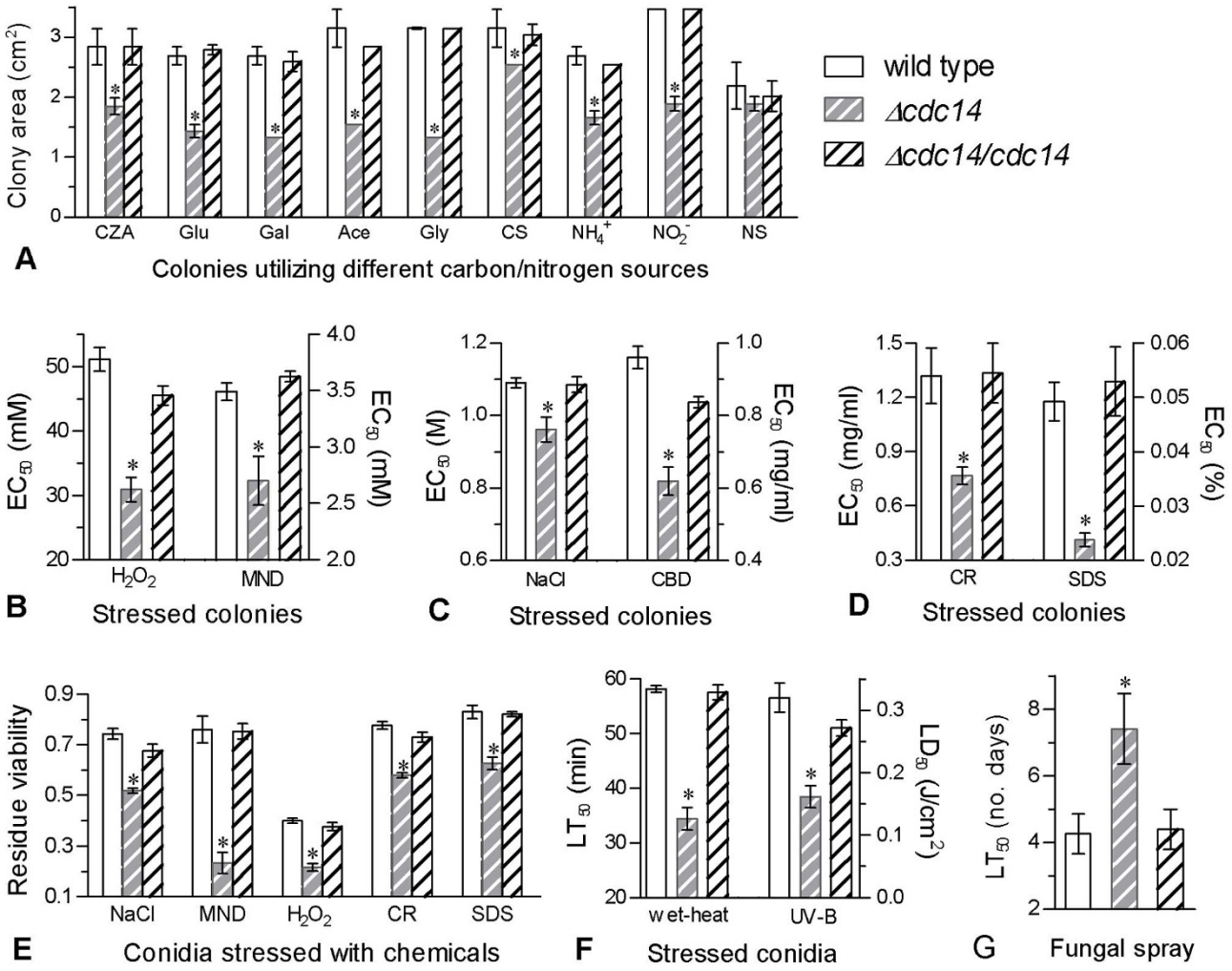

Figure 3 | Multi-phenotypic defects observed in Δ*cdc14* versus two control strains. (A) Colony sizes after 6-day growth on minimal CZA (3% sucrose as mere carbon and 0.3% $NaNO_3$ as mere nitrogen) and CZA-derived media [altered carbon: glucose (Glu), galactose (Gal), glycerol (Gly), acetate (Ace) or carbon starvation (CS); altered nitrogen: $NaNO_2$, NH_4Cl or nitrogen starvation (NS)]. (B −D) Effective concentrations (EC_{50}s) of stressful chemicals required for the suppression of 50% colony growth after 6-day incubation at 25°C on the plates of 1/4 SDAY supplemented with the gradients of H_2O_2, menadione (MND), NaCl, carbendazim (CBD), Congo red (CR) and SDS respectively. (E) Residue viability of conidia after 24 h incubation at 25°C on germination medium supplemented with NaCl (1.2 M), MND (0.2 mM), H_2O_2 (4 mM), CR (0.5 mg/ml) or SDS (0.04%). (F) Median lethal time (LT_{50}) for conidial tolerance to wet-heat stress at 45°C and median lethal dose (LD_{50}) for conidial UV-B resistance. (G) LT_{50} (no. days) for conidial virulence to *S. litura* second-instar larvae under a uniform spray. The asterisked bar in each group differs significantly from those unmarked (Tukey's HSD, $P < 0.05$). Error bars: SD from three repeated assays.

Cloning and analysis of *cdc14* in *B. bassiana*. The Cdc14 gene was blasted from the genome of Bb2860 under the NCBI accession ADAH00000000[20]. Its coding region (Tag code: BBA_07962) was amplified from Bb2860 via PCR and verified by sequencing at Invitrogen. The deduced Cdc14 sequence was compared with the Cdc14 homologues of other fungal and yeast species in NCBI protein database via online structural and phylogenetic analyses.

Cellular localization of Cdc14. To localize Cdc14 in wild-type cells, a cassette with multiple restriction enzyme sites of 5′-*Pme*I-*Spe*I-*Eco*RV-*Eco*RI-*Bam*HI-3′ (x) was constructed and introduced to the C-terminus of P*trpC* promoter via PCR with designed primers using the template of p0380-bar[43]. The resultant fragment was digested with *Not*I/*Bam*HI and ligated into pAN52-1N[46] to remove original P*gpdA*, forming pAN52-x. The *bar* marker cut from p0380-bar was digested with *Xba*I/*Hin*dIII and ligated into the same enzyme sites of pAN52-x, yielding pAN52-x-bar. The coding sequence of *cdc14* was amplified from WT cDNA with