G-protein subunit Ga_i in mitochondria, MrGPA1, affects conidiation, stress resistance, and virulence of entomopathogenic fungus *Metarhizium robertsii*

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Abstract

G proteins are critical modulators or transducers in various transmembrane signaling systems. They play key roles in numerous biological processes in fungi, including vegetative growth, development of infection-related structures, asexual sporulation, and virulence. However, their function in entomopathogenic fungi remains unclear. Here, we characterized the roles of MrGPA1, a G-protein subunit Gα i , in conidiation, stress resistance, and virulence in Metarhizium robertsii . MrGPA1 was localized in the mitochondria. MrGpa1 deletion resulted in a significant reduction (47%) in the conidiation capacity, and reduced expression of several key conidiation-related genes, including fluG , flbD , brlA , wetA , phiA , and stuA . Further, MrGpa1 disruption resulted in decreased fungal sensitivity to UV irradiation and thermal stress, as determined based on conidial germination of ΔMrGpa1 and wild-type strains. Chemical stress analysis indicated that MrGpa1 contributes to fungal antioxidant capacity and cell wall integrity, but is not involved in antifungal ability and osmotic stress. Importantly, insect bioassays involving (topical inoculation and injection) of Galleria mellonella larvae revealed decreased virulence of ΔMrGpa1 strain after cuticle infection. This was accompanied by decreased rates of appressorium formation and reduced expression of several cuticle penetration-related genes. These observations suggest that MrGpa1 contributes to the regulation of conidiation, UV irradiation, thermal stress response, antioxidant capacity and cell wall integrity in M. robertsii . This gene is also involved in insect cuticle penetration during infection. These findings raise the possibility of designing powerful strategies for genetic improvement of M. robertsii conidiation capacity and virulence for killing pests.

Key Points

MrGPA1 is a G-protein subunit $G\alpha_i$ of Metarhizium robertsii.
MrGPA1 is a mitochondria protein involved in fungal stress responses and virulence.
MrGPA1 deletion affects the expression of conidiation and cuticle-penetration genes.

Introduction

G protein with GTP-hydrolase activity is a type of signaling protein that binds to guanine nucleotides (Robishaw and Berlot 2004). It participates in signal transduction pathways linking activated cell-surface receptors with intracellular effectors, including adenylate cyclase and phospholipase through a series of signaling cascades involved in the regulation of physiological and biochemical processes (Chakravorty and Assmann 2018; Ortiz-Urquiza and Keyhani 2015). In fungi, G protein is associated with sexual and asexual reproduction, virulence and response to external signal stimuli (Guo et al. 2016b; Ivey et al. 1996; Liu and Dean 1997).

The heterotrimeric G protein is composed of three subunits α, β, and γ, wherein α subunit binds to GDP, and β and γ subunits from a heterodimers (Birnbaumer 2007; Lambert 2008; McIntire 2009). When the heterotrimeric G protein is stimulated by a G-protein-coupled receptor (GPCR) that senses external signals, GDP is exchanged for GTP, and Gα and Gβγ complexes dissociate (Wedegaertner 2012). Then, Gα-GTP

and Gβγ act on the respective downstream effectors (Barren and Artemyev 2007). The cycle is reset by the hydrolysis of GTP to GDP, and Gα recombining with Gβγ and GPCR (Gilchrist et al. 1999; Slessareva and Dohlman 2006).

In mammals, G-protein α subunits are divided into four classes, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, based on the amino acid sequence identity (Neer 1995). Further, the $G\alpha_i$ family is composed of four subfamilies, $G\alpha_i$, $G\alpha_o$, $G\alpha_t$ and $G\alpha_z$. The functions of $G\alpha_i$ family proteins are diverse, and include regulation of adenylyl cyclase, K^+ and Ca^+ channels, and cGMP phosphodiesterase activities (Simon et al. 1991). The conserved functional motif of $G\alpha_i$ protein is characterized by possession of N-myristoylation and ADP-ribosylation (Buss et al. 1987).

The functions of the G-protein α subunit have been characterized in some fungi. In Saccharomyces cerevisiae, two kinds of Gα proteins have been identified (Gpa1 and Gpa2). Gpa1 is involved in pheromone regulation (Jahng et al. 1988), while Gpa2 regulates pseudohyphal development via cyclic AMP (cAMP)-dependent pathways and heat resistance (Kubler et al. 1997). However, these proteins have different functions in other filamentous fungi. For example, Fusarium oxysporum f. sp. cubense possesses three Gα proteins (Gα-fga1, -fga2, and -fga3), and the deletion of encoding genes leads to phenotypic defects in colony morphology, reduced conidiation, increased heat tolerance, reduced virulence, and decreased intracellular cAMP levels (Guo et al. 2016a, b; Jain et al. 2002). Further, three different Gα proteins control unique signal transduction pathways in Magnaporthe grisea, influencing fungal vegetative growth, conidiation, conidium attachment, appressorium formation, mating, and pathogenicity (Liu and Dean 1997; Zhang et al. 2012). However, it is unclear whether the Gα proteins are involving in vegetative growth, conidiation, stress resistance, and virulence in entomopathogenic fungi, such as Metarhizium robertsii.

M. robertsii, an important entomopathogenic fungus, has been developed as an environmentally friendly alternative to chemical insecticides (Frazzon et al. 2000; Lord 2005; Wang and Wang 2017). Unfortunately, commercialized broad application of M. robertsii formulations is limited by the low conidiation rate, failure of conidia germination under high-temperature and UV stress, slow killing speed, and inconsistent field performance (Fang et al. 2012; Faria and Wraight 2007; Muniz-Paredes et al. 2017). Genetic improvements of this mycoinsecticide require extensive understanding of the molecular mechanisms and M. robertsii genes involved in stress tolerance and virulence (Zhang and Feng 2018).

In the current study, we aimed to investigate the role of Gα proteins in M. robertsii. BLASTP search of against the assembled draft genome sequence of M. robertsii identified four putative Gα proteins. Among these Gα proteins, MrGPA1 (EFZ00892) shared the highest identity (96.32%) with GNA-1 protein of Neurospora crassa, which is required for the extension of basal hypha, growth, conidiation, and formation of female reproductive structures (Ivey et al. 1996; Yang and Borkovich 1999). We then, characterized the biological function of MrGPA1 by constructing and analyzing MrGpa1 gene deletion mutant. We show that MrGpa1 influences conidiation, stress resistance, and virulence in M. robertsii.

Materials And Methods

Fungal strains and culture

In the present study, *M. robertsii* strain ARSEF 23 was the wild-type (WT) strain. All *M. robertsii* strains were inoculated onto potato dextrose agar (PDA, 20% potato, 2% glucose, and 2% agar, w/v), and cultured at 25 °C for 10 days. Conidial suspensions were obtained by vortex-mixing in 0.05% (v/v) Tween-80, and filtration through sterile non-woven fabric to remove mycelial debris.

