The Magas1 Gene is Involved in Pathogenesis by Affecting Penetration in Metarhizium acridum

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Received: November 22, 2011 / Revised: February 21, 2012 / Accepted: February 22, 2012

Appressorium is a specialized infection structure of filamentous pathogenic fungi and plays an important role in establishing a pathogenic relationship with the host. The Egh16/Egh16H family members are involved in appressorium formation and pathogenesis in pathogenic filamentous fungi. In this study, a homolog of Egh16H, Magas1, was identified from an entomopathogenic fungus, Metarhizium acridum. The Magas1 protein shared a number of conserved motifs with other Egh16/Egh16H family members and specifically expressed during the appressorium development period. Magas1-EGFP fusion expression showed that Magas1 protein was not localized inside the cell. Deletion of the Magas1 gene had no impact on vegetative growth, conidiation and appressorium formation, but resulted in a decreased mortality of host insect when topically inoculated. However, the mortality was not significant between the Magas1 deletion mutant and wild-type treatment when the cuticle was bypassed by injecting conidia directly into the hemocoel. Our results suggested that Magas1 may influence virulence by affecting the penetration of the insects’ cuticle.

Keywords: Metarhizium acridum, virulence, appressorium, penetration, Magas1, pathogenic fungus

The entomopathogenic fungus Metarhizium acridum has been used as an important biocontrol agent against various insects [10, 11]. It has also been regarded as a model organism for studying the insect–fungus interactions [5]. Like most of the pathogenic fungi, M. acridum is a dimorphic fungus with two life cycles, including the saprophytic cycle in the artificial media and the parasitic cycle in insects [13]. The saprophytic phase involves conidia germination, growth of hyphae, and production of conidia. The parasitic phase of M. acridum is very complicated, encompassing a series of developmental events, including attachment of conidia to the insect cuticle, germination of conidia, development of appressorium, formation of the penetration peg to penetrate the insect’s cuticle, insect mortality resulting from the combination of the growth of invasive hyphae and toxin produced by the fungus, and finally development of new conidia on the surface of the cadaver [4].

Many genes associated with these processes have been cloned and their functions have been explored. The Egh16/Egh16H family members are identified in the highly expressed infection stage and involved in the pathogenesis in many pathogenic filamentous fungi. In the saprophytic cycle of Blumeria graminis, the Egh16 gene was highly up-regulated in germinating conidia and greatly declined after germination, possibly playing a role in hyphal growth [8]. In the parasitic phase, Egh16 is expressed during all development stages since appressorium formation [6, 8]. Two Egh16H1 homologs, GAS1 and GAS2, are specifically expressed during the appressorium formation stage and function as virulence factors in Magnaporthe grisea [15]. The gks1 gene, a GAS1 homolog from Monacrosporium haptotylum, is also involved in the development of the infecting structure [1]. Until now, no Egh16/Egh16H homolog has been reported from entomopathogenic fungi.

In this study, we characterized the Magas1 gene from M. acridum, explored its distinct expression pattern and localization, and investigated its influence on growth, conidiation, and pathogenesis.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**

Metarhizium acridum CQMa102 and engineered strains generated in this study were grown on 1/4 strength Sabouraud’s dextrose agar medium [1/4 SDAY: 1% dextrose, 0.25% mycological peptone, 0.5% yeast extract, and 2% agar (w/v)] at 28°C for 14 days to produce conidia.
Gene Cloning and Sequence Analysis
A full-length cDNA library of M. acridum germinating and differentiating on locust wing was constructed by He and Xia [7]. One Egh16H1 homolog, Magas1, was found through random sequencing of the cDNA library. Homolog searches of DNA/protein sequence databases were performed using the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov). The deduced amino acid sequence of the Magas1 gene was analyzed by DNAMAN and Mega 4.0. The characteristics of the deduced Magas1 protein were predicted using various Web-based programs (http://www.expasy.org).

