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Miro GTPase controls mitochondrial behavior affecting stress tolerance and virulence of a fungal insect pathogen

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ABSTRACT

Miro homologues are small mitochondrial Rho GTPases belonging to the Ras superfamily across organisms and are generally unexplored in filamentous fungi. Here we identified a Miro orthologue (bMiro) in *Beauveria bassiana*, a filamentous fungal insect pathogen as a classic biological control agent of insect pests. This orthologue was proven to anchor on mitochondrial outer membrane in a manner depending completely upon a short C-terminal transmembrane domain. As a result of *bmiro* deletion, mitochondria in hyphal cells were largely aggregated, and their mass and mobility were reduced, accompanied with a remarkable decrease in ATP content but little change in mitochondrial morphology. The deletion mutant became 42%, 37%, 19% and 10% more tolerant to Ca^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+} than wild-type, respectively, during cultivation in a minimal medium under normal conditions. The deletion mutant also showed mild defects in conidial germination, vegetative growth, thermotolerance, UV-B resistance and virulence despite null response to oxidative and osmotic stresses. All these phenotypic changes were restored by targeted gene complementation. Our results indicate that bMiro can control mitochondrial distribution and movement required for the transport of ATP-form energy and metal ions and contributes significantly to the fungal potential against insect pests through the control.

1. Introduction

Mitochondria are organelles that undergo dynamic changes in shape and distribution for correct cellular assignment at appropriate time and location (Reis et al., 2009). Such changes are important for a supply of ATP-form energy that is consumed in cellular events and hence for a range of vital cellular processes including Ca^{2+} homeostasis (Niescier et al., 2013). As integral parts of signaling cascades, mitochondria participate not only in the regulation and execution of programmed cell death and cellular differentiation but in the control of cell cycle and cell growth (McBride et al., 2006). Changes in both morphology and intracellular distribution take place in the processes of mitochondrial fusion, fission and cytoskeleton-dependent transport, which are considered to sustain their functional homeostasis (Yamaoka and Hara-Nishimura, 2014).

Miro, a conserved mitochondrial Rho, is a small GTPase belonging to the Ras superfamily. The Miro GTPase is anchored to the mitochondrial outer membrane by its C-terminal transmembrane domain (TMD) with its N-terminus being exposed to the cytoplasm, where two distinct GTPase domains are separated by a pair of helix-loop-helix motifs (EF hands 1 and 2) to bind Ca^{2+} (Lewit-Bentley and Rety, 2000; Niescier et al., 2013). There are two Miro GTPases (Miro1/RhoT1 and Miro2/RhoT2) in human (MacAskill et al., 2009), three (MIRO1-3) in *Arabidopsis thaliana* (Yamaoka and Leaver, 2008) but only a single homologue (Gem1p) exists in *Saccharomyces cerevisiae* (Frederick et al., 2004) and *Dictyostelium discoideum* (Vlahou et al., 2011). Mammalian Miro proteins have been shown to regulate mitochondrial morphology and apoptosis (Fransson et al., 2003). Function loss of a Miro homologue (dMiro) in *Drosophila synapses* also altered the distribution of mitochondria in neurons and muscles (Guo et al., 2005). Miro1 mutations in *A. thaliana* resulted in abnormally enlarged or tube-like mitochondrial morphology and hence a disruption of continuous mitochondrial stream in the developing pollen tube although Miro2 exerted little influence on the plant development (Yamaoka and Leaver, 2008). In the *S. cerevisiae* cells devoid of Gem1p, mitochondria were collapsed or became globular or grape-like in morphology, accompanied with altered distribution (Frederick et al., 2004).

Miro homologues also act as key regulators of mitochondrial dynamics (Niescier et al., 2013). Mammalian mitochondria move along cytoskeletal tracks to areas in the cell, where the energy demand is high, while actins serve as tracks for short range transport of mitochondria to areas where the microtubules do not reach

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(Liu and Hajnoczky, 2009; Reis et al., 2009). In plant and yeast cells, mitochondrial movement takes place primarily on actin filaments (Frederick and Shaw, 2007). Elevated Ca^{2+} levels in the cytoplasm of several cell types may arrest mitochondrial motility (Rintoul et al., 2003; Yi et al., 2004; Brough et al., 2005; Quintana et al., 2006), resulting in a homeostatic feedback circuit to position the organelles near Ca^{2+} sources for Ca^{2+} buffering and ATP production where the energy demand is high (Hoth et al., 2000; Park et al., 2001; Malli et al., 2003). Miro is likely involved in mitochondrial Ca^{2+} homeostasis due to a negative correlation of mitochondrial Ca^{2+} content with the velocity of mitochondrial movement (Chang et al., 2011). Miro is a Ca^{2+}-sensing element of the molecular complex controlling mitochondrial motility (Saotome et al., 2008). Overexpression of nonfunctional EF hand-mutated Miro in axons decreased Ca^{2+} entry into mitochondria, suggesting an involvement of Miro in the regulation of mitochondrial Ca^{2+} influx and homeostasis, which, in turn, affects mitochondrial transport (Chang et al., 2011).

However, little is known about the roles of Miro homologues in filamentous fungi. A single Miro protein (bMiro) orthologous to the *S. cerevisiae* Gem1p exists in the annotated genome of *Beauveria bassiana* (Xiao et al., 2012), a filamentous fungal insect pathogen that has been widely applied for arthropod pest control (Wang and Feng, 2014). The goal of this study was to elucidate its subcellular localization and possible role in mitochondrial morphology, distribution and movement and to explore its contribution to the biological control potential of the insect pathogen against arthropod pests.

