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A Group III histidine kinase (mhk1) upstream of high-osmolarity glycerol pathway regulates sporulation, multi-stress tolerance and virulence of *Metarhizium robertsii*, a fungal entomopathogen

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Summary

The role of *Metarhizium robertsii* Group III histidine kinase (mhk1) in regulating various phenotypes of the fungal entomopathogen and the transcripts of 25 downstream genes likely associated with the phenotypes were probed by constructing Δ*mhk1* and Δ*mhk1/mhk1* mutants. All examined Δ*mhk1* phenotypes except unchanged sensitivity to fungicide (dimethachlon) differed significantly from those of wild type and Δ*mhk1/mhk1*, which were similar to each other. Significant phenotypic changes in Δ*mhk1* included increased conidial yields on two media, increased tolerance to H_2O_2, decreased tolerance to menadione, increased tolerance to hyperosmolarity, increased conidial thermotolerance, decreased conidial UV-B resistance and reduced virulence to *Tenebrio molitor* larvae. The *mhk1* disruption elevated the transcripts of nine genes, including two associated with conidiation (*flbC* and *hymA*) and three encoding catalases but decreased seven other gene transcripts, including three for superoxide dismultases, under normal conditions. The high-osmolarity glycerol pathway MAPK phosphorylation level in Δ*mhk1* culture was increased 1.0- to 1.8-fold by KCl, sucrose and menadione stresses but reduced drastically by H_2O_2 or heat (40°C) stress, accompanied with different transcript patterns of all examined genes under the stresses. Our results confirmed the crucial role of mhk1 in regulating the expression of the downstream genes and associated phenotypes important for the fungal biocontrol potential.

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Introduction

The active ingredients of most mycoinsecticides are the formulated unicellular conidia of fungal entomopathogens, such as *Metarhizium anisopliae* (de Faria and Wraight, 2007). Upon application, such fungal cells often suffer from damages caused by high temperature, UV irradiation and fungicides applied for plant disease control (Roberts and St Leger, 2004). Thus, fungal tolerance to environmental stresses is of special value for the field persistence and efficacy of a fungal formulation against insect pests.

A two-component phosphorelay signalling system is generally involved in fungal response to a stress (Wolanin *et al*., 2002). This system consists of hybrid-type histidine kinase (HK), histidine-inclusive phosphor-transfer protein and response regulator (Motoyama *et al*., 2008). Of those, HK can autophosphorylate on a conserved histidine residue in response to a stress, yielding a phosphoryl group which can be transferred by the transfer protein to an aspartic acid residue of the response regulator so as to alter the cascade of conventional downstream signalling modules such as mitogen-activated protein (MAP) kinases (Thomason and Kay, 2000; West and Stock, 2001). All fungal HKs with N-terminal extracellular sensing domain and C-terminal cytosolic signalling domain constitute a large family of multifunctional signal-transduction enzymes (Wolanin *et al*., 2002). They act as autokinase, phosphor-transfer or phosphatase (Marina *et al*., 2005) and are classified to 11 groups in filamentous fungi (Catlett *et al*., 2003).

Much effort has been made to elucidate the HK role in regulating yeast or fungal osmosensitivity. *Saccharomyces cerevisiae* Sln1 was activated by low osmolarity, forming the phosphoryl group to be transferred, while the enzyme was inactivated by high osmolarity, resulting in the unphosphorylation of the response regulator and thus the activation of the MAP kinase (Santos and Shiozaki, 2001; Saito and Tatebayashi, 2004). Involved in the osmosensitive signal transduction pathway of *Aspergillus nidulans* is also the fungal HK gene *tcsB* (Furukawa *et al*., 2002). Recently, fungal phenotypes more than osmosensitivity have been found to be

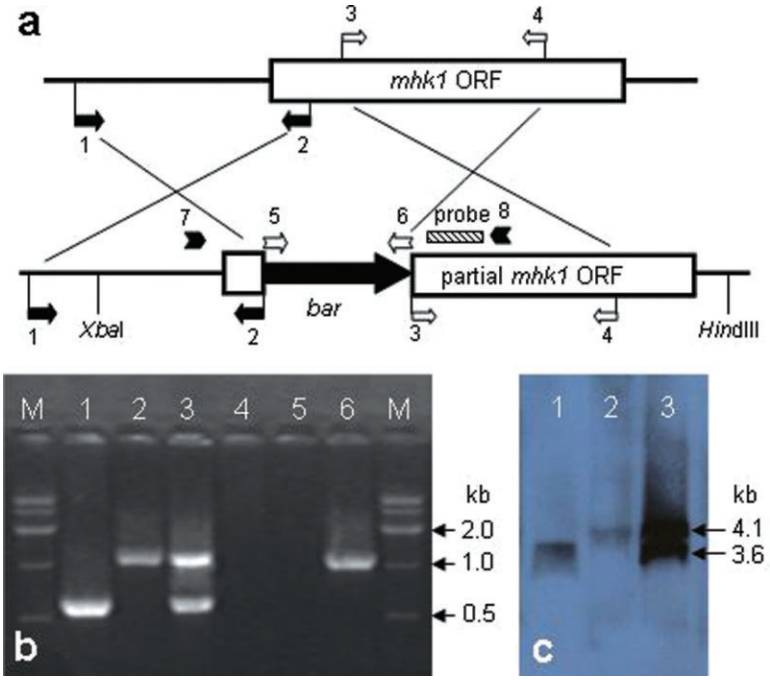

Fig. 1. Disruption and complement of *mhk1* gene encoding the Group III hestidine kinase of *M. robertsii*.
A. Diagram for *mhk1* knockout. Digits represent numbered primers in Table 2.
B. PCR detection for the presence of *mhk1* in the genomes of wild type (lanes 1 and 4), Δ*mhk1* (lanes 2 and 5) and Δ*mhk1/mhk1* (lanes 3 and 6) using primers HKd1F/HKd1R (lanes 1–3) and Sur1F/Sur1R (lanes 4–6).
C. Southern blotting of DNA extracts (digested with XbaI/HindIII) from wild type (lane 1), Δ*mhk1* (lane 2) and Δ*mhk1/mhk1* (lane 3) using the probe amplified with HKd2F/ HKd2R primers.

HK-mediated. The inactivation of Fhk1 in *Fusarium oxysporum* resulted in fungal resistance to fungicides as well as increased sensitivity to hyperosmotic and oxidative stresses and reduced virulence (Rispail and Di Pietro, 2010). *Magnaporthe oryzae* pathogenicity was reduced by disrupting the HK gene *MoSLN1* (H. Zhang *et al*., 2010). BOS1, a Group III HK in *Botrytis cinerea*, was proven to be necessary for macroconidiation and full virulence of the fungus besides its effect on osmosensitivity and fungicide resistance (Viaud *et al*., 2006). The decrease of *Penicillium marneffei* PmHHK1 expression by double-strand RNA interference led to significant changes in morphogenesis, sporulation and cell wall composition (Liu *et al*., 2008). These studies indicate that a fungal HK mediates not only cellular responses to environmental stresses, such as hyperosmolarity and fungicides, but also growth, sporulation and virulence. However, fungal entomopathogens were not considered in the previous HK studies. Neither was an effort made to probe possible HK role in regulating fungal thermotolerance and UV-B resistance, two traits important for the field persistence of a mycoinsecticide after application, and to show the effect of altered HK expression on other genes, particularly those in the high-osmolarity glycerol (HOG) pathway and associated with phenotypic changes.