Sequence analysis

To construct the phylogenetic tree of GPA proteins and analyze the structural domains of guanine nucleotide-binding site, amino acid sequences of the G-protein a subunits were downloaded from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), and phylogenetic analysis was performed using MEGAX software (https://www.megasoftware.net/).

Construction of MrGPA1-GFP fusion vector and analysis of subcellular localization of MrGPA1

To monitor subcellular localization of *MrGpa1*, *gfp* and *MrGpa1* gene fragments were amplified by polymerase chain reaction (PCR), using *gfp*-F/*gfp*-R, and *gfpMrGpa1*-F/*gfpMrGpa1*-R primers (Supplementary Table S1), high-fidelity Taq DNA polymerase (KOD Plus Neo, Toyobo, Osaka, Japan) and *M. robertsii* genomic DNA as a template. The amplification products were inserted into the *Eco*RI restriction site in pDHt-SK-*bar* vector (kindly provided by Dr. ChengshuWang; the vector conferred resistance against glufosinate-ammonium) (Fang et al. 2006) containing a strong promoter and terminator to generate vector pDHt-*MrGpa1-gfp* for *Agrobacterium tumefaciens* transformation. The corresponding transformants resistant to glufosinate ammonium were obtained, and verified by PCR using the primers *gfp*-F and *gfp*-R (Supplementary Table S1).

The MrGPA1-GFP strain was cultured on sabouraud dextrose agar medium containing yeast extract (SDAY 4% glucose, 1% peptone, 2% agar, and 1% yeast extract powder, w/v) at 25 °C for 2 days. The hyphae were then washed off the plate with sterile water and mixed with 500 nM MitoTracker Red CMXRos (Invitrogen, Shanghai, China), a dye specific to mitochondria. Subcellular localization of MrGPA1 was evaluated using a laser scanning confocal microscopy (LSCM, Zeiss LSM880). Before using laser scanning confocal microscopy, Wolf PSORT software (https://wolfpsort.hgc.jp) was used for prediction subcellular localization by analysing protein sequence of MrGPA1.

Gene deletion and complementation

To disrupt *MrGpa1* gene, the 5'- and 3'-flanking regions of *MrGpa1* were obtained by using *MrGpa1*-5F/*MrGpa1*-5R and *MrGpa1*-3F/*MrGpa1*-3R primers, genomic DNA (using the Plant GenomicDNAKit; Tiangen, Beijing, China) extracted as a PCR template and high-fidelity Taq DNA polymerase (KOD Plus Neo, Toyobo, Osaka, Japan). The amplification products were then inserted into the pDHt-SK-*bar* vector (containing glufosinate resistance gene) (Fang et al. 2006) digested with the *Sma*I、*Bam*HI、and *Xba*I restriction enzymes to generate vector pDHt-*MrGpa1-bar* for *A. tumefaciens* transformation (Fang et al. 2006). *ΔMrGpa1* strains were obtained by selection for glufosinate resistance, and subsequently verified by PCR and reverse-transcription (RT) - PCR using primer pairs *MrGpa1*-F/*MrGpa1*-R, up*MrGpa1*-F/up*MrGpa1*-R, dn*MrGpa1*-F/dn*MrGpa1*-R, *gpd*-F/*gpd*-R, and *bar*-F/*bar*-R (Supplementary Table S1).

For gene complementation, the entire *MrGpa1* gene and the 1000-bp upstream sequence and 600-bp downstream sequence were inserted into vector pDHt-SK-*ben* (containing benomyl resistance gene) digested with the *Spe*I restriction enzyme for fungal transformation. The 3050-bp fragment was ectopically integrated into *ΔMrGpa1* strain by the same method as that used for gene deletion. Complemented strains (cp*ΔMrGpa1*) were obtained by selection for benomyl resistance, and verified by PCR using primer pairs *MrGpa1*-F/*MrGpa1*-R and *ben*-F/*ben*-R (Supplementary Table S1).

Phenotype assays

For phenotype assays, these experiments were performed with 3 technical and biological replicates per strain (WT, *ΔMrGpa1*, and cp*ΔMrGpa1*).

Fungal conidiation ability was evaluated as previously described (Meng et al. 2017). Briefly, 30 ml of conidial suspension(1×10^6 conidia /ml) was spread on PDA plate (35-mm diameter). After culturing at 25 °C for 14 days, the conidia on each plate were collected into 30 mL of 0.05% Tween-80 by vortex-mixing, and conidial density was determined using a hemocytometer and converted to the number of conidia per square centimeter of colony.

To evaluate the fungal vegetative growth, 1 ml of WT, *ΔMrGpa1*, and cp*ΔMrGpa1* conidial suspensions (1×10^7 conidia /ml) was spotted on PDA and 1/4 SDAY (1/4 dilution of SDAY) media, and incubated in the dark at 25 °C for 10 days. Colony diameters were then measured.

For conidial germination assay, 10 ml of conidial suspension (5×10^6 conidia /ml) were spread on PDA medium. The conidial germination was observed by microscope (Olympus BX 51, Tokyo, Japan) at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 hours after incubated at 25 °C. Conidia are considered to be germinated when the length of the germ tube reaches or longer than the length of the conidia (Wang et al. 2014). Three hundred conidia were counted at least by per plate and the germination rates were calculated by comparing the number of germinated conidia with the 300 counted conidia, and the median germinate time (GT_{50}) was calculated using the SPSS software

For heat stress tolerance assays, 1 ml of conidial suspensions (5 ´ 10^6 conidia /ml) of WT, *ΔMrGpa1*, and cp*ΔMrGpa1* strains were placed in 1.5-ml Eppendorf tubes, and then incubated in a water bath at 42 °C or 28 °C (as control) for 1 h. Then 10 ml of the suspension were spread on PDA medium, incubated at 25 °C. Conidial germination was observed under a microscope (Olympus BX 51, Tokyo, Japan) after 16 h and 24 h. Three hundred conidia were counted at least by per plate and the relative germination rates were calculated by comparing the number of germinated conidia with had not been heat stressed (Wang et al. 2019).

To determine fungal tolerance to ultraviolet B (UV-B) light, 10 ml of conidial suspensions (5 ´ 10^6 conidia /ml) of WT, *ΔMrGpa1*, and cp*ΔMrGpa1* was taken to PDA medium. The plates were then exposed to UV-B irradiation (312-nm wavelength at 100 mJ cm^{-2}) using HL-2000 Hybrilinker (UVP, CA, USA) (Yao et al. 2010) or exposed to sunlight (as control). Relative UV-B tolerance was assessed and calculated by aforementioned methods to assess relative germination rate of tolerance to UV-B.

