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Molecular cloning, characterization and expression of *PmRsr1*, a Ras-related gene from yeast form of *Penicillium marneffei*

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Abstract GTP-binding proteins such as Ras act as molecular switches in a large number of signal pathways. In this report