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Characterization of three mitogen-activated protein kinase kinase-like proteins in *Beauveria bassiana*

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ABSTRACT

Pbs2, Mkk1 and Ste7 orthologs are three mitogen-activated protein kinase (MAPK) kinases (MAPKKs) acting as checkpoints of the Hog1, Slt2 and Fus3 MAPK cascades that constitute major parts of fungal signaling network. Here, we show that three other MAPKK-like proteins (Mkk4/5/6) exist in *Beauveria bassiana* and other entomopathogenic or non-entomopathogenic fungi but lack in yeasts and aspergilli, and elucidate how they function in the fungal insect pathogen. Based on phenotypic defects of single-, double- and triple-deletion mutants, *Mkk4*, *Mkk5* and *Mkk6* played collaborative or independent roles in sustaining radial growth on various media, conidiation capacity, conidial germination, conidial UV-B resistance, and/or virulence. In stress assays, three single-deletion Δ*mkk* mutants showed increased tolerance to cell wall stress but null response to a 3-h heat shock at 40 °C during normal incubation. Only did Δ*mkk6* exhibit increased sensitivity to either menadione or H_2O_2 oxidation. Intriguingly, Δ*mkk5* and Δ*mkk6* displayed a remarkable increase in cellular sensitivity to a high osmolarity of NaCl or KCl instead of non-salt sorbitol, suggesting a link of their increased sensitivity to the toxicity of a high Na^+/K^+ concentration rather than to the plausible osmotic stress of either salt. However, all of the deletion mutants showed no resistance to fludioxonil, a phenylpyrrole-type fungicide. A discussion is provided on whether Mkk4, Mkk5 and Mkk6 could be likely associated with or without the MAPK cascades in *B. bassiana*.

1. Introduction

Mitogen-activated protein kinases (MAPKs), MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs) are cascaded into several signaling pathways that regulate invasive growth, development, multiple stress responses and pathogenicity in fungi (Gustin et al., 1998; Saito and Tatebayashi, 2004; Rispail et al., 2009). Main MAPKKK-MAPKK-MAPK cascades include Ste11-Ste7-Fus3/Kss1 responsible for mating pheromone response and filamentation-invasion growth, Bck1/2-Mkk1/2-Slt2 required for maintenance of cell wall integrity (CWI), and Ssk2/Ssk22/Ste11-Pbs2- Hog1 that functions in high-osmolarity glycerol (HOG) pathway. Each of the MAPK modules possesses an evolutionarily conserved MAPKK, which is represented by Ste7 in the Fus3/Kss1 cascade, Mkk1/2 in the Slt2 cascade and Pbs2 in the Hog1 cascade, but may have more MAPKKKs or MAPKs that function in model yeast. For instance, the HOG pathway is characterized by two upper branches converging on the MAPKK Pbs2. One branch is activated by a two-component phosphorelay signaling system, which perceives and transmits hyperosmotic cue to Ssk2/Ssk22, Pbs2 and Hog1 through sequential phosphorylation (Posas and Saito, 1997; O'Rourke et al., 2002; Hohmann et al., 2007). Another branch relies upon Sho1, a plasma membrane protein that acts as a signal sensor and activates Ste11 via the small G protein Cdc42, the adaptor protein Ste50 and the PAK kinase Ste20 (Raitt et al., 2000) or via the PAK kinase Cla4 in parallel with Ste20 (Tatebayashi et al., 2006). Aside from the checkpoint role in the Sho1 branch of the Hog1 cascade, Ste11 also acts as the unique MAPKKK to sequentially activate Ste7 and Fus3 or Kss1. In filamentous fungi, the Hog1 cascade is activated by the branch of the two-component system to regulate the hallmark phenotypes osmotolerance and phenylpyrrole-type fungicide resistance and other non-hallmark phenotypes (Zhang et al., 2002; Fujimura et al., 2003; Furukawa et al., 2005; Noguchi et al., 2007) but rarely linked to the Sho1 branch existing in yeast. Instead, previous studies have often uncovered a unique linkage of Ste11 with the Kss1-absent Fus3 cascade that regulates vegetative growth, asexual/sexual development and pathogenicity in filamentous fungal pathogens (Wei et al., 2003; Sakaguchi et al., 2010; Schamber et al., 2010; Kitade et al., 2015).