The HOG pathway consists of MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and the MAP kinase (MAPK) to be activated by dual phosphorylation of conserved threonine and tyrosine residues within the activation loop (Chang and Karin, 2001), and can be activated by environmental stresses, such as hyperosmolarity and fungicides, causing changes in fungal morphology, growth and virulence (Jones *et al*., 2007; Rispail and Di Pietro, 2010). A null mutant of *Cochliobolus heterostrophus* MAPKKK (ChSte11) showed defects in conidiation, sexual development, melanization, appressorium formation, virulence and tolerance to the stresses of hydrogen peroxide and heavy metals (Izumitsu *et al*., 2009). Knockout of *B. cinerea* MAPKK gene (*bos5*) led to reduced growth, impaired conidiation and increased sensitivity to iprodion and hyperosmolarity (Yan *et al*., 2010). A null mutant of *Beauveria bassiana* MAPK hog1 became more sensitive to hyperosmolarity, oxidation and heat, and more resistant to fludioxonil (Zhang *et al*., 2009).

Metarhizium robertsii is an entomopathogenic fungus recently separated from *M. anisopliae* (Bischoff *et al*., 2009). The present study sought to elucidate the role of *M. robertsii* Group III HK (mhk1) in regulating colony growth, conidiation, virulence and tolerance to various stresses. Disrupting the *mhk1* gene from the fungal genome resulted in the increased expression of some MAP kinases and several interesting phenotypic changes.

Results

Knockout and complement mutants of mhk1

The *mhk1* gene (3879 bp ORF) was disrupted from the genome of *M. robertsii* wild-type strain (WT) by inserting the *bar* cassette into its upstream (Fig. 1A) and complemented by ectopic insertion of the cassette of its full-

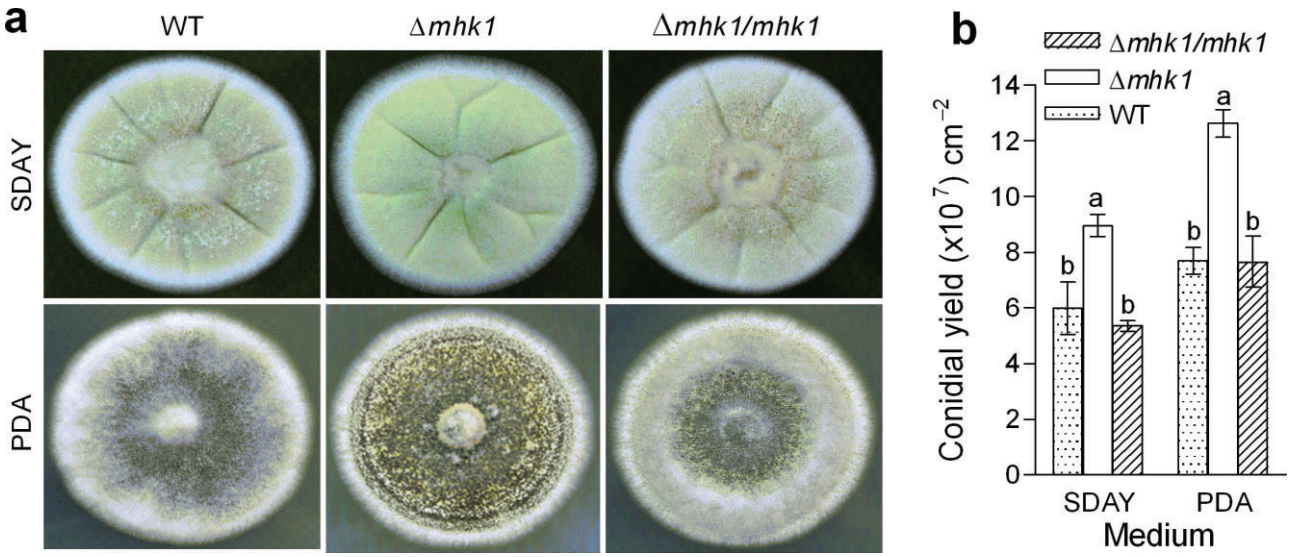

Fig. 2. Growth and conidiation features of *M. robertsii* wild-type strain (WT), Δ*mhk1* and Δ*mhk1/mhk1* on SDAY and PDA plates.
A. Colony morphology after 15 day growth at 25°C and 12:12 h.
B. Conidial yields on the colonies of the three strains. Different letters on grouped bars denote significant difference (Tukey's HSD, $P < 0.05$). Error bars: SD of the mean from three replicates.

length fragment into the Δ*mhk1* genome via *Agrobacterium*-mediated transformation (Fang *et al*., 2006; Duarte *et al*., 2007). As a result, a 630 bp fragment of partial *mhk1* ORF was PCR-amplified from WT and Δ*mhk1/mhk1* but not from Δ*mhk1* while a 1100 bp fragment bearing *bar* (marker 1) was present in the two mutants but not in WT (Fig. 1B). Another 1100 bp fragment (partial *sur* as marker 2) was detected only from Δ*mhk1/mhk1* by PCR. In Southern blotting, the *bar*-inclusive fragment (4.1 kb) was hybridized only to the XbaI/HindIII-digested genomes of both mutants (Fig. 1C) whereas the *bar*-free fragment (3.6 kb) was present in WT and Δ*mhk1/mhk1*. These data indicate that the disruption and complement of the *mhk1* gene were successful.

Phenotypic changes of Δmhk1 *and* Δmhk1/mhk1

Improved sporulation under normal conditions. The colonies of Δ*mhk1* grown for 15 days on SDAY and PDA plates at 25°C and 12:12 h showed some difference from those of WT and Δ*mhk1/mhk1* (Fig. 2A) although their colonies were similar in size. The Δ*mhk1* colonies were darker, particularly on PDA, indicating more pigment production.

Under the normal culture conditions, Δ*mhk1* produced the yields of 9.0 ± 0.4 and $12.6 \pm 0.5 \times 10^7$ conidia cm^{-2} on the SDAY and PDA plates respectively. These conidial yields were 50–67% higher than the similar yields from WT and Δ*mhk1/mhk1* (Fig. 2B).

Enhanced hyperosmotolerance. The sizes of Δ*mhk1* colonies grown for 6 days at 25°C on the plates of 1/4 SDAY supplemented with different stressful agents differed from the WT and Δ*mhk1/mhk1* counterparts (Table 1). Based on the per cent growth inhibition of the strains relative to unstressed controls (~ 20 mm diameter), Δ*mhk1* was significantly more tolerant to or less inhibited by the hyperosmotic stress of KCl, NaCl, glucose

Table 1. Relative growth inhibition (RGI) of WT, Δ*mhk1* and Δ*mhk1/mhk1* colonies grown on 1/4 SDAY plus stressful agents at 25°C.