Construction of the Magas1 Gene Disruption Strain (Magas1)
A 0.7 kb EcoRV-EcoRI fragment of downstream flanking sequences of the Magas1 gene [amplified with primer R1GasF 5'-TAGATA TCAAGAAGCCTGATGAAATCAGC-3' (EcoRV) and R1GasR 5'- CCTCGAGCTTGAATTCCTTGGA-3' (EcoRI)] was inserted into the pK2-PB vector harboring a bar cassette to generate pK2-PB-R. The pK2-PB-Magas1 vector was constructed by insertion of a 0.95 kb XbaI-BamHI fragment of upstream flanking sequences of the Magas1 gene [amplified with primer L1GasF 5'-GGCTTACAG GCACAGTCTATTTCCTTACA-3' (XbaI) and L1GasR 5'-CAT TAGGCCCTTGATCCT-3' (BamHI)] into the XbaI-BamHI site of pK2-PB-R. The construct pK2-PB-Magas1 was transformed into M. acridum mediatred Agrobacterium tumefaciens and transformants were screened according to Cao et al. [2].

The transformants were analyzed by PCR. If the Magas1 gene was replaced by the deletion module within the genome, the transformants would have the 1.1 kb and 0.9 kb PCR products with primer pairs LF/PTR2 (5'-TGGCTTCAACAAACAGGTTCCTCA-3' and 5'-CAG CCAAGCCCAAAAGTG-3') and BarF/RR (5'-ACTGGCATGACG TGGGTT-3' and 5'-ACCGAAGACAGCAAGCCGA-3'), respectively. The transformants were further confirmed by Southern blot analysis.

TGGGTT-3' and 5'-ACCGAAGACAGCAAGCCGA-3'), respectively.

Subcellular location of the Magas1 protein was predicted using Euk-mPlc2.0 [3]. Programs of SignalP, SIG-Pred, and PRED-SIGNAL were used for predicting the signal peptide of Magas1. The location of the Magas1 protein was further analyzed and mediated Magas1-EGFP fusion expressed. The Magas1–EGFP fusion and EGFP non-fusion strains were constructed. For the PMagas1-Magas1–EGFP fusion construction, the Magas1 coding region and a 1.2 kb upstream fragment (PMagas1) were amplified with primers PMagas1F 5'-CGAGATCTTGTGACCGAGTCTCCAGA CGT-3' (BglII) and PMagas1R 5'-CAGGCCCCGAGATAGGG TGGGTTTGGGA-3' (Apal). The PMagas1–Magas1–EGFP fusion and PMagas1–EGFP non-fusion cassettes were inserted into a pK2 binary vector containing a T-DNA harboring a bar cassette, and then transformed into M. acridum and screened as mentioned above.

Appressorium was induced on locust hind wings as described previously by He and Xia [7]. The locust wings inoculated with conidia was put on a slide and then incubated in a plastic container with moist filter paper at 28°C. After 20 h incubation, the locust wings were moved away and the appressoria attached to the slide were used for inspection of fluorescence with Leica SP5 Confocal Laser Scanning Microscopy (CLSM).

Analysis of Growth Characteristics and Appressorium Formation
Growth, percentage of germination, and conidial production of wild-type strain (WT) and ΔMagas1 were measured as described by Liu et al. [9]. The appressorium formation rates were determined from 300 conidia after a 36 h induction on locust hind wings according to He and Xia [7]. Appressorial turgor pressure was measured using serial solutions of PEG-8000 as reported previously [14]. The assay was replicated at least three times.

Insect Bioassay
Bioassays were conducted in two ways (topical infection and injection) against the fifth-instar nymphs of L. migratoria manilensis (Meyen). Mortality was recorded every day over a period of 10 days. The mean 50% lethal times (LT50) were estimated using the program Data Processing System (DPS) [12]. For each treatment, 10 additional insects were inoculated as described above and bled 3.5 days later to observe the hyphal body differentiation in the insect hemolymph.

Statistical Analysis
Data from growth rate, germination percentage, conidial production, appressorium formation rate, turgor pressure, and virulence were analyzed with the one-way ANOVA model. Tukey's Honestly Significant Difference (HSD) test was used to separate means at α = 0.05.

RESULTS AND DISCUSSION

Molecular Characteristics of Magas1
The full-length cDNA of the Magas1 gene was obtained. Alignment between cDNA and the M. acridum genomic sequence (Acc. No. ANDI00000000.1) revealed that the Magas1 gene contained two introns of 53 bp and 90 bp with an open reading frame (ORF) of 954 bp, encoding a protein of 317 amino acid residues with a calculated molecular mass of 31.6 kDa and theoretical isoelectric point of 8.61.