To examine the fungal tolerance to chemical stress, 1 ml conidial suspensions (1 ´ 10^7 conidia /ml) of WT, *ΔMrGpa1*, and cp*ΔMrGpa1* strains were spotted onto PDA medium containing carbendazim (2 mg/ml), NaCl (0.5 M), H_2O_2 (2 mM) or Congo red (2 mg/ml), and incubated in the dark for 10 days at 25 °C. Colony diameter was then measured and the relative inhibition rate calculated (Wang et al. 2017; Ying and Feng 2011).

To assess the effects of *MrGpa1* on virulence, bioassays with *Galleria mellonella* larvae (RuiQing Bait, Shanghai, China) were performed as described previously (Zhou et al. 2018). The larvae were immersed in conidial suspension (1 ´ 10^6 conidia /ml) for 90 s or injected (into the hemocoel) with 10 ml of conidial suspensions (1 ´ 10^5 conidia /ml) and incubated at 25 °C, Each treatment was performed in triplicate, with 18 larvae in each group. The experiment was repeated three times. Larva mortality was evaluated every 24 h, and the median lethal time (LT_{50}) was calculated using the SPSS software.

The appressorium formation assay was performed as described previously (Gao et al. 2013). Briefly, to test the appressorium formation on a hydrophobic surface, 1 ml of conidial suspension (1 ´ 10^6 conidia /ml) in MMGly (minimal medium amended with 1% glycerol) was spread on a sterile plastic Petri dishes (3.5-cm diameter), followed by 24 h incubation at 25 °C. At least 300 conidia of each strain were evaluated microscopically, and the induction rates of appressorium formation were quantified by observing different microscopic fields (inverted microscope, Olympus IX 71, Tokyo, Japan).

Quantitative RT-PCR (RT-qPCR)

To analyze the expression of conidiation-related genes, 200 ml of conidial suspensions (1 ´ 10^7 conidia/ml) of WT, *ΔMrGpa1*, and cp*ΔMrGpa1* strains were plated on PDA medium, and cultured in the dark at 25 °C for 2.5 days. The samples were collected and milled in liquid nitrogen to extract total RNA.

To analyze the expression of virulence genes related to cuticle infection, *G. mellonella* larvae were dipped in conidial suspensions (5 ´ 10^7 conidia/ml) of WT, *ΔMrGpa1*, and cp*ΔMrGpa1* strains for 1.5 min, transferred to 25 °C for 48 h, and then placed in liquid nitrogen for total RNA extraction. Total RNA was extracted by using Trizol reagent (Invitrogen, Foster City, CA, USA). cDNA was obtained by using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China), and used as a template for RT-qPCR. The gene expression analysis was performed by using the CFBR96^{TM} Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and SYBR® PremixEx TaqTM II (TaKaRa). Three biological repeats of each treatment were analyzed. The qPCR primers are listed in Supplementary Table S2. The expression of the *gpd* gene (MAA_07675, encoding glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control (Fang and Bidochka 2006). The relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

All data are presented using GraphPad Prism version 6.0. Data are expressed as the mean ± standard error (SE) of the mean, from three biological replicates. Statistical analysis was performed by one-way analysis of variance (ANOVA). For multiple comparisons, Tukey's multiple comparison test was used to analyze statistical the significance. $p < 0.05$ was considered to be significant, and $p < 0.01$ was considered to be extremely significant.

Results

Sequence characteristics of MrGPA1 from *M. robertsii*

We chose the sequences of GNA-1 proteins of the model fungus *N. crassa* as reference sequences to retrieve their *M. robertsii* orthologs. We thus identified four genes encoding G-protein a subunit (GPA) (GenBank accession numbers EFZ00892.1, EFY98464.1, EFY99066.2, and EFZ00060.2, named *MrGpa1*, *MrGpa2*, *MrGpa3*, and *MrGpa4* (i.e., the *M. robertsii Gpa1*, *Gpa2*, *Gpa3*, and *Gpa4* genes), respectively) in the genome of *M. robertsii* ARSEF 23.

Further bioinformatics analysis indicated that *MrGpa1* (MAA-03488) is a single copy gene encoding G-protein a subunit (353-aa protein) in *M. robertsii*. A BLASTP search of MrGPA1 homologs in NCBI revealed that the protein shares 100% amino acid similarity with GPA from *M. acridum* (XP_007811324), *M. anisopliae* (KFG82129), *M. brunneum* ((XP_014542423) *and M. rileyi* (OAA44756). Phylogenetic tree of GPA proteins from *Metarhizium* spp, and related fungal species was constructed with *Saccharomyces cerevisiae*as an outgroup (Fig. 1a). All GPA proteins from the genus *Metarhizium* formed an independent branch (100% support value). These *Metarhizium* GPA proteins are closely related to GPA proteins from *Pochonia chlamydosporia*, *Moelleriella libera* and *Purpureocillium lilacinum*.

A conserved domain database search demonstrated that MrGPA1 contains a highly conserved guanine nucleotide-binding site (34-347-aa protein), which is the key identification domain of the G-protein a subunit (Fig. 1b). Furthermore, homologous alignment revealed the presence of *N*-myristoylation and ADP-ribosylation sites (two conserved positions in the G-protein a_i subunit) in the conserved functional motif of MrGPA1. Hence, MrGPA1 is a member of the Ga_i family.

MrGPA1 is a mitochondria protein

To investigate the subcellular localization of MrGPA1, the Wolf PSORT software was first used. The analysis predicted that the protein is localized in the mitochondrial inner membrane. To verify this prediction, we generated *MrGpa1-gfp* strain (Supplementary Fig. S1a, b). As shown in LSCM images in Fig. 2, the mitochondria in hyphal is stained with a mitochondrial dye (red), in a punctate patterns, while punctate green fluorescence was also observed in vegetative hyphae. Red and green fluorescence was detected and overlapped (Fig. 2), suggesting that MrGPA1 is a mitochondria protein.

Construction of *MrGpa1* knockout and complementation

To investigate the function of MrGPA1 in detail, we next generated *MrGpa1* gene replacement and complemented strains (Supplementary Fig. S1a). The nature of the generated mutant strains was confirmed by using genomic DNA PCR. The analysis indicated the presence of a 1, 222-bp fragment corresponding to the partial *MrGpa1* gene sequence in the WT and cp*ΔMrGpa1* stains, but not in the *ΔMrGpa1* strain. In addition, a partial 806-bp *bar* gene fragment was detected in *ΔMrGpa1* and cp*ΔMrGpa1* strains, and a partial 785-bp *ben* gene fragment was detected in the cp*ΔMrGpa1* strain. Furthermore, PCR analysis indicated the presence of a fragment containing upstream sequence of *MrGpa1* and a partial *bar* gene (2689-bp) and a fragment containing downstream sequence of *MrGpa1* and a partial *bar* gene (1975-bp), and detected by using the primer sets up *MrGpa1*-F/up*MrGpa1*-R and dn*MrGpa1*-F/dn*MrGpa1*-R, respectively, in the *ΔMrGpa1* strain (Supplementary Fig. S1c). Finally, RT-PCR analysis verified the loss or regain of the *MrGpa1* gene expression in *ΔMrGpa1* and cp*ΔMrGpa1* strains, accordingly (Supplementary Fig. S1d)**.** These observations indicated a successful construction of the *MrGpa1* knockout and complementation strains.