	RGI ± SD (%)[a]			
Stressful agents	WT	Δ*mhk1*	Δ*mhk1/mhk1*	$F_{2,6}$ value[b]
KCl (40 mg ml^{-1})	70.9 ± 2.7 a	38.0 ± 4.4 b	65.8 ± 3.1 a	78.41**
NaCl (40 mg ml^{-1})	77.7 ± 3.6 a	50.0 ± 1.3 b	71.7 ± 3.3 a	75.84**
Glucose (240 mg ml^{-1})	78.6 ± 0.5 a	62.0 ± 3.6 b	71.8 ± 3.1 a	27.74**
Sucrose (240 mg ml^{-1})	62.5 ± 1.3 a	54.3 ± 1.9 b	59.8 ± 3.7 ab	8.27*
Menadione (10 mM)	11.0 ± 5.9 b	33.6 ± 4.2 a	15.3 ± 4.2 b	18.55**
H_2O_2 (100 mM)	24.7 ± 4.2 a	15.4 ± 4.3 b	24.8 ± 3.0 a	5.81*
Dimethachlon (200 μg ml^{-1})	45.3 ± 2.8 a	44.8 ± 3.5 a	44.4 ± 2.8 a	0.06

a. Means with different letters in each line differed significantly (Tukey's HSD, $P < 0.05$).
b. *F*-test in one-way ANOVA: $P < 0.05$ (*) or 0.01 (**).

or sucrose (Tukey's HSD, $P < 0.05$) than two other strains, which showed similar osmosensitivity. The *mhk1* disruption caused an increase of 9–42% (vs. Δ*mhk1/mhk1*) or 13–46% (vs. WT) in hyperosmotolerance, varying with the hyperosmotic agents tested.

Altered antioxidative capability and no change in fungicide sensitivity. The Δ*mhk1* colonies responded differently to the oxidants menadione and H_2O_2. The relative growth inhibition of its colonies was significantly increased by adding 10 mM menadione (a compound generating superoxide) to 1/4 SDAY but reduced by 100 mM H_2O_2 compared to the estimates from WT and Δ*mhk1/mhk1* (Table 1). However, the three strains displayed similar sensitivity to the fungicide dimethachlon ($F_{2,6} = 0.06$, $P = 0.94$).

Increased conidial thermotolerance. The inverted sigmoid surviving trends of the conidia of the three strains over the time lengths of wet-heat stress at 48°C (Fig. 3A) and the gradient doses of UV-B irradiation (Fig. 3C) fit well the modified logistic equation ($r^2 \geq 0.985$, $P < 0.001$ in *F*-tests for the fitness). Based on the fitted trends, the medial lethal time (LT_{50}) of the heat stress towards Δ*mhk1* conidia (Fig. 3B) was estimated as 60.7 min. Compared to the heat LT_{50}s for WT (44.4 min) and Δ*mhk1/mhk1* (47.7 min), conidial thermotolerance increased 27.1–36.6% after the *mhk1* deletion.

Reduced UV-B resistance. The median lethal dose (LD_{50}) of the UV-B irradiation (Fig. 3D) estimated from the fitted trends were averaged as 0.158, 0.148 and 0.156 J cm^{-2} ($F_{2,6} = 9.7$, $P = 0.013$) for the WT, Δ*mhk1* and Δ*mhk1/mhk1* strains respectively. The LD_{50}s indicate that the gene disruption caused a significant decrease of 5.4–6.6% in conidial UV-B resistance.

Increased content of conidial formic-acid-extractable (FAE) proteins. The FAE protein content in the conidial extract of Δ*mhk1* was assessed as 37.5 ± 0.12 μg mg^{-1} conidia and enhanced significantly by 41.2–51.4% compared to the contents of 24.8 ± 0.34 and 26.5 ± 1.05 μg mg^{-1} from WT and Δ*mhk1/mhk1* (Tukey's HSD, $P < 0.01$). Interestingly, the trend of the contents from the three strains coincided well with that of their heat-responsive LT_{50}s.

Decreased virulence to Tenebrio molitor *larvae.* Bioassay data (Fig. 3E) of the three strains against the third-instar larvae of *T. molitor* topically inoculated under a standardized conidial spray were subjected to probit analysis, generating acceptable time-mortality trends ($\chi^2 \leq 10$, d.f. = 6, $P \geq 0.12$ for heterogeneity tests for fitness). The LT_{50}s estimated from the fitted trends of three repeated bioassays were averaged as 4.5 ± 0.5, 5.6 ± 0.2 and 4.4 ± 0.4 days (Fig. 3F) for the WT, Δ*mhk1* and Δ*mhk1/mhk1* respectively. The results indicate that the Δ*mhk1* virulence was reduced by ~ 25% (i.e. the percentage of prolonged LT_{50}).

The transcript levels of related genes and the level of MAPK phosphorylation

The transcript levels of 25 genes in the WT, Δ*mhk1* and Δ*mhk1/mhk1* cultures were assessed by quantitative real-time PCR (qRT-PCR) using the fungal 18S rRNA as internal standard. Under normal and stressful conditions, all the genes showed similar transcript levels between Δ*mhk1/mhk1* and WT (= 1) but different expression patterns in Δ*mhk1* (Fig. 4), whose *mhk1* transcript was not detected throughout the study. In the Δ*mhk1* culture with no stress, *hymA* (conidiophore development protein gene) and *flbC* (conidiation transcription factor) were upregulated respectively by 1.6- and 4.7-fold (Fig. 4A), well in agreement with the enhanced conidial yields of Δ*mhk1* on two substrates. All MAP kinase genes except *mapk2* were upregulated by 2.2- to 8.5-fold or downregulated to 26–85% of the transcripts in WT. Other affected genes included the catalase (CAT) genes *cat3*, *cat6* and *cat7* (upregulated by 1.6- to 15.1-fold) and the superoxide dismutase (SOD) genes *sod4*, *sod5* and *sod6* (downregulated to 11–33% of the transcripts in WT). Exceptionally, *cat4* seemed to be a pseudogene because its transcript was not detected in any cDNAs of the tested strains.

KCl and sucrose-incuded hyperosmotic stresses caused the upregulation of several MAP kinases genes in the HOG pathway of Δ*mhk1*, accompanied with the upregulaition of *cat3*, *cat5*, *cat6*, *sod4*, *sod5* and *sod6* by KCl (Fig. 4B) and the downregulation of all CAT genes by sucrose (Fig. 4C). This and the drastic increase of MAPK phosphorylation (1.0- to 1.8-fold) by either agent (Fig. 5) indicated different HOG pathways involved in the regulation of the fungal response to different hyperosmotic agents.

The menadione stress elevated the gene transcripts of two MAPKKKs, one MAPKK and four SODs in Δ*mhk1* but decreased those of three MAPKs, all CATs and two SODs (Fig. 4D), accompanied by 100% increase in the level of MAPK phosphorylation (Fig. 5). This implied that the fungal response to menadione was likely associated with the upregulated CAT and SOD genes but not regulated by the MAPKs. Although H_2O_2 increased significantly the genes transcript levels of two MAPKs (Fig. 4E), a significant decrease of 34% in the level of MAPK phosphorylation (Fig. 5) indicated that the HOG pathway was not involved in regulating the fungal response to H_2O_2 oxidation. Two CAT genes and four SOD genes upregulated by H_2O_2 were likely to take part in the regulation.