Beauveria bassiana is a filamentous fungal insect pathogen that has

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been widely applied in insect pest management programs. Our recent study has unveiled that the Hog1 cascade of *B. bassiana* is compositionally simplified with the unique MAPKKK Ssk2 whereas orthologous Ste11 functions only in the fungal Fus3 cascade (Liu et al., 2017a). Intriguingly, the simplified Hog1 cascade is activated by a more complicated two-component system, in which Groups III and VIII histidine kinases (HK3 and HK8) act as the sensors of fungicidal and hyperosmotic cues respectively (Liu et al., 2017b). The Hog1-like MAPK Mpk3 was found to collaborate with Hog1 in response to heat shock and contribute to phenotypes associated with the biological control potential of *B. bassiana* against insect pests (Liu et al., 2017c). In addition, the Slt2 cascade was shown to interplay with the Hog1 cascade and hence regulate virulence and cellular responses to not only cell wall perturbation but also nutritional, hyperosmotic, fungicidal, thermal and UV-B irradiative stresses in the fungal insect pathogen (Chen et al., 2014). These studies indicate that singular MAPK, MAPKK or MAPKKK works in the Fus3, Slt2 and Hog1 cascades of *B. bassiana*. However, there exist six MAPKK-like proteins in the annotated genome of *B. bassiana* (Xiao et al., 2012). Aside from Ste7, Pbs2 and Mkk1 characterized in the previous studies, three other putative MAPKKs (Mkk4/5/6), which are also present in some other filamentous fungi but absent in yeasts, are functionally unknown. This study sought to explore possible roles of Mkk4, Mkk5 and Mkk6 in sustaining radial growth, asexual development, stress tolerance and virulence of *B. bassiana* by multi-phenotypic analyses of their single, double and triple deletion mutants. We found that these putative MAPKKs contributed significantly to several phenotypes relevant to the pest control potential of the fungal insect pathogen although none of them was essential for the fungal life.

2. Materials and methods

2.1. Bioinformatic analysis of Mkk4, Mkk5 and Mkk6 in B. bassiana

The putative Mkk4, Mkk5 and Mkk6 sequences (NCBI accession codes: EJP69695, EJP65257 and EJP63408 respectively) were located in the genomic database of *B. bassiana* under the NCBI accession NZ_ADAH00000000 (Xiao et al., 2012) and structurally compared with the sequences of *B. bassiana* Ste7, Pbs2 and Mkk1 by online blast at http://blast.ncbi.nlm.nih.gov/Blast.cgi and sequence alignment with online ClustalW software at http://www.genome.jp/tools/clustalw. Each of the located sequences was used as a query to search for the homologs in the genomic databases of other representative fungi, followed by phylogenetic analysis with a neighbor-joining method in MEGA7 software at http://www.megasoftware.net.

2.2. Creating deletion mutants of Mkk4, Mkk5 and Mkk6

The 3′ and 5′ coding/flanking fragments of *Mkk4* (1404 and 1376 bp), *Mkk5* (1479 and 1471 bp) and *Mkk6* (1669 and 1647 bp) were separated cloned from the genomic DNA of the wild-type strain *B. bassiana* ARSEF2860 (designated WT herein) via PCR with paired primers (Table S1) and inserted into p0380-ura3 (Liu et al., 2017a), yielding p0380-5′*x* -ura3-3′*x* (*x* = *Mkk4*, *Mkk5* or *Mkk6*) for targeted single-gene deletion. The full-length coding sequence of each gene with flanking regions (4133, 4747 and 2836 bp for *Mkk4*, *Mkk5* and *Mkk6* respectively) were amplified from the WT DNA and ligated into p0380-sur-gateway to exchange for the gateway fragment, forming the complement plasmids p0380-sur-*x*. Each *Mkk* gene was deleted by transforming p0380-5′*x* -ura3-3′*x* into Δ*ura3* and complemented by ectopic integration of p0380-sur-*x* into the corresponding deletion mutant as described previously (Ying et al., 2013). To construct double-deletion mutants of *Mkk4*, *Mkk5* and *Mkk6*, the plasmid p0380-ura3 was digested at *Nco*I/*Nco*I sites and then inserted by the 3′ and 5′ fragments of *Mkk4* or *Mkk5*, yielding p0380-5′*x*-3′*x* (*x* = *mkk4* or *mkk5*) for *ura3* deletion. The new plasmid p0380-5′*x*-3′*x* was then transformed into Δ*mkk4* or Δ*mkk5*, forming the mutant Δ*mkk*4Δ*ura3* or Δ*mkk*5Δ*ura3*. Subsequently, the plasmid p0380-5′mkk4-ura3-3′mkk4 was transformed into Δ*mkk*5Δ*ura3*, forming the double deletion mutant Δ*mkk*4Δm*kk5* (abbreviated as Δ4Δ*5*). The same method was used to transform p0380-5′mkk6-ura3-3′mkk6 into Δ*mkk*4Δ*ura3* or Δ*mkk*5Δ*ura3*, yielding the other two double-deletion mutants Δ*mkk*4Δm*kk6* (Δ4Δ*6*) or Δ*mkk*5Δm*kk6* (Δ5Δ*6*). The triple deletion mutant Δ*mkk*4Δm*kk*5Δm*kk6* (Δ4Δ5Δ*6*) was created by transforming p0380-5′mkk4-3′mkk4 into Δ4Δ*5* for generation of Δ4Δ5Δ*ura3* and then transforming p0380-5′mkk6-ura3-3′mkk6 into Δ4Δ5Δ*ura3*. Putative deletion mutant colonies were screened based on their ability to grow in the absence of exogenous uridine or resist 5′-fluoroorotate (1 mg/ml) in the presence of uridine auxotrophy (Ying et al., 2013). Putative complement colonies were screened in terms of their *sur* resistance to chlorimuron ethyl (10 μg/ml) in a selective medium. Expected recombination events were examined via PCR and Southern blot analyses with paired primers and amplified probes (Table S1). Positive single, double, triple deletion mutants of *Mkk4*, *Mkk5* and *Mkk6* (Fig. S1) were evaluated in parallel with their control strains (WT and complemented strains) in phenotypic experiments of three replicates as follows.