MrGpa1 contributes to fungal conidiation but is not involved in vegetative growth

To examine the effect of *MrGpa1* on the growth and development of *M. robertsii*, we evaluated mycelial growth and conidial yield of WT and mutant strains on PDA and 1/4 SDAY medium, respectively. The 14-day-old colonies of WT, Δ*MrGpa1*, and cp*ΔMrGpa1* strains formed 6.18×10^7, 3.28×10^7, and 6.55×10^7 conidia /cm^{-2}, respectively (Fig. 3a). The loss of *MrGpa1* caused a significant, 47% reduction in

conidiation, but little difference in the growth rate of WT, Δ*MrGpa1*, and cp*ΔMrGpa1* strains on PDA and 1/4 SDAY media was apparent (Fig. 3a, b). We also examined the expression of genes involved in conidiation in *M. robertsii* by RT-qPCR. The expression of *fluG*, *flbD*, *brlA*, *wetA*, *phiA* and *stuA* genes in the *MrGpa1* strain was significantly reduced compared with that in the WT and cp*ΔMrGpa1* strains (Fig. 3c). Collectively, these observations indicate that while MrGPA1 plays an important role in the conidiation of *M. robertsii*, it is not involved in vegetative growth.

MrGpa1 is important for heat and UV stresses tolerance, and is involved in antioxidant capacity and cell wall integrity of *M. robertsii*

We observed that conidial germination rate of Δ*MrGpa1* strain on PDA medium was significantly higher than that of WT and cp*ΔMrGpa1* strains. The GT_{50} values for WT, *ΔMrGpa1*, and cp*ΔMrGpa1* were 11.99 h, 6.16 h ($p < 0.01$, compared with WT strain), and 12.16 h, respectively (Fig. 4a).

To investigate the effects of *MrGpa1* deletion on UV irradiation and thermal stress, the relative germination rate of conidia exposed to these stresses was determined 16-h or 24-h after stress exposure. We found that the sensitivity of *ΔMrGpa1* strain to 42 °C heat-stress was reduced. For example, compared with the WT, *ΔMrGpa1* germination rates at 16 h and 24 h increased by 51% ($p < 0.01$) and 24% ($p < 0.05$), respectively (Fig. 4b). Similar results were obtained for conidial tolerance of UV irradiation; compared with the WT, *ΔMrGpa1* germination rates at 16 h increased by 83% ($p < 0.01$), but only by 8% ($p < 0.05$) at 24 h (Fig. 4b). Hence, it appears that MrGPA1 plays an important role in conidial tolerance of both UV irradiation and thermal stress.

To evaluate the role of *MrGpa1* in fungal growth under different chemical stress conditions, we investigated the mycelial growth of the WT and mutant strains on PDA containing carbendazim, NaCl, H_2O_2, or Congo red. The antioxidant capacity and cell wall integrity of Δ*MrGpa1* strain were significantly different than those of the WT and cp*ΔMrGpa1* strains. For instance, compared with the WT, the relative inhibition of *ΔMrGpa1* growth was decreased by 68.6% ($p < 0.01$) on PDA containing H_2O_2, while the sensitivity to Congo red was increased by 47.4%($p < 0.01$). However, the relative inhibition of *ΔMrGpa1* growth in the presence of carbendazim and NaCl was not markedly different from that of the control strains (Fig. 4c). These observations indicate that *MrGpa1* contributes to fungal antioxidant capacity and cell wall integrity, but is not involved in antifungal ability and osmotic stress.

MrGpa1 plays an important role in insect cuticle penetration via appressorium formation

We next used *G. mellonella* bioassays to assess the consequences of *MrGpa1* deletion on fungal virulence. In topical infection bioassays, the mean lethal times to death (LT_{50}) in insects infected with *ΔMrGpa1*, WT and cp*ΔMrGpa1* strains were 7.2 ± 0.45, 11.8±0.54 and 8.3 ± 0.71 days, respectively, with

a significant ($p < 0.05$) attenuation of virulence in *G. mellonella* (Fig. 5a, b). The treatment with *ΔMrGpa1* also resulted in a increased survival rate of the larvae compared with the WT and cp*ΔMrGpa1* strains treatment. By contrast, in the injection bioassays, we did not observe any differences in LT_{50} values between larvae infected with *ΔMrGpa1* (3.69 ± 0.15 days), and WT (3.53 ± 0.14 days) or cp*ΔMrGpa1* strains (3.57 ± 0.12 days) (Fig. 5c, d).

We then determined the expression of insect virulence-related genes during cuticle penetration by RT-qPCR. Indeed, the expression of several genes involved in the adhesion (*mad1*, 45% expression in *ΔMrGpa1* strain compared with the WT strain), appressorium formation (*mpl1*, 72%, and *gpa*, 52%) and cuticle penetration (*pr1A*, 93%, and *pr1C*, 96%) was significantly decreased in the *ΔMrGpa1* strain compared with their expression in the WT strains (Fig. 6a).

To determine the mechanism of the virulence defect of *ΔMrGpa1* strain, we then assayed appressorium formation on a hydrophobic surface. We observed that the loss of *MrGpa1* impaired appressorium differentiation, compared with the control strains. Specifically, 24 h after induction, *ΔMrGpa1* strain did not form appressoria, while, the appressorium formation rate of the WT strain was 80% (Fig. 6b). Further, 48 h after induction, the appressorium formation rate of *ΔMrGpa1* was only approximately 20% and were significantly reduced (by 76.5%) compared with WT strains (Fig. 6c). Therefore, *MrGpa1* plays an important role in cuticle penetration by impacting appressorium formation.

Discussion

G-proteins are key components of various signal transduction pathways and play show important biological roles function in the control of cell proliferation, behavior, and development in higher organisms (Harashima and Heitman 2005; Ivey et al. 1996; Regenfelder et al. 1997). In the current study, we described the identification of *MrGpa1* gene encoding the Ga_i subunit in *M. robertsii*, and showed that this Ga_i protein plays an important role in conidiation, stress resistance, and virulence in the host fungus.