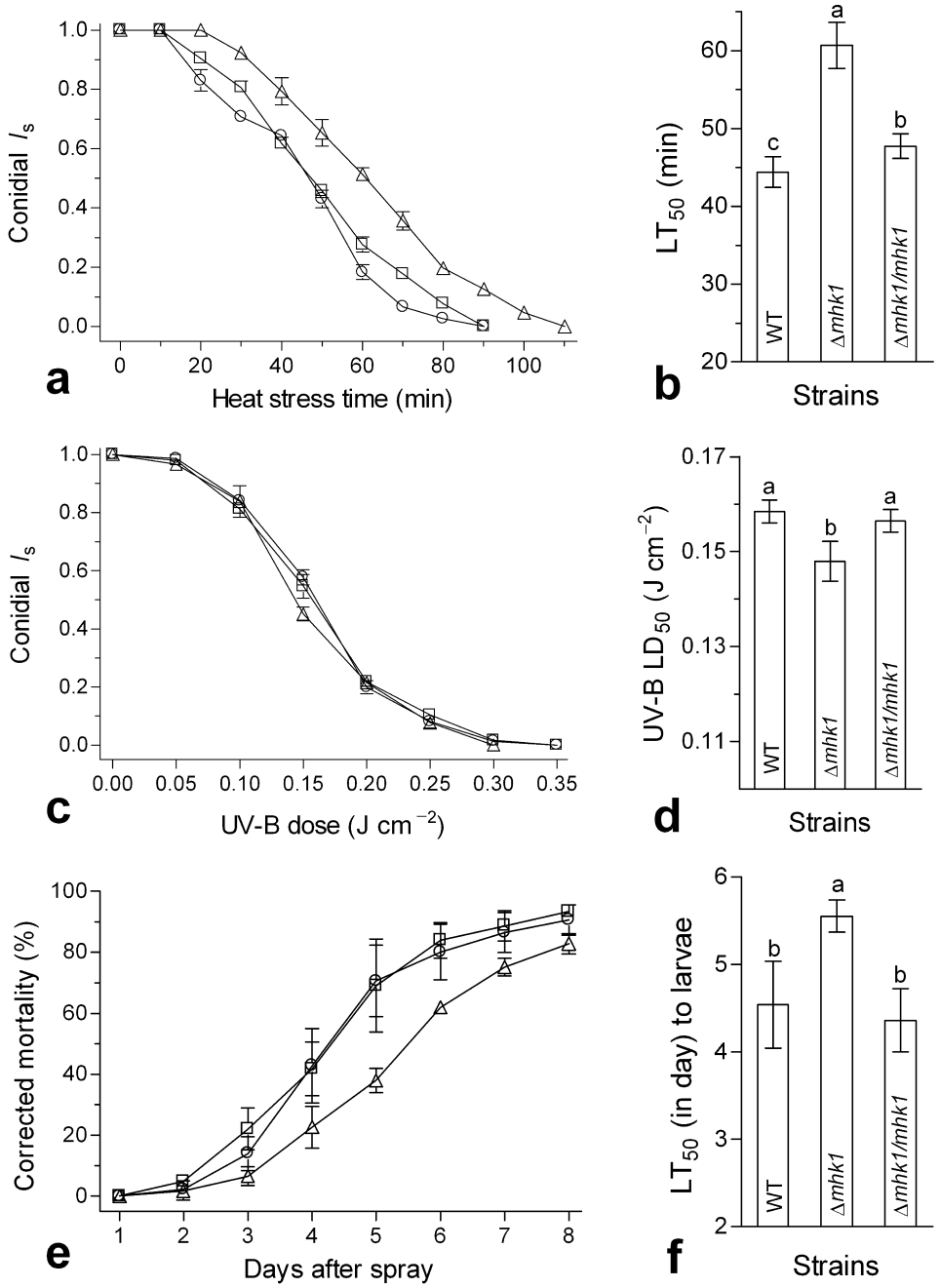

Fig. 3. Comparison of phenotypic parameters between Δ*mhk1* (△) and wild type (WT) (○) or Δ*mhk1/mhk1* (□) of *M. robertsii*.
A. Trends of conidial survival indices (I_s) over the time of the wet-heat stress at 48°C.
B. LT_{50} estimates from the fitted trends.
C. The observed I_s trends over the gradient doses of UV-B irradiation.
D. LD_{50} estimates from the fitted trends.
E. Time–mortality trends of the three strains against the third-instar larvae of *T. molitor* after spraying the standardized suspension of 10^7 conidia ml^{-1}.
F. LT_{50}s estimated by probit analysis.
Different letters on bars denote significant difference (Tukey's HSD: $P < 0.05$). Error bars: SD of the mean from three repeated assays.

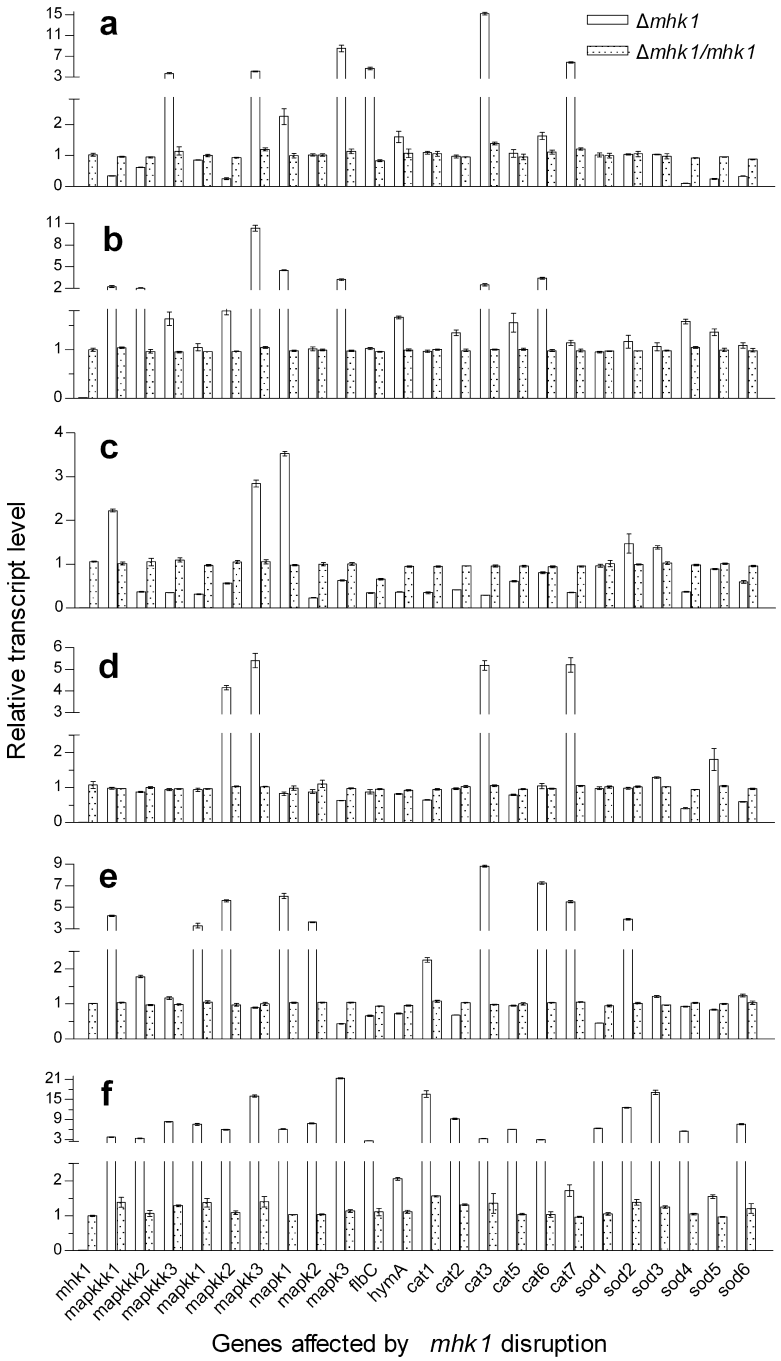

Fig. 4. Relative transcript levels of selected genes in the cDNAs of Δ*mhk1* and Δ*mhk1/mhk1* versus wild type. Quantitative real-time PCR was performed with paired primers (Table 3) using the 18S rRNA as internal standard and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Total RNAs were extracted from the cultures grown for 4 days at 25°C on 1/4 SDAY as control (A) or supplemented with 40 mg KCl ml^{-1} (B), 240 mg sucrose ml^{-1} (C), 0.2 mM menadione (D) or 4 mM H_2O_2 (E), or from the control cultures exposed to 40°C for 1 h (F) after 4 day growth at 25°C. Error bars: SD.