The Ala-Gly-rich characteristic of the Egh16/Egh16H1 family was also found in the Magas1 protein (14.2% Ala and 16.7% Gly). The multiple sequence alignment of the Magas1 homologs was performed with DNAMAN. Two conserved motifs, DGAGP and GPFGG, throughout the Egh16/Egh16H family members [1, 6, 8, 15] were identified.
in Magas1 (Fig. 1A). Other conserved amino acids were also shadowed in Fig. 1A. Homology analysis showed that Magas1 shared 33.9%, 53.4%, 32.4%, and 33.2% identities to GAS1 and GAS2 from M. grisea, Egh16 from B. graminis, and gks1 from M. haptotylum, respectively. Fig. 1B shows the phylogenetic relationship of Magas1 and 11 other Egh16 homologs. The phylogenic tree was constructed using neighbor-joining algorithm with the Mega 4 program. Numbers in parentheses are the GenBank accession numbers.

**Magas1** is Specifically Expressed in Appressorium Formation Stage

The expression of Magas1 gene was explored in both the saprophytic cycle and the parasitic cycle of M. acridum. The Magas1 expression could not be detected in the saprophytic cycle (conidium, germinated conidium and mycelium), hyphal body inside insect and conidia emerged from insect cuticle. Consistent with other Egh16/Egh16H family members [1, 6, 8, 15], strong expression of the Magas1 gene was found during appressorium formation (Fig. 2A), indicating a possible role for Magas1 in the pathogenesis of M. acridum.

**Fig. 1.** Sequence characteristics of the Magas1 gene. (A) Multiple sequence alignment. Magas1: M. acridum; GAS1 and GAS2: M. grisea; gks1: M. haptotylum; Egh16H1: B. graminis. The identical amino acid residues in five Egh16 homologs are indicated by solid black boxes. Asterisks indicate the Egh16/Egh16H family signature sequence motifs. Short dash lines (-----) represent non-homologous region. (B) Phylogenetic analysis of Magas1 and 11 other Egh16 homologs. The phylogenic tree was constructed using neighbor-joining algorithm with the Mega 4 program. Numbers in parentheses are the GenBank accession numbers.

**Fig. 2.** Analyses of the Magas1 protein. (A) Expression pattern of Magas1 by semiquantitative RT-PCR analysis. Total RNAs were extracted from M. acridum at six developmental stages using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. Lanes 1–6 indicate the transcription of Magas1 in the following samples, respectively. Conidia, germinated conidia, mycelia, germinated conidia with appressoria generated on locust hind wing, hyphal body collected from the hemolymph of the infected locusts, and conidia emerged from the cuticle of infected locusts. The *gpd* gene was used as the endogenous control. (B) Schematic map of PMagas1–Magas1–EGFP (Fusion) and PMagas1–EGFP (Non-fusion) expression vector. PMagas1, the Magas1 gene promoter; Trpc, A. nidulans trpC terminator; bar cassette, a bar gene cassette resistant to phosphinothricin (PPT). RB and LB are Right Border and Left Border, respectively. (C) Confocal microscopy of PMagas1–EGFP non-fusion proteins (left) and PMagas1–Magas1–EGFP fusion proteins (right). The images were acquired with the same field under bright-field (Light) and UV light (UV). A merged picture was generated from Light and UV. Bar = 5 µm. Abbreviations in the figures: CO, Conidium; AP, Appressorium.
**Magas1 was Secreted out of the Cell**

All the three programs (PRED-SIGNAL, SignalP, and PSORTII) showed a signal sequence, but different potential cleavage sites, at the N-terminal sequence in Magas1 protein (PRED-SIGNAL: 36–37 amino acid; SignalP: 16–17 amino acid; PSORTII: 16–17 amino acid). Euk-mpLoc 2.0 analysis showed that Magas1 was possibly secreted out of the cell or located in the peroxisome. Cellular location of the Magas1 protein was confirmed by Magas1–EGFP fusion expression. Fig. 2B shows the PMagas1–Magas1–EGFP fusion and PMagas1–EGFP non-fusion expression vector map. The two vectors were transformed into M. acridum. The fluorescence of transformants at various developmental stages was observed under CLSM. Intense fluorescence was spread evenly throughout the cytoplasm when the EGFP gene was driven directly by promoter PMagas1, indicating that PMagas1 could strongly initiate gene expression during the appressorium formation stage. However, only a few appressoria had visible green fluorescence in the cytoplasm of Magas1–EGFP fusion constructs (Fig. 2C). Most Magas1–EGFP fusion construct cells had no detectable EGFP signal. The bioinformatics analysis and Magas1–EGFP fusion expression results suggested that Magas1 was mainly secreted out of the cell. These results were inconsistent with two other Egh16H1 homologs in M. grisea, GAS1 and GAS2, which were mainly localized in the cytoplasm of the appressorium [15]. We also predicted the signal peptide of other Egh16H1 family members. However, inconsistent prediction results were obtained for these Egh16H1 homologs. It is reasonable to deduce that Egh16H1 homologs may have different subcellular locations in different fungi.