In filamentous fungi, genes encoding three G-protein a subunits were reported in *N. crassa*, *F. oxysporum* f. sp. *cubense*, *Aspergillus nidulans* and *M. grisea* (Guo et al. 2016b; Hicks et al. 1997; Ivey et al. 1996; Liu and Dean 1997). However, while genes for four G protein a subunits were identified in *M. robertsii*, two of the open reading frames (ORF) are highly similar (*MrGpa2* and *MrGpa4*). Further, the existence of Ga_i has been demonstrated in filamentous fungi including the *M. robertsii* in this study, but not in the yeasts *S. cerevisiae* or *Schizosaccharomyces pombe* (Kallal and Fishel 2000; Kubler et al. 1997).

Previously, to be activated by GPCR that sense external signals in plasma membrane (PM), G protein was considered to locate at the PM (Marrari et al. 2007). However, recent studies revealed G-protein localization beyond the plasma membrane, e.g., in the mitochondrion, endoplasmic reticulum, and Golgi apparatus (Michaelson et al. 2002; Nair et al. 2017). Wolf PSORT prediction in the current study indicated a possible location of MrGPA1 in the mitochondrion. Indeed, by using a fluorescent protein fusion, we

showed here that MrGPA1 is located in the mitochondria, which is consistent with location of $G\alpha_i$ in HEK293T cells (Lyssand and Bajjalieh 2007).

According to multiple studies, G-protein a subunits play a role in the conidial yield in some fungi. For example, the conidiation of *magB* deletion mutant of *M. grisea* and *Δfga1* mutant of *F. oxysporum* f. sp. *cubense* is impaired (Guo et al. 2016b; Liu and Dean 1997). A similar phenomenon was also observed in the current study. Further, some key genes involved in conidiation were down-regulated in the *MrGpa1* deletion mutant, suggesting that the *MrGpa1* gene is involved in the conidiation of *M. robertsii* by regulating the expression of conidiation-related genes.

We also observed that *ΔMrGpa1* strain is more tolerant to UV irradiation and thermal stress than the WT and cp*ΔMrGpa1* strains. This observation was consistent with findings for *F. oxysporum* f. sp. *cubense* and *N.crassa* (Guo et al. 2016b; Yang and Borkovich 1999). According to the two cited studies, intracellular cAMP levels are reduced in the $G\alpha_i$ deletion mutants, suggesting that the cAMP pathway might be involved in the response to thermal stress and tolerance of UV irradiation in some fungi.

The virulence of plant pathogenic fungi is regulated by multiple pathways, such as the mitogen activated protein kinase (MAPK) cascades and the cAMP-PKA pathway (Dean 1997). Further, the $G\alpha_i$ family contributes to pathogenicity in many plant pathogenic fungi. For example, the pathogenicity of the deletion strains *M. grisea ΔmagB*, *Botryils cinerea Δbcg1*, and *F. oxysporum* f. sp. *cubense Δfga1* is markedly reduced (Gronover et al. 2001; Guo et al. 2016b; Liu and Dean 1997). In the entomopathogenic fungus *M. robertsii*, *MrGpa1* deletion resulted in a marked attenuation of virulence in the *G. mellonella* model, with an increased LT_{50} value (by 63.9%) compared with that of the WT strain. Further analysis indicated that the reduced virulence is associated with an impaired appressorium differentiation in the mutant. This was similar to the effect of *magB* deletion in *M. grisea*, namely, blocked appressorium formation in a deletion strain (Liu and Dean 1997). To the best of our knowledge, the current study constitutes the first report of virulence decrease resulting from a deletion of a G protein a subunit gene in an entomopathogenic fungus.

In conclusion, MrGPA1 is located in the mitochondria of *M. robertsii* cell and is a member of $G\alpha_i$ family. MrGPA1 controls unique signal transduction pathways, and thus plays important role in conidiation, stress resistance, and virulence in that fungus. These findings raise the possibility of designing powerful strategies for genetic improvement of *M. robertsii* conidiation capacity and virulence for killing pests.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

BH and ZW conceived and designed the study. YT and HW wrote the manuscript, conducted the experiments, and analyzed the data. ZL did a part of the experiments. BH edited the manuscript and supervised the project. All authors read and approved the manuscript.