Interestingly, in the Δ*mhk1* culture exposed to 40°C for 1 h, all the genes except *f1bC* and *cat7* were upregulated (Fig. 4F) and the MAPK phosphorylation level was remarkably decreased, suggesting a complexity in the regulation of the fungal thermotolerance.

Discussion

As presented above, mhk1 was proven to mediate various phenotypes of *M. robertsii* by affecting the expressions of some HOG pathway MAP kinases. Such phenotypes are important for the biocontrol potential of the fungal entomopathogen and discussed below in comparison with phenotypic changes in other fungal HK knockout or knockdown mutants.

First of all, the *mhk1* knockout resulted in improved conidiation on both PDA and SDAY and thus 50–67% increase in conidial yield despite inconspicuous effect on colony growth. This phenotypic change is opposite to defected sporulation in the mutants of *B. cinerea* BOS1

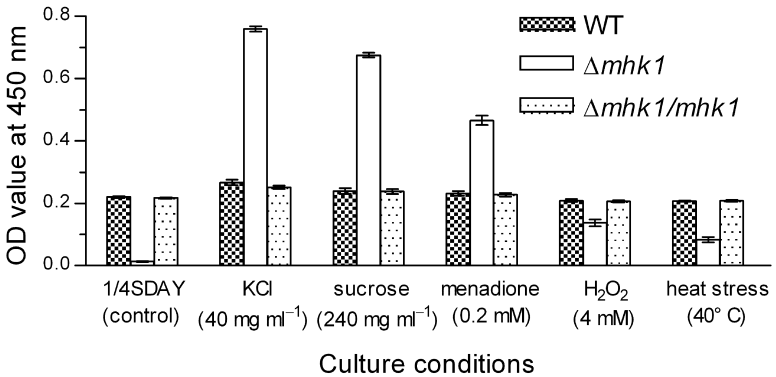

Fig. 5. Comparison of MAPK phosphorylation levels in the Δ*mhk1* cultures under normal (control) and stressful conditions. Error bars: SD.

knockout and *P. marneffei* PmHHK1 knockdown (Viaud *et al*., 2006; Wang *et al*., 2009) but well in agreement with the reduction of sporulation by the inactivation of *B. bassiana* MAPK hog1 and *M. grisea* osm1 (Dixon *et al*., 1999; Zhang *et al*., 2009) because the transcript levels of MAPK1 and MAPK3 in Δ*mhk1* was enhanced by 2.2- to 8.5-fold. The upregulated expressions of the *flbC* and *hymA* genes associated with conidiation also support an increase of conidial yield in Δ*mhk1*. Our results indicate that mhk1 is required in regulating the sporulation of *M. robertsii* although possible cross-talk between mhk1 and the downstream MAP kinases is not understood at present. Regulators of G protein signalling (RGS) pathway also could be involved in the cross-talk. Previously, the inactivation of the RGS protein cag8, orthologous to the RGS protein FlbA in *A. nidulans*, resulted in poor sporulation of *M. anisopliae* on agar plates or mycosed insect cadavers (Fang *et al*., 2007).

The *mhk1* deletion facilitated colony growth on 1/4 SDAY supplemented with all tested hyperosmotic agents, particularly KCl, suggesting tha mhk1 negatively regulate the fungal hyperosmotolerance. This is opposite to the increase of osmosensitivity by HK inactivation or suppression in other fungi (Lin and Chung, 2010; Rispail and Di Pietro, 2010; H. Zhang *et al*., 2010). Previously, fungal HK was found regulating negatively the downstream hog1-like MAPK (Yoshimi *et al*., 2005; Liu *et al*., 2008; Lin and Chung, 2010), a phenomenon also confirmed in our study. The elevated transcript levels of some MAP kinase genes in the control and stressed cultures of Δ*mhk1* suggest that mhk1 mediate cellular hyperomosensitity to sucrose through the mapkkk1–mapkk2–mapk1 HOG pathway and to KCl through the pathways of possible combinations of MAPKKK1, MAPKKK2, MAPKK2, MAPKK3 and MAPK1. This hypothesis is partially evident with the fact that KCl induced higher MAPK phosphorylation level in the Δ*mhk1* culture than sucrose.

Moreover, Δ*mhk1* became much more sensitive or less tolerant to menadione oxidation, which reduced the gene transcripts of three MAPKs but increased greatly their phosphorylation level. The increased sensitivity was well in accordance with the responses of previous HK knockout mutants to the same stress (Nathues *et al*., 2007; Rispail and Di Pietro, 2010). The genes *sod3* and *sod5* (encoding MnSOD) were likely responsible for the fungal tolerance to menadione-produced superoxide anions because their transcript levels were enhanced by 2.6- and 7.2-fold in the Δ*mhk1* culture under the chemical stress. Two other Cu/Zn-SOD genes, *sod4* and *sod6*, were less likely involved in the response to menadione, as indicated by smaller increases of their transcripts in the stressed culture. On the other hand, Δ*mhk1* was more tolerant to the H_2O_2 oxidation, which remarkably increased the gene transcripts of two MAPKs but significantly reduced their phosphorylation level. This phenomenon is very different from most previous reports (Viaud *et al*., 2006; Rispail and Di Pietro, 2010; H. Zhang *et al*., 2010). The enzyme genes *cat6* and four SOD genes (*sod2*, *sod4*, *sod5* and *sod6*) were most likely involved in regulating the fungal response to H_2O_2 because their transcripts in the stressed Δ*mhk1* culture were 4.5- and 3.3- to 8.2-fold of those in the control respectively. Since menadione and H_2O_2 act as SOD and CAT inducers (Jeong *et al*., 2001; Gonzalez-Parraga *et al*., 2003; Angelova *et al*., 2005), the different responses of Δ*mhk1* to the two oxidants indicate that mhk1 may affect the anti-oxidative capability of *M. robertsii* by mediating the expression of some SODs positively or some CATs negatively and that the mediation is likely independent of the HOG pathway because the two oxidants caused different changes in MAPK transcripts and phosphorylation levels. However, mhk1 was not involved in mediating the fungal sensitivity to dimethachlon fungicide, a phenotype often reported to be HK-mediated in phytopathogenic fungi (Nathues *et al*., 2007; Liu *et al*., 2008; Dongo *et al*., 2009; Lin and Chung, 2010).

More interestingly, mhk1 wad found for the first time to negatively regulate conidial FAE protein content and thermotolerance, which were enhanced by 41–51% and 27–37% in Δ*mhk1* respectively. This finding provides further evidence for the previous correlation of the two

Table 2. The primers designed for *mhk1* cloning, disruption, complement and mutant identification.