paired primers (5′-AAAAAACTAGTATGGCTCCCGCCTCCCGCC-3′/5′-AAAAAGAATTCT-GCGCCCGACACCTTGCGGG-3′). The reporter gene of enhanced green fluorescent protein (eGFP) was amplified from pAN52-eGFP with designed primers[47]. The fragments of *cdc14* and *eGFP* were digested with *Spe*I/*Eco*RI and *Eco*RI/*Bam*HI and introduced to the respective enzyme sites in pAN52-x-bar. The resultant plasmid pAN52-cdc14-eGFP-bar was linearized with *Not*I and then integrated into Bb2860 using the method of blastospore transformation[47].

A positive transformant expressing the fusion BbCdc14::eGFP was grown in SDB (i.e., agar-free SDAY) containing10^6 conidia/ml. After 2-day shaking at 25°C, mycelia were collected, rinsed with PBS buffer (pH 7.0), stained with the nuclear stain DAPI (4′,6′-diamidine-2′-phenylindole dihydrochloride) (Sigma) and visualized for their microscopic images of bright field and fluorescence at the excitation/emission wavelengths of 358/460 nm and 488/507 nm for the location of DAPI-stained Cdc14 and expressed eGFP in cells respectively.

Disruption and complementation of *cdc14*. The 5′ and 3′ ends (1500 and 1500 bp) of *cdc14* were amplified from Bb2860 via PCR with paired primers (Fig. S1E) and La*Taq* DNA polymerase (Promega, Madison, MI, USA) and inserted into the respective sites of *Eco*RI/*Bam*HI and *Bgl*II/*Spe*I in p0380-bar, forming disruption plasmid p0380-bar-cdc14D, which vectors phosphinothricin (PPT) resistant *bar* gene as marker 1. To rescue the target gene, p0380-sur-gateway[43] was used as a backbone. The full-length *cdc14* sequence with flanking regions (6450 bp in total) was cloned from Bb2860 with rCdc14-F/R (Fig. S1E) and ligated into the backbone to replace the gateway fragment, yielding the plasmid p0380-sur-cdc14C, which vectors chlorimuron ethyl resistance gene *sur* as marker 2.

The two plasmids were transformed into Bb2860 and Δ*cdc14* mutant respectively through *Agrobacterium*-mediated transformation[42]. Putative mutant colonies grown on selective medium were screened in terms of their *bar* and *sur* resistance to PPT (200 μg/ml) and chlorimuron ethyl (10 μg/ml) and identified sequentially through PCR, qRT-PCR and Southern blotting with paired primers and amplified probe (Fig. S1E). Positive Δ*cdc14* mutant was evaluated together with WT and Δ*cdc14*/*cdc14* (control strains) in triplicate experiments below.