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Figures

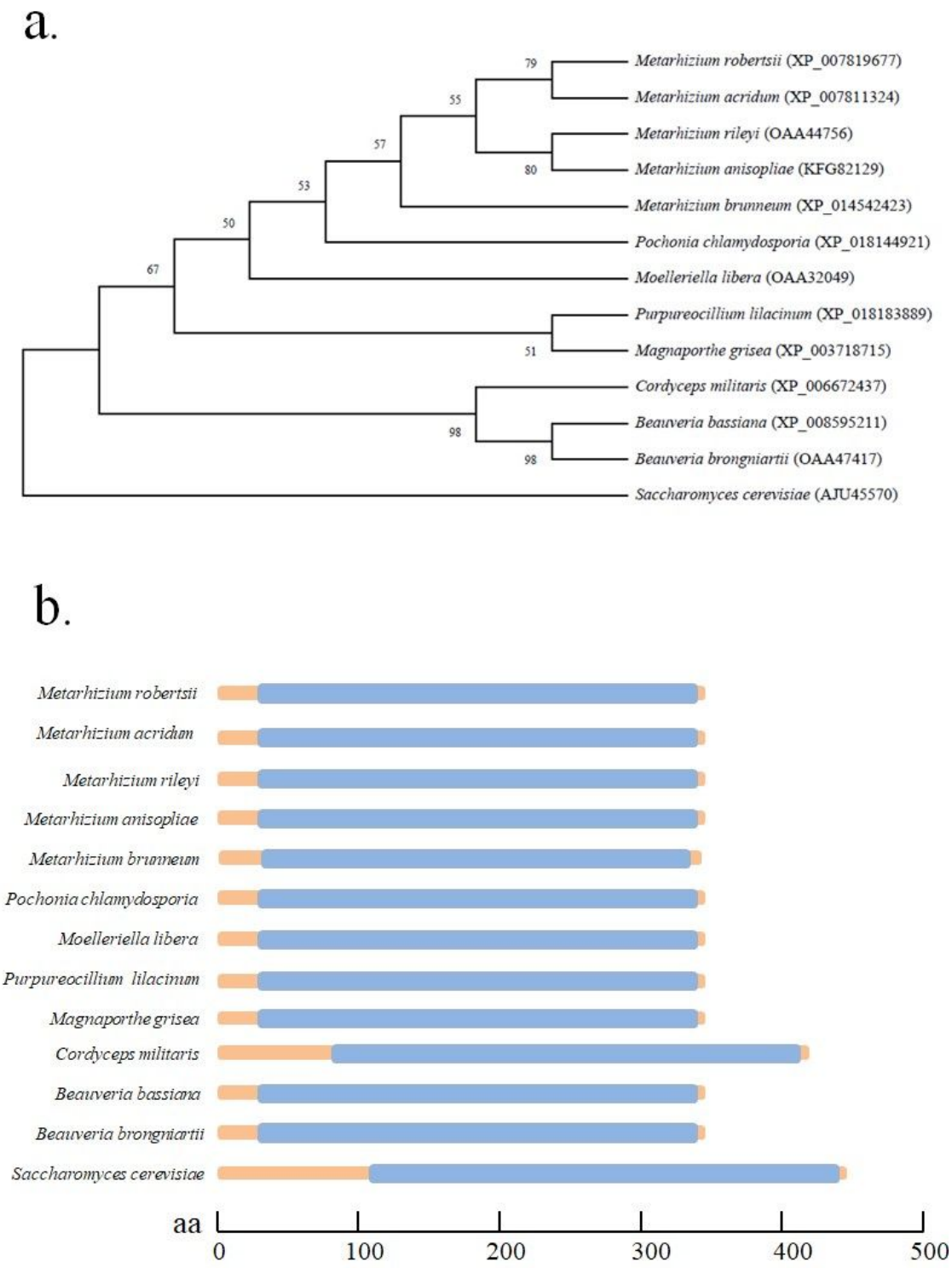

Figure 1

Phylogenetic and conserved domain analysis of MrGPA1. (a) Phylogenetic analysis of MrGPA1 and its orthologs from different fungi. The National Center for Biotechnology Information accession numbers of MrGPA1 and related proteins are given in brackets following each fungal name. (b) Conserved domain

analysis of MrGPA1 and its orthologues from different fungi. The guanine nucleotide-binding domain of the G-protein subunit is shown in blue. aa, amino acid.

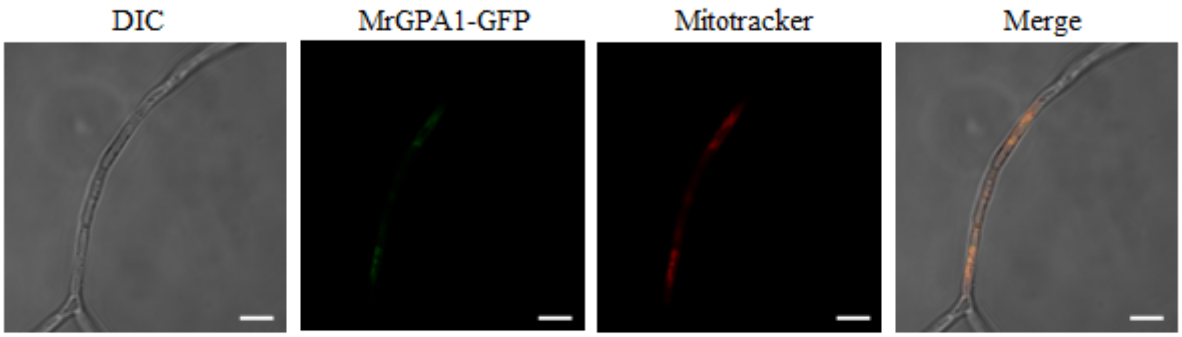

Figure 2

MrGPA1 is a mitochondria protein. LSCM images (scale bars: 10 m) of the cellular location of MrGPA1, showing that MrGPA1 is located in the mitochondria, with the GFP fluorescence signal apparent in hyphae.