No.	Primer	Sequence (5′–3′)[a]	Purpose
1	HK5F	CGGAATTCTCTTATCCCTCCTCTCGCTTC	Cloning 5′ fragment
2	HK5R	CGGGATCCGCCCAACAAAAGAAAGAAGATG	
3	HK3F	AAAACTGCAGGGCGAAATCTTTGAACTCAAGTC	Cloning 3′ fragment
4	HK3R	GGACTAGTTTGCCCACAAGGTTGAGAATAAT	
5	BarF	CGGGATCCTCGACAGAAGATGACATTGAAGGAGC	Cloning the first marker *bar*
6	BarR	AACTGCAGTCAGATCTCGGTGACGGGCAGGACCG	
7	HKd1F	CGTAATGGCTCAAAACCTCAC	PCR for knockout mutant
8	HKd1R	CTCCAGGTTCCTTCAACATCAT	
9	HKd2F	ATCAAATGGTAGATCGTCTGGGC	Probe for Southern blotting
10	HKd2R	GGTAAAGTCTCCGTCAGTCGCTAAAT	
11	HKfF	GGGGACAAGTTTGTACAAAAAAGCAGGCT GTCCGACTCCGTGCGTACTACATAC	Cloning the full-length *mhk1*
12	HKfR	GGGGACCACTTTGTACAAGAAAGCTGGGT GCAGATCTCGCTCAGGTATATCAAC	
13	Sur1F	CGGAATTCGTCGACGTGCCAACGCCACAGTG	Cloning the second marker *sur*
14	Sur1R	CGGGATCCGTCGACGTGAGAGCATGCAATTCC	
15	Sur2F	CCAAGGTGTCATTTTGTCGGAAG	PCR for complement mutant
16	Sur2R	GCCACAGGAGTGCATTGGATATATT	

a. The enzyme sites are underlined while the gateway exchange sequences are framed.

traits in fungal biocontrol agents (Ying and Feng, 2004) and also for a significant decrease of conidial thermotolerance by suppressed expression of the FAE protein CP15 in the knockdown mutants of *B. bassiana* (Ying and Feng, 2011). Thus, we propose that mhk1 is likely involved in conidial thermotolerance by mediating the expression of at least some thermosensitive FAE proteins, such as CP15, in *M. robertsii*. Moreover, most of the examined genes were upregulated in the Δ*mhk1* culture after exposure to 40°C for 1 h. Of those, the remarkably upregulated genes include *mapkkk1* (10.9-fold), *mapkk2* (22.9-fold), *mapk2* (7.7-fold), *cat1* (15.2-fold), *cat2* (9.5-fold), *cat5* (5.6-fold), *sod2* (12.2-fold), *sod3* (16.6-fold) and *sod5* (48.9-fold). These suggest a great complexity in mediating the fungal response to the heat stress and the involvements of at least some SODs and CATs in the mediation.

However, the effect of the *mhk1* disruption on conidial UV-B resistance is somewhat complicated due to different SOD and CAT expression patterns in the Δ*mhk1* responses to the two oxidants. The genes *sod4* and *sod5* are more likely associated with the fungal UV-B sensitivity because their transcripts in Δ*mhk1* drastically decreased to 11 and 25% of those in WT but increased 2.6- and 6.2-fold under the menadione stress. In another study, the overexpression of *B. bassiana* MnSOD in a WT lacking the enzyme resulted in 61% increase in conidial tolerance to menadione and 23% increase in conidial UV-B resistance (Xie *et al*., 2010). All CAT genes except *cat6* are unlikely associated with the reduced UV-B resistance because the H_2O_2 stress downregulated most of them in the Δ*mhk1* culture despite the upregulation of *cat3* and *cat7* in the unstressed control. Unfortunately, possible interactions between the two types of antioxidant enzymes are currently unknown. More pigment production in Δ*mhk1* did not seem to increase its UV-B resistance, as reported previously for *M. anisopliae* (Braga *et al*., 2006).

Finally, *M. robertsii* virulence to *T. molitor* larvae was decreased by ~ 25% after the *mhk1* disruption. This was in agreement with most previous reports on the reduced virulence of the HK knockout mutants of several plant pathogens (Viaud *et al*., 2006; Nathues *et al*., 2007; H. Zhang *et al*., 2010). However, the Group III HK of *Alternaria alternata* was considered completely dispensable for the fungal pathogenicity (Lin and Chung, 2010).

Conclusively, mhk1 is a crucial enzyme upstream of the HOG pathway to regulate the sporulation, multi-stress tolerance and virulence of *M. robertsii*. Particularly, its effects on the fungal sporulation and tolerance to hyperosmolarity are distinct from the HK effects observed in other fungi.

Experimental procedures

Microbial strains and cultures

The WT *M. robertsii* ARSEF2575 (RW Holley Center for Agriculture and Health, Ithaca, NY, USA), denoted as WT strain in this report, was used as a recipient of gene knockout and cultured on SDAY (Sabouraud's dextrose agar supplemented with 1% yeast extracts) plates at 25°C. *Escherichia coli* Top10 and *E. coli* DH5α from Invitrogen (Shanghai, China) used for vector propagation were cultured at 37°C in LB broth supplemented with kanamycin (100 μg ml^{-1}). *Agrobacterium tumefaciens* AGL-1 used for fungal transformation was cultured in YEB broth (Fang *et al*., 2006) at 28°C.

Disruption and complement of mhk1

Two pairs of primers, HK5F/HK5R and HK3F/HK3R (Table 2), were designed to amplify the 5′ and 3′ regions of

the *mhk1* gene from the WT strain in terms of its open reading frame (3879 bp) encoding for a 1293 amino acid protein (GenBank: EFY98542.1) in the sequenced genome (GenBank: GL698718) of *M. robertsii* ARSEF23 (Gao *et al*., 2011). The deduced protein was predicted to share 74–100% sequence identity with 99 fungal HKs in the NCBI protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and to have five methyl-accepting protein and phosphatase (HAMP) domains, one HK A domain, one two-component VirA-like sensor kinase domain and one HK-like ATPase domain, and thus identified as a Group III HK based on the structural features (Catlett *et al*., 2003). The phosphinothricin (PPT) resistance gene *bar* was amplified as the first selective marker from pAN52-bar (Ying and Feng, 2006) using paired primers BarF/BarR. The *bar* fragment was digested with BamHI/PstI and then ligated into pCAMBIA-0380 (p0380 herein), forming p0380-bar1. The amplified 5′ fragment (1540 bp) was inserted into the EcoRI/BamHI sites of p0380-bar1, yielding the plasmid p0380-bar1-mhk1up. The 3′ fragment (1320 bp) was then cloned into this plasmid cut with PstI/SpeI, resulting in the disruption vector p0380-bar1-mhk1. This vector was introduced into *A. tumefaciens* AGL-1 for fungal transformation using a previous protocol (Fang *et al*., 2006; Duarte *et al*., 2007) with slight modification. Briefly, the WT strain was co-cultivated with the vector-carrying AGL-1 on the plates of induced medium for 48 h at 25°C in dark, followed by washing with ~ 5 ml of 0.02% Tween 80. The resultant suspension was spread onto M-100 plates containing 300 μM cefotaxime and 200 μM PPT and the incubated for 6 days at 25°C and 12:12 h (light : dark cycle). The disruption mutant Δ*mhk1* was identified from the colonies grown on the selective plates via PCR and Southern blotting.