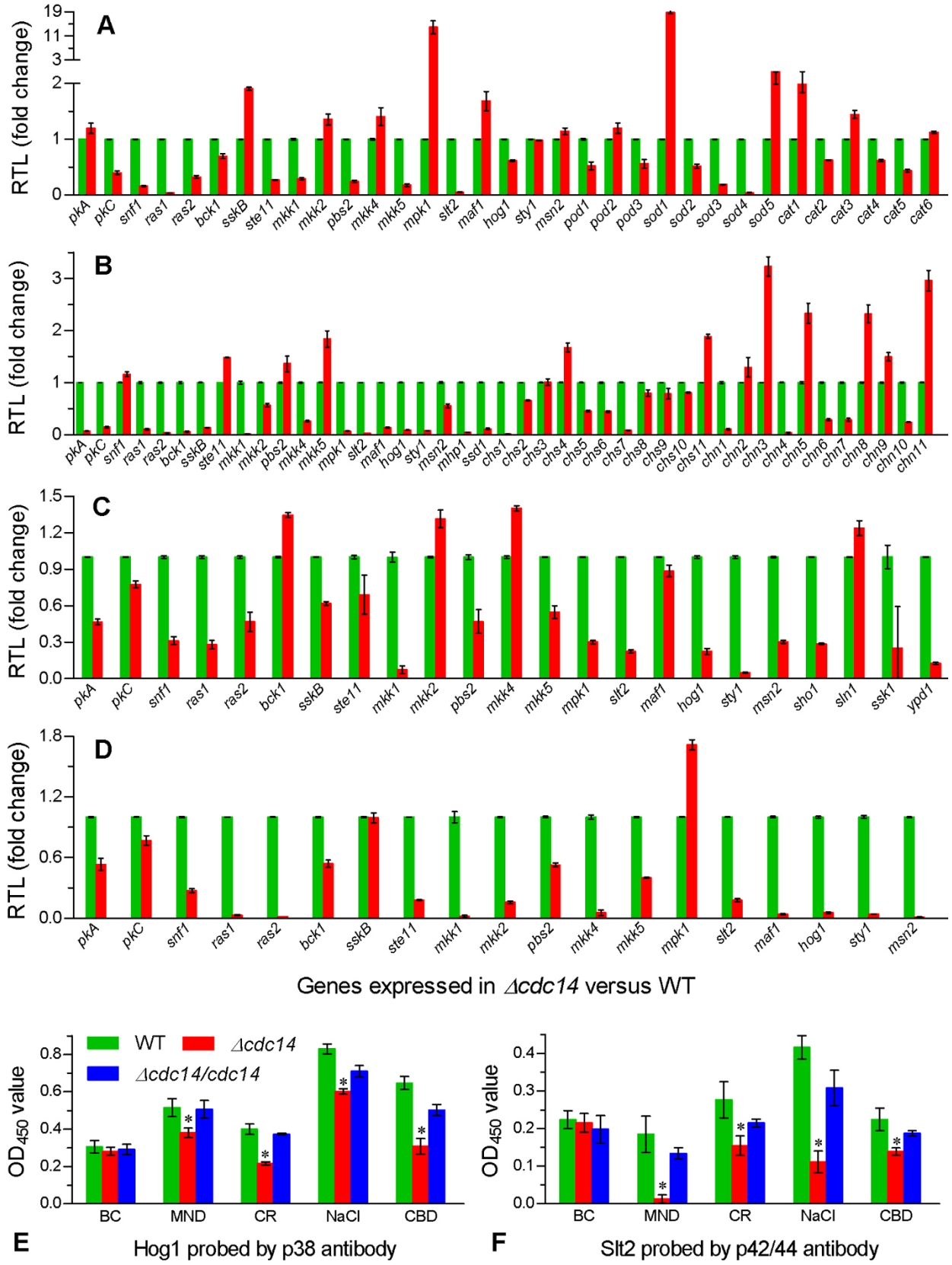

Figure 4 | Disruption of *cdc14* altered the transcripts of stress-responsive genes and the phosphorylation of Hog1 and Slt2. (A−D) Relative transcript levels (RTL) of stress-responsive genes in the 1/4 SDAY cultures of Δ*cdc14* versus wild type supplemented with MND (menadione 0.2 mM), CR (Congo red 0.5 mg/ml), NaCl (0.8 M) and CBD (carbendazim 0.5 μg/ml) for 3-day growth at 25°C respectively (assessed via qRT-PCR with paired primers in Table S1). (E and F) OD_{450} values for the respective phosphorylation levels of Hog1 and Slt2 in the protein extracts of fungal cultures grown under the same stresses (BC: unstressed blank control). The extracts were probed with phospho-p38 and phospho-p42/44 antibodies. The asterisked bar in each group differs significantly from those unmarked (Tukey's HSD, $P < 0.05$). Error bars: SD from three samples.