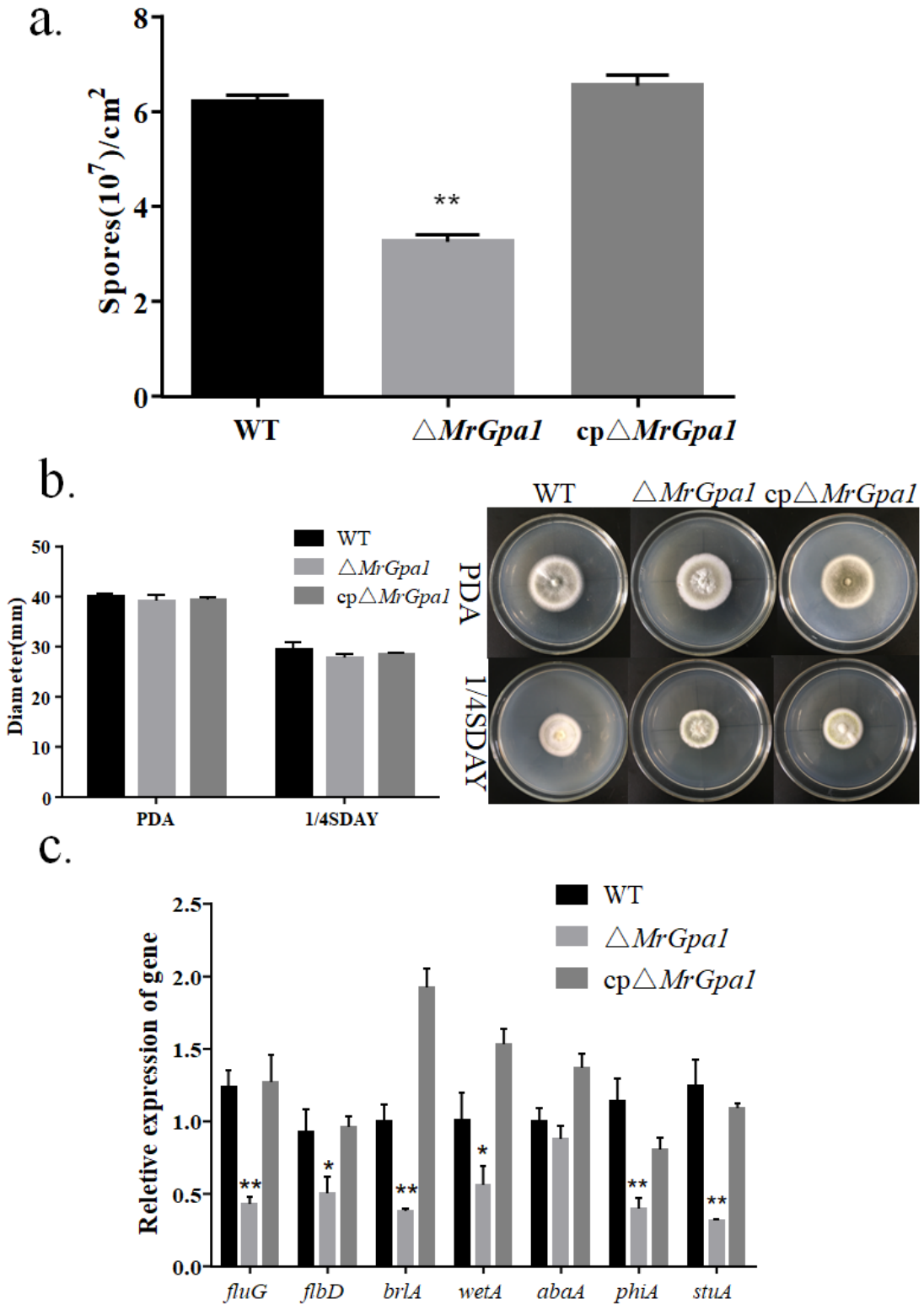

Figure 3

MrGpa1 contributes to fungal conidiation but is not involved in vegetative growth. (a) Conidial yields of three fungal strains after 14-days, growth on PDA at 25°C. (b) Colony diameters and phenotype of three fungal strains on PDA and 1/4SDAY media after 10-days, growth at 25°C. (c) RT-qPCR analysis of the relative expression of conidiation-related genes in 2.5-day-old PDA cultures of three fungal strains. * $p < 0.05$, ** $p < 0.01$.

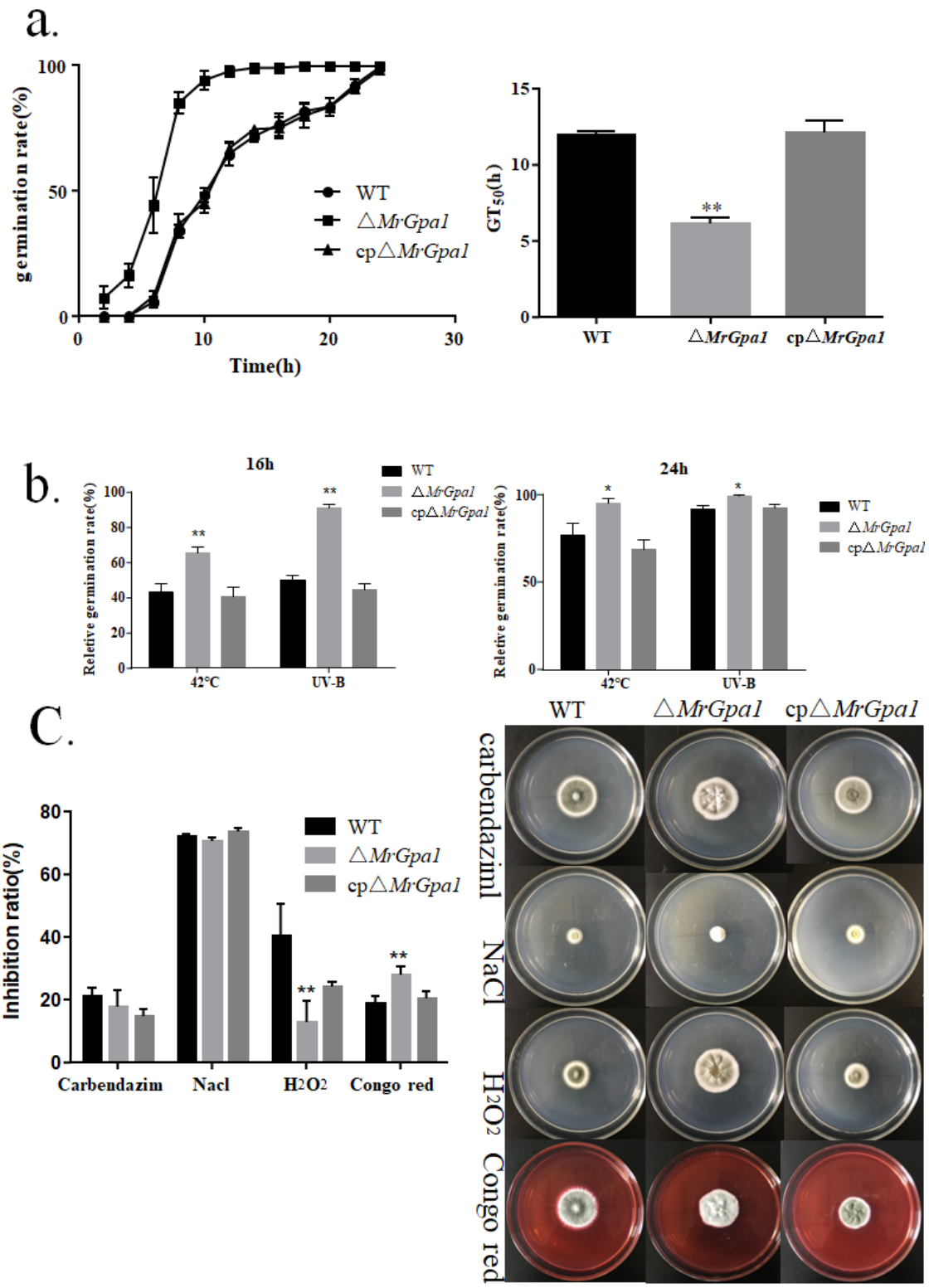

Figure 4

MrGpa1 is required for fungal stress response. (a) Germination rate and GT50 values for three fungal strains after growth on PDA. (b) Relative germination rate of three fungal strains after exposure to heat stress and UV-B treatment. (c) Inhibition ratio and colony phenotype values for three fungal strains, on PDA medium containing carbendazim (2 μg/ml), NaCl (0.5 M), H2O2 (10 mM), or Congo red (2 mg/ml). * $p < 0.05$, ** $p < 0.01$.