To complement the target gene into Δ*mhk1*, the *Magnaporthe grisea* sur gene cassette was amplified as the second marker from pCB1536 (S. Zhang *et al*., 2010) using SurF/SurR primers (Table 2). The purified fragment was digested with EcoRI/BamHI and ligated into p0380-bar2, which was constructed by adding more enzyme sites to p0380 as above. The resultant plasmid was designated as p0380-bar2-sur. A gateway fragment cut from pGK02 (Khang *et al*., 2005) with XbaI/HindIII was inserted into the *sur* plasmid digested with the same enzymes. The *bar* gene in p0380-bar2-sur was then replaced with the fragment, forming p0380-sur-gateway. The full-length *mhk1* fragment including the upstream and downstream regions of 1981 and 1338 bp was amplified from the WT genome using paired primers HKfF/HKfR (Table 2) and LA Taq® Polymerase (TaKaRa, Dalian, China). The gateway fragment in the plasmid was then exchanged for the amplified fragment (7550 bp) under the action of Gateway® BP ClonaseTM II Enzyme Mix (Invitrogen). The new plasmid p0380-sur-mhk1_7550 was finally transformed into Δ*mhk1* for the gene complement through *A. tumefaciens* AGL-1 as detailed above. After co-cultivation, the suspension washed off from the induced medium plate was spread onto the M-100 plates containing not only 300 μM cefotaxime and 200 μM PPT but also 10 μM chorimuron ethyl (ChemService, West Chester, PA, USA). After 6 day incubation at 25°C and 12:12 h, the complement mutant Δ*mhk1/mhk1* was identified from the fungal colonies grown on the selective plates via PCR and Southern blotting.

The PCR for verification of *mhk1* knockout and complement was performed using the primers HKd1F/HKd1R (Table 2), which were designed to amplify the fragments of 630 bp, 1100 bp and both from WT, Δ*mhk1* and Δ*mhk1/mhk1* respectively. In Southern blotting with HKd2F/HKd2R primers, 30 μg genomic DNA extracted from SDAY colonies of each strain was digested with XbaI/HindIII, separated by electrophoresis in 0.7% agarose gel, and then transferred to Biodyne B nylon membrane (Gelman Laboratory, Shelton, WA, USA) in Trans-Blot SD Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Probe preparation, membrane hybridization and visualization were carried out using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany).

Phenotypic assays of WT and mutant strains

After *mhk1* gene was disrupted and complemented, a series of phenotypic parameters of the WT, Δ*mhk1* and Δ*mhk1/mhk1* strains were assessed in the following triplicate experiments.

Growth and conidiation under normal conditions. Filter paper discs (5 mm diameter) immersed with suspension (10^7 conidia ml^{-1} 0.02% Tween 80) were placed on the centre of SDAY and PDA (potato dextrose agar) plates (60 mm diameter), followed by 15 day incubation at 25°C and 12:12 h. All colonies were observed for possible phenotypic changes in colony colour and size. The conidial yields of each strain on the plates were assessed by taking three 5-mm-diameter samples from each plate, washing each sample in 1 ml of 0.02% Tween 80 via 10 min vibration, making microscopic counts on a haemocytometer to determine the concentration of the suspension, and converting the concentration to no. conidia cm^{-2}.

Assays for cell osmosensitivity, antioxidative capability and fungicide sensitivity. Aliquots of 200 μl suspension (10^7 conidia ml^{-1}) were evenly smeared onto cellophane attached to SDAY plates. After 3 day incubation at 25°C and 12:12 h, 5-mm-diameter discs were cut from the colony of each strain and the cellophane discs with growing mycelia were gently attached to the 9-cm-diameter plates of 1/4 SDAY (amended SDAY with all components except agar reduced to 1/4) supplemented with the agents: (i) NaCl (40 mg ml^{-1}), KCl (40 mg ml^{-1}), glucose (240 mg ml^{-1}) and sucrose (240 mg ml^{-1}) for osmosensitivity assay respectively; (ii) menadione (10 mM) and H_2O_2 (100 mM) for oxidative stress assay; and (iii) dimethachlon (200 μg ml^{-1}) for fungicide sensitivity assay. All the plates were incubated for 6 days at the same regime and the resultant colonies were cross-measured for their diameters (mm). For each strain, relative growth inhibition was calculated as $(C - N)/(C - 5) \times 100$, where C is the control (not stressed) colony diameter and N the colony diameter under a given stress.

Assays for conidial tolerance to heat and UV-B irradiation. For thermotolerance assay, aliquots of 50 mg conidia were suspended in 1 ml of 0.02% Tween 80 in 1.5 ml glass vials and then exposed to heat stress by immersing the vials in

Table 3. The primers used in qRT-PCR for assessing the transcript levels of 25 genes in the disruption and complement mutants versus wild type.

GenBank code	Gene	Sequences (5′–3′) of paired primers
AY755540.1	18S RNA	GAGCCAGCGAGTAATTCC / AGCCATTCAATCGGTAGTAG
EFY98542.1	*mhk1*	GATTGAACGACCAGCCAGAG / CTTGACCACCCAGAATACCTTC
EFY99654.1	*mapkkk1*	TCGTCACCACCATCTTCC / TTGCCTCCTTCCTCTTAGC
EFZ00933.1	*mapkkk2*	CACGAGATTCACAGAGATG / ATCAGAGGCGATGAGTTC
EFZ00513.1	*mapkkk3*	GTGTTGCTGTTGGTGTTG / TGTATTGGACGGAGTTCTG
EFY98774.1	*mapkk1*	TGCTATGGAGTTCTGTGAAGG / GAGTGTGGAGGTAAGTGAGTC
EFZ00644.1	*mapkk2*	GGCGGCAGAGATGATAAC / AACCTTGCGAGCCATTAC
EFZ03782.1	*mapkk3*	AATCTCTGGCGGAGCAATG / CCTTCTACAATGGCGTTCAAC
EFY99068.1	*mapk1*	ACCCTCAAATGACTGGATAC / TCGGTGATAATGGAGAACTG
EFZ01952.1	*mapk2*	CGCAAGGTCTTCAAGGTCTTC / CGCTCGTCTGGTTGTTCAC
EFY99574.1	*mapk3*	TTAGCGAGCAATACGACATTC / CGTGATTGAAGTAGCGAAGG
EFY96136.1	*flbC*	GAACCTCTACTACCAACAAC / TCTTGACTCTGCGTGTAG
EFZ01462.1	*hymA*	GTCGTATGTCGTATGTAACC / CCGTCGTCATAGAGGATG
EFY98740	*cat1*	ACCTCTACTCTTCATACCTTGTTC / GCTTAGTGTCACCATTCTCATTC
EFZ01974.1	*cat2*	CAAGACGCCATCAAGTTC / GAAGTCCCAGAAGTTGTTG
EFZ04187.1	*cat3*	GATTGATGGATGGACATATACTG / AATACCTTGACTGCCTGTTC
EFY94110.1	*cat4*	AGATTCGCTACGGTAACAG / GGTGAGGAACTTGGCATC
EFY98646	*cat5*	ACAATAACACGCCAATCTTCTTC / CTGGTGAGTTGAGAGGTAATCC
EFY97957	*cat6*	ATCTGAATGAGAGCCTTGAAC / AACGCCTTGATGGAATGAC
EFY94986	*cat7*	TGGAGTCTGTTGGCTATC / GTCGTAGTTGGTGTTCAG
EFZ03762.1	*sod1*	GCTGGACCTCACTTCAAC / GGAACCCTTGGCATTACC
EFZ00595.1	*sod2*	GCTGCCTCACGACTACTC / TGGTCTTGGTGTCCTTGTC
EFY99820.1	*sod3*	CTGCGGATCGTGACTACC / CAGTTGATGACCTTCCAGATG
EFZ00365.1	*sod4*	CTTGCGATGCTGGCTCTC / GTTGGCGAGGTGGATGAC
EFY99375.1	*sod5*	AGGTCCTCTTCTGAACTTCC / GCGTTGGTCTGCTTCTTC
EFY94215.1	*sod6*	ATCTGAGCGGCAAGTATG / AATTGGCACAGGTGATCC

water bath at 48°C for up to 90 min. During the exposure, 100 μl samples taken from each vial at 10 min interval were spread onto the plates of TPYA germination medium (0.2% trehalose, 0.25% peptone, 0.25% yeast extract and 2% agar). After 48 h incubation at 25°C, the ratio of the per cent germination of a stressed sample (determined with three microscopic counts per sample) over that of an unstressed control was defined as conidial survival index (I_s).

To assay conidial UV-B resistance, 60 μl aliquots of fungal suspension (10^7 conidia ml^{-1}) were spread onto TPYA plates (6 cm diameter), followed by ~ 20 min drying in air (not covered with the lids of Petri dishes). The plates were then placed in the sample tray of Bio-Sun^{++} UV irradiation chamber (Vilber Lourmat, Marne-la-Vallée, France) and exposed to the UV-B irradiation of weighted wavelength of 312 nm at the gradient doses of 0.05–0.35 J cm^{-2}. After exposure, the plates were covered with lids and incubated for 24 h germination at 25°C. Conidial survival indices (I_s) on the plates were determined using non-irradiated plates as controls, as described above.

Extraction of conidial proteins. Proteins were extracted from the conidia of each strain using the formic acid method detailed elsewhere (Ying and Feng, 2004) since the formic-acid-extractable (FAE) proteins may contribute to conidial thermotolerance (Ying and Feng, 2011). The FAE protein contents (μg mg^{-1} conidia) in the extracts were quantified with the BCA Protein Assay Kit (KeyGen Biotech, Nanjing, China) following the user's guide.

Bioassay for fungal pathogenicity. Aliquots of 1 ml suspension (10^7 conidia ml^{-1}) of each strain were sprayed onto ~ 30 third-instar larvae of *T. molitor* in lid-free Petri dish (9 cm diameter) from the top nozzle of an Automatic Potter Spray Tower (Burkhard Scientific, Uxbridge, UK) at the working pressure of 0.7 kg cm^{-2}. The same volume spray of 0.02% Tween 80 was used as blank control. Dried for ~ 10 min in air, the sprayed larvae were supplied with wheat bran and reared for 8 days at 25°C. Mortalities were monitored daily during the rearing period.

Assays for the transcript levels of possibly related genes in mhk1 mutants

Aliquots of 200 μl suspension (10^7 conidia ml^{-1}) were spread onto cellophane attached plates of 1/4 SDAY alone (control) or supplemented with KCl (40 mg ml^{-1}), sucrose (240 mg ml^{-1}), menadione (0.2 mM) or H_2O_2 (4 mM) and incubated for 4 days at 25°C and 12:12 h under the hyperosmotic and oxidative stresses. The cultures grown on 1/4 SDAY only were then exposed to the heat stress of 40°C for 1 h. Total RNAs were separately extracted from the control and stressed cultures with RNAisoTM Plus Reagent (TaKaRa) and translated into cDNA using PrimeScript® RT reagent Kit (TaKaRa). Tenfold dilutions of the cDNAs of WT, Δ*mhk1* and Δ*mhk1/mhk1* were used as templates for assessing the transcript levels of 25 selected genes via qRT-PCR with paired primers (Table 3) and the fungal 18S rRNA as internal standard. The selected genes were those encoding nine MAP kinases in the HOG pathway (*mapkkks* 1–3, *mapkks* 1–3 and *mapks* 1–3) or associated with conidiation (*flbC* and *hymA*) and anti-oxidation (*cat1* to *cat7* encoding CATs; *sod1* to *sod6* encoding SODs). All qRT-PCR experiments were performed with SYBR® Premix Ex TaqTM (TaKaRa) on Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) following the user's guide. The transcript level of each gene in cDNA was

assessed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The ratio of a gene transcript from Δ*mhk1* or Δ*mhk1/mhk1* over that from WT was defined as the relative transcript level for each mutant.

Indirect ELISA for the level of MAPK phosphorylation

The method of indirect enzyme-linked immunosorbent assay (ELISA) (Kashyap *et al*., 2007) was adopted to assess the levels of MAPK phosphorylation in Δ*mhk1*, Δ*mhk1/mhk1* and WT. Protein extracts from the 4 day cultures on 1/4 SDAY plates grown under normal and stressful conditions, as mentioned above for qRT-PCR, were suspended in 1 ml aliquots of PBS (pH 7.5). After 10 min homogenization on ice, the mixture was centrifuged at 10000 *g* for 10 min at 4°C and the supernatant was centrifuged for another 10 min. The protein concentration (μg mg^{-1}) in each extract was assessed with the BCA Protein Assay Kit (KeyGen Biotech, Nanjing, China). For indirect ELISA, aliquots of 100 μl each supernatant (5 μg ml^{-1} in 0.05 M carbonate buffer, pH 9.6) were uploaded onto 96-well ELISA plate (Jet Biofil, Guangzhou, China), followed by blocking overnight at 4°C. Subsequently, the plate was washed three times with PBS and then reacted with 2000× dilution [0.5% bovine serum albumin (BSA) in PBS] of the rabbit anti-phosphate-p38 MAPK kinase antibody (Cell Signaling Technology, Boston, MA, USA), followed by incubation for 1 h at 37°C. Washed again with PBS, 5000× dilution of the second antibody goat anti-rabbit IgG-HRP (Sigma) was added to the wells and incubated for 1 h at 37°C, followed by washing. Finally, 100 μl aliquots of TMB/H_2O_2 substrate (Amresco, Solon, OH) were added to the wells for 20 min incubation at 37°C. The reaction was terminated with 2 M H_2SO_4 (100 μl per well). The OD value of each well was read at 450 nm. The assay was repeated three times.

Modelling and statistical analyses

The declining I_s trends of each strain over the ranges of the heat stress time (t) and the UV-B doses (D) were separately fitted to the modified logistic equation $I_s = 1/[1 + \exp(a + rx)]$, where $x = t$ or D, a is the intercept for the fitted curve, and r the decline rate of conidial viability over the intensity of a given stress. When $I_s = 0.5$, the fitted equation gave a solution ($-a/r$) to median lethal time (LT_{50} for the heat stress) or dose (LD_{50} for the UV-B irradiation). This solution provided a quantitative index for conidial thermotolerance or UV-B resistance of each strain. Time-mortality trends from the bioassays were subjected to probit analysis, yielding the LT_{50} of each strain against *T. molitor* larvae. All measurements and fitted results from three repeated assays were subjected to one factor (strain) analysis of variance. All modelling and statistical analyses were performed using DPS software (Tang and Feng, 2007).

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