Cytokinesis assay. For WT and mutant strains, 50 ml cultures were initiated by suspending 10^6 conidia in 1 ml SDB. After 3-day shaking at 25°C, hyphal cells were harvested, washed twice and resuspensed in 50 ml dd-H_2O. Three 1 ml aliquots of the suspension were stained with 1 μg DAPI and 200 μl calcofluor white (Sigma), a stain specific to cell wall. The stained cells were microscopically examined for the images of bright field and fluorescence as above to reveal cytokinesis change in Δ*cdc14* versus control strains and their nuclei were counted.

Measurement of growth and conidiation parameters. Aliquots (1 μl) of 10^6 conidia/ml suspension were spotted centrally onto the plates (9 cm diameter) of nutrition-rich SDAY, minimal CZA and eight CZA-derived media. The derived media were prepared by deleting 3% sucrose (carbon starvation) or 0.3% $NaNO_3$ (nitrogen starvation) from standard CZA, replacing 3% sucrose with 3% of glucose, galactose, glycerol or acetate (NaAc) as sole carbon source, and replacing 0.3% $NaNO_3$ with 0.3% of $NaNO_2$ or NH_4Cl as sole nitrogen source respectively. During 8-day incubation at 25°C and 12 : 12 h, colony diameters were daily cross-measured to calculate colony area as an index of growth rate.

To assess sporulation capacity of each strain, 100 μl aliquots of 10^7 conidia/ml suspension (the same below unless specified) were evenly spread on SDAY plates, followed by 7-day incubation at 25°C and 12 : 12 h. From day 3 onwards, colony discs (5 mm diameter) were daily cut off from the plates and conidia on each disc were washed into 1 ml of 0.02% Tween 80 by vibration. After removal of hyphal debris by filtration, the conidial concentration was determined with microscopic counts and converted to the number of conidia per cm^2 colony. During the incubation, fungal mass samples taken from the colonies were examined under microscope to reveal possible morphological changes in their conidiophores and conidia.

Assaying cellular responses to chemical and environmental stresses. SDAY plates overlaid with cellophane were spread with 100 μl aliquots of conidial suspension to produce uniform cultures after 3-day incubation at 25°C. Fungal mass discs (5 mm diameter) cut from the culture of each strain were placed centrally onto the plates (90 mm diameter) of 1/4 SDAY supplemented with the gradients of NaCl (0.4−2 M), menadione (2−8 mM), H_2O_2 (20−80 mM), Congo red (0.5−3 mg/ml), SDS (0.02−0.12%) and carbendazim (0.4–2 μg/ml) respectively. After 6-day stressful incubation at 25°C and 12 : 12 h, diameters of all colonies in the control (free of any chemical stress) and stress treatments were cross-measured to compute their net area increases.

Conidial tolerances to oxidation, hyperosmolarity and cell wall disturbance were assayed by spreading 100 μl aliquots of conidial suspension onto the plates of germination medium (GM: 2% sucrose and 0.5% peptone plus 1.5% agar) supplemented with menadione (0.2 mM), H_2O_2 (4 mM), NaCl (1.2 M), Congo red (1 mg/ml) and SDS (0.04%) respectively. After 24 h incubation at 25°C, conidial germination on each of the plates stressed or not (control) was assessed using three counts of germinated and ungerminated conidia under microscope. Residue viability was calculated as the ratio of percent germination under each stress over that in the control. Conidial tolerances to wet-heat stress of 15−120 min at 45°C and UV-B irradiation (weighted 312 nm) of 0.1–0.8 J/cm^2 in Bio-Sun^{++} chamber (Vilber Lourmat, Marne-la-Vallée, France) were assayed using our previous protocols[48,49]. After exposure to a given intensity of heat or UV-B stress, conidia were incubated for 24 h at 25°C under saturated humidity and percent germination was determined using microscopic counts.