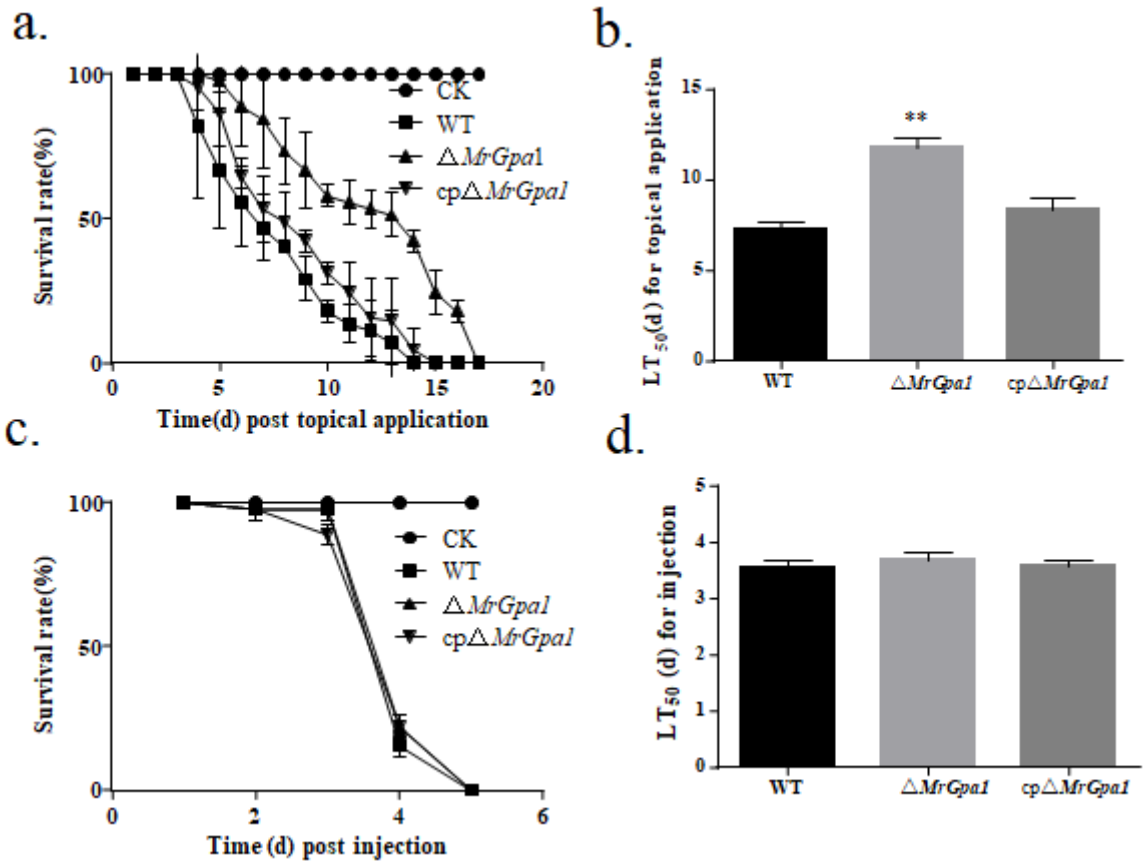

Figure 5

MrGpa1 deletion impacts fungal virulence. (a) Survival of G.mellonella after topical application of conidial suspensions from three fungal strains. The control insects were treated with sterile water. (b) LT50 (days) of three fungal strains after topical inoculation of larvae. (c) Survival of G.mellonella after injection of conidial suspension of three fungal strains. The control insects were treated with sterile water. (d) LT50 (days) of three fungal strains after injection. * $p < 0.05$, ** $p < 0.01$.

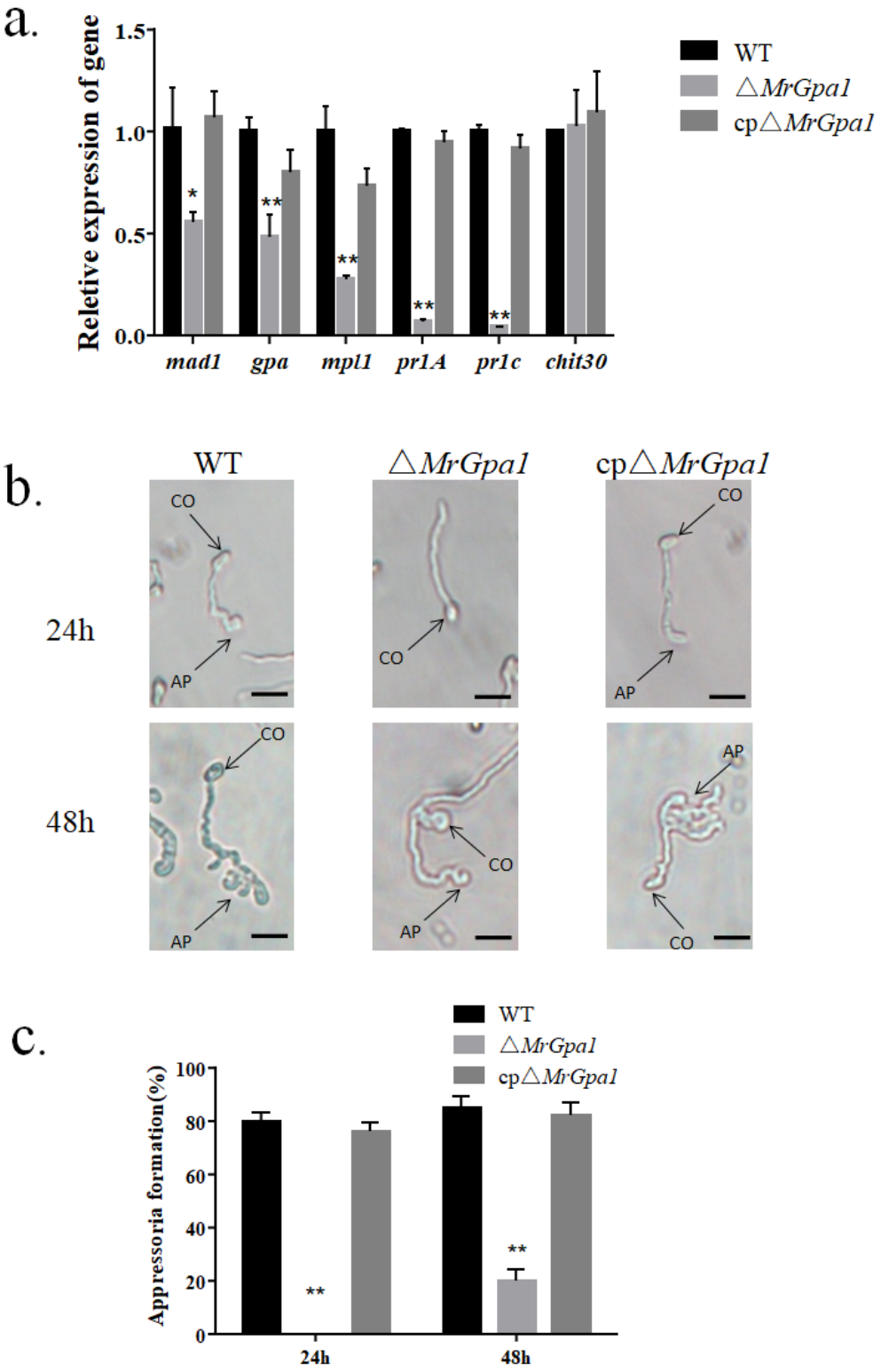

Figure 6

MrGpa1 deletion affects appressorium formation. (a) RT-qPCR analysis for the relative expression of virulence-associated genes of three fungal strains in vivo (24 h after inoculation of G. mellonella larvae). (b) Microscopic analysis and percentage appressorium formation by fungal strains induced on a plastic hydrophobic surface. Scale: 10μm. (c) Percentage of appressorium formation in vitro. * $p < 0.05$, ** $p < 0.01$. CO, conidium, AP, appressorium

Supplementary Files

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