**Magas1 Affects Virulence, but not Growth, Conidiation, and Appressorium Formation**

Magas1 deletion mutants were generated by homologous replacement (Fig. 3A). Ten transformants were screened by PCR analysis (data not shown). Southern-blot analysis revealed that WT had a 700 bp band, whereas ΔMagas1 D2 and D3 had a 2.4 kb band but lost the 700 bp-band when hybridized to the probe (Fig. 3B), indicating that the Magas1 gene was correctly replaced by the bar cassette. The transformant D1 had both 700 bp and 2.4 kb bands, resulting from ectopically integrated pK2-PB-Magas1.

Consistent with GAS1 and GAS2, Magas1 did not affect the growth, conidiation, and appressorium formation (data not shown). Some appressorium formation associated genes were reported to contribute to virulence by regulating appressorial turgor pressure [14]. We also measured the turgor pressure of WT and ΔMagas1. However, ΔMagas1 did not show significant difference with the WT (p>0.05, data not shown), indicating that Magas1 did not affect the mechanical pressure and might possibly be involved in the interaction with the host.

Different mortality was recorded from the two bioassays. Topical infection on the pronotum revealed that ΔMagas1 had a significant reduction in mortality compared with WT (Fig. 4A). Mean LT50 values of 4.25 ± 0.52 and 5.46 ± 0.17 day for WT and ΔMagas1, respectively, differed significantly (p<0.05). However, there was no significant difference in virulence between WT (LT50 = 4.16 ± 0.04 day) and ΔMagas1 (LT50 = 4.29 ± 0.07 day) when the conidia were injected directly into the hemocoel. Similar results were obtained for the growth characteristic of hyphal body inside insects for the two bioassays. For topical infection assay, the number of the hyphal body in hemolymph differed significantly between the WT (15 ± 3.2 × 10^5 hyphal body/ml hemolymph) and ΔMagas1 (8.2 ± 3.2 × 10^5 hyphal body/ml hemolymph) (p<0.05). Yet, the growth of ΔMagas1 (16 ± 3.5 × 10^5 hyphal body/ml hemolymph) and WT (17.9 ± 2.7 × 10^5 hyphal body/ml hemolymph) was not significantly different (p>0.05) when the cuticle was bypassed by injecting conidia directly into the hemocoel. These results indicated that Magas1 contributed to virulence by affecting the penetration of the insect cuticle, not by affecting the fungal growth inside the insect.

Alignment against the nucleic acid database of the NCBI revealed that Magas1 homologs were all from filamentous pathogenic fungi, but none from nonpathogenic fungi, indicating that Egh16H1 homologs may be a virulent factor.
of filamentous fungi [1, 8, 15]. However, owing to different subcellular locations of Egh16/Egh16H homologs, they may contribute to pathogenesis in different ways in different fungi. Further studies would be required to clarify the mechanism of Magas1 in penetration of the host cuticle.

Note: The GenBank accession number of the sequence reported in this paper is JF780914.

Acknowledgments

We gratefully thank Sultan Mahmud (Chongqing University, China) for critical reading of this manuscript. This research was supported by grants from the Natural Science Foundation of China (No. 30800736) and the New Teachers of Doctoral Program of Higher Education of China (No. 20070611043).

Fig. 4. Insect bioassays of WT and ΔMagas1. Survival rates were calculated of L. migratoria following topical application of conidium on the pronotum (A) or injection conidium directly into the hemocoel (B). Cottonseed oil and sterile water without conidia were used as blank controls in the infection assay and injection assay, respectively. Error bars are standard deviations of three trials. A 3 μl aliquot of conidia suspension was applied either by topically inoculating the conidia (10^7 conidia/ml suspended in cottonseed oil) or by injecting the conidia (10^6 conidia/ml in sterile water) directly into hemocoel through the second abdominal segment of the locust. Control consisted of locusts treated with cottonseed oil or water only. Each treatment had three replicates with 20 insects. Inoculated insects were maintained at 28°C and 75% relative humidity.

REFERENCES