Bioassay of fungal virulence. A standardized cabbage leaf disc system[19] was used to bioassay the virulence of each strain to the second-instar larvae of *S. litura*. Briefly, batches of 30−40 larvae on a cabbage leaf disc were separately exposed to a spray of 1 ml conidial suspension (treatment) or 0.02% Tween 80 (control) in automatic Potter Spray Tower (Burkard Scientific Ltd, Uxbridge, UK) at uniform working pressure. After spray, all larvae were reared in Petri dishes (with leaf discs changed daily for their feeding) for 8 days at 25°C and 12 : 12 h and monitored daily for mortality records.

Transcriptional analysis of phenotype-associated genes. Aliquots of 100 μl conidial suspension were spread onto cellophane-overlaid plates of 1/4 SDAY alone (control) or supplemented with NaCl (0.8 M), menadione (0.2 mM), carbendazim (0.5 μg/ml), and Congo red (0.5 mg/ml) respectively, followed by 3-day incubation at 25°C and 12 : 12 h. Total RNAs were extracted from the stressed and unstressed (control) cultures with RNAiso^{TM} Plus Reagent (TaKaRa, Dalian, China) and reversely transcribed into cDNAs with PrimeScript® RT reagent Kit (TaKaRa). Each cDNA (10 × dilution) was used as template to assess the transcript levels of 90 phenotype-associated genes via qRT-PCR with paired primers (Table S1) under the action of SYBR® Premix Ex TaqTM (TaKaRa). The transcript level of each gene in cDNA was assessed using the $2^{-\Delta\Delta CT}$ method[50] and the fungal 18 S rRNA as internal standard. The relative transcript level of each gene under a given stress was calculated as the ratio of its transcript in each mutant over that in WT.

Assaying the phosphorylation levels of Hog1 and Slt2. The method of enzyme-linked immunosorbent assay (ELISA)[51] was adopted to assess the phosphorylation levels of Hog1 and Slt2 in the HOG and CWI pathways of Δ*cdc14* and control strains using their commercial phospho-antibodies respectively. Protein extracts from the 4-day cultures grown on 1/4 SDAY plates under normal conditions and chemical stresses were suspended in 1 ml PBS (pH 7.5). After 10 min homogenization on ice, the mixture was centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant was centrifuged for another 10 min. The protein concentration in each extract was assessed with the BCA Protein Assay Kit (KeyGen Biotech, Nanjing, China). For ELISA, 100 μl aliquots of each supernatant (5 μg/ml 0.05 M carbonate buffer, pH 9.6) were uploaded onto 96-well ELISA plate (Jet Biofil, Guangzhou, China) for overnight blocking at 4°C. Subsequently, the plate was repeatedly washed with PBS and added with 100 μl confining liquid [1% bovine serum albumin (BSA) in 0.05 M carbonate buffer] for 1 h incubation at 37°C. Washed again, the plate reacted with the rabbit phospho-p38 antibody (200 × dilution) against Hog1 or phospho-p44/42 antibody (500 × dilution) against Slt2 (Cell Signaling Technology, Boston, MA, USA) for 1 h at 37°C and then with the second antibody goat anti-rabbit IgG-HRP (Sigma) for 1 h at 37°C, followed by rinsing. Finally, 100 μl aliquots of TMB/H_2O_2 substrate (Amresco, Solon, OH, USA) were added to the wells on the plate for 20 min incubation at 37°C and the reaction was terminated with 2 M H_2SO_4 (100 μl per well). The phosphorylation level of Hog1 or Slt2 in each supernatant sample was measured as an OD reading at 450 nm (i.e., OD_{450}). Each assay included three samples as replicates.

Data analyses. The ratio of colony size or conidial germination rate under a given stress over that in the control was defined as relative viability (V_R). For each of the tested strains, the V_R trends over the concentrations (C) of each chemical, the time lengths (T) of wet-heat stress, and the doses (D) of UV-B irradiation were fitted to the equation $V_R = 1/[1 + \exp(a + bx)]$, where x is C, D or T, a and b are parameters to be estimated. When $V_R = 0.5$, the fitted equations gave solutions $(-a/b)$ to effective concentration (EC_{50}) of each stressful chemical required to suppress 50% colony growth and median lethal responses of conidia to heat (LT_{50}, min) and UV-B (LD_{50}, J/cm^2) stresses. Time-mortality trends from the bioassay were subjected to probit analysis, yielding LT_{50} (no. days) of each strain against the larvae. All the solutions or observations from three repeated assays were differentiated among the tested strains by one-way analysis of variance.

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Author contributions

M.G.F. and J.W. designed the research. J.W. and M.G.F. analyzed the data. J.W., J.L., Y.H. and S.H.Y. performed the experiments. M.G.F. and J.W. wrote the paper. All authors reviewed the manuscript.

Additional information

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