2. Materials and methods

2.1. Microbial strains and culture conditions

The wild-type strain *B. bassiana* ARSEF 2860 (WT herein) and its mutants were cultured on rich Sabouraud dextrose agar (SDAY; 4% glucose, 1% peptone, 1.5% agar and 1% yeast extract) for normal growth or on minimal Czapek agar (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) for phenotypic assays at 25 °C in a light/dark cycle of 12:12 h. *Escherichia coli* Top10 and *E. coli* DH5α from Invitrogen (Shanghai, China) were cultivated for vector propagation at 37 °C in Luria-Bertani medium plus ampicillin (100 μg/ml) or kanamycin (50 μg/ml). *Agrobacterium tumefaciens* AGL-1 incubated in YEB medium (Fang et al., 2004) was used as a T-DNA donor for fungal transformation.

2.2. Structural analysis of bMiro

The full-length sequence of *S. cerevisiae* Gem1p was used as a query to locate bMiro in the *B. bassiana* genome (Xiao et al., 2012). The coding sequence of the located bMiro [NCBI accession number: BBA_04570 (gene) or EJP66630 (protein)] was amplified from the WT DNA with a pair of primers (Table S1) and sequenced for verification at Invitrogen. The protein sequence deduced from the verified nucleotide sequence was subjected to online blast analysis for its structural features and aligned with the Gem1p sequence of *S. cerevisiae* using SMART program (Letunic et al., 2012). Phylogenetic analysis was then performed for the Miro homologues in human and several fungi using a neighbor-joining method in MEGA5 software (Tamura et al., 2011).

2.3. Subcellular localization of bMiro

To construct a plasmid for subcellular localization of intact, head-free or tail-free bMiro, the plasmid pAN52-bar (Ying and Feng, 2006) was modified, resulting in pGMB-bar vectoring the promoter P*trpC*, the multiple restriction enzyme sites 5′-*Nco*I-*Nde*I-*Xma*I-*Sma*I-*Spe*I-*Eco*RV-*Eco*RI-*Bam*HI-3′ and the phosphinothricin-resistant *bar* marker. The reporter gene encoding enhanced green fluorescence protein (eGFP) was amplified from pEGFP-C1 (BD Biosciences Clontech, CA, USA) and inserted into pGMB-bar at the *Xma*I/*Spe*I sites, forming pGMB-eGFP-bar. The coding fragments of the intact bMiro (1884 bp), the head-deleted bMiro TMD (*bmiroF1*; 138 bp) and the tail/TMD-deleted bMiro (*bmiroF2*; 1746 bp) were amplified from the WT cDNA with paired primers (Table S1) and individually inserted into pGMB-eGFP-bar at the *Spe*I/*Bam*HI sites. The new plasmid pGMB-eGFP-*x*-bar (*x* = *bmiro*, *bmiroF1* or *bmiroF2*) was transformed into the WT via blastospore transformation (Ying and Feng, 2006). Positive transformants expressing *eGFP*-tagged *bmiro*, *bmiroF1* and *bmiroF1* were selected under a two-photon laser confocal microscope (Carl Zeiss AG, Germany) and then incubated in SDAY for conidial production. The conidia were suspended in Sabouraud dextrose broth (SDB, i.e., agar-free SDAY), followed by 3 days of shaking (150 rpm) at 25 °C. The hyphal cells of each transformant were stained with 500 nM MitoTracker Red CMXRos (Molecular Probes, Invitrogen, Karlsruhe, Germany), a dye specific to mitochondria, and visualized in the same bright/fluorescent field of view under the confocal microscope.

2.4. Generation of bmiro mutants

The backbone plasmids p0380-bar and p0380-sur-gateway (Xie et al., 2012) were used to construct plasmids for *bmiro* deletion and complementation. Briefly, the 5′ and 3′ fragments (1763 and 1765 bp respectively) of *bmiro* comprising partial coding and flanking regions were amplified from the WT with paired primers (Table S1) and inserted into p0380-bar at the *Eco*RI/*Hin*dIII and *Xba*I/*Bgl*II sites respectively, forming p0380-5′bmiro-bar-3′bmiro. The full-length sequence of *bmiro* and its flanking regions (4191 bp in total) was amplified from the WT and inserted into p0380-sur-gateway to exchange for the gateway fragment under the action of Gateway BP Clonase™ II Enzyme Mix (Invitrogen), yielding p0380-sur-bmiro vectoring the *sur* marker. The two plasmids were propagated in *E. coli* Top10 and *E. coli* DH5α and transformed respectively into the WT and the Δ*bmiro* mutant via *Agrobacterium*-mediated transformation (Fang et al., 2004). Putative mutants were screened in terms of the *bar* resistance to phosphinothricin (200 μg/ml) or the *sur* resistance to chlorimuron ethyl (15 μg/ml) in a selective medium, followed by identification through PCR and Southern blotting analyses with paired primers and amplified probe (Table S1). The genomic DNA of each strain used for the Southern blot analysis was digested with *Bgl*I/*Bgl*I. Positive Δ*bmiro* mutant and its control strains (WT and complementary mutant Δ*bmiro::bmiro*) were used in triplicate phenotypic experiments.

2.5. Analysis of mitochondrial morphology, distribution and motility

The aliquots of 50 ml 2×10^5 conidia/ml SDB were shaken for 2 days at 25 °C. Hyphal cells were collected from the WT and Δ*bmiro* cultures and stained in 500 nM MitoTracker Red for 15 min in darkness, followed by washing three times with the warm water. The stained hyphal cells were resuspended in dd-H_2O. The morphology and distribution of mitochondria in 300 stained hyphae of each strain were observed under the confocal microscope. Additionally, mitochondrial morphology in the ultrathin sections of hyphal cells was examined via transmission electronic microscopy (TEM) as described previously (Chen et al., 2014).