2.3. Phenotypic experiments

For all mutants and control strains, 1 μl aliquots of a 10^6 conidia/ml suspension were spotted centrally onto the plates of rich SDAY (Sabouraud dextose agar: 4% glucose, 1% peptone and 1.5% agar plus 1% yeast extract), minimal CZA (Czapek agar: 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 amended CZAs, which were prepared by replacing 3% sucrose with 3% of glucose, trehalose or sodium acetate (NaAc) as sole carbon source and 0.3% $NaNO_3$ with 0.3% of glycine, glutamine, histidine or arginine as sole nitrogen source respectively. After 10-day incubation at 25 °C in a light/dark (L:D) cycle of 12:12 h, the diameter of each colony was assessed as an index of growth rate using two measurements taken perpendicular to each other across the colony center.

Conidiation capacity of each strain was assessed from the SDAY cultures during 7-day incubation at the regime of 25 °C and L:D 12:12. Each of the cultures was initiated by spreading 100 μl of a 10^7 conidia/ml suspension onto the SDAY plate (9 cm diameter). From day 4 onwards, three culture plugs (5 mm diameter) were bored daily from each of the plate cultures, and each plug was washed in 1 ml of 0.02% Tween 80 for the release of conidia by 10-min vibration. Conidial concentration in the suspension was estimated using a haemocytometer and converted to the number of conidia per cm^2 culture as a daily estimate of conidial yield.

Several properties indicative of conidial quality were examined using our previous protocols (Liu et al., 2017a-c). Median germination time (GT_{50}; h) required for 50% germination at optimal 25 °C was estimated as an index of conidial viability from the trend of conidial germination on a germination medium (2% sucrose, 0.5% peptone and 1.5% agar). Conidial resistance to UV-B irradiation was estimated as median lethal dose (LD_{50}; J/cm^2) from the survival trend of conidia exposed to the irradiation of the weighted UV-B wavelength of 312 nm (280–320 nm) at the gradient doses of 0–0.6 J/cm^2 in a Bio-Sun^{++} UV chamber (Viber Lourmat, Marne-la-Vallée, France). Conidial virulence of each strain to *Galleria mellonella* larvae (~300 mg per capita) was assayed by immersing cohorts of 30–40 larvae for ~10 s in 30 ml of a 10^7 conidia/ml suspension for normal cuticle infection or injecting 5 μl of a 10^5 conidia/ml suspension into the haemocoel of each larva in each cohort for cuticle-bypassing infection. All immersed or injected cohorts were maintained in Petri dishes (15 cm diameter) at 25 °C and examined every 12 h for mortality records until no more mortality change in continuous two days. LT_{50} (no. days) was estimated from the time-mortality trend of each strain in each bioassay through probit analysis.

To assess cellular responses of each strain to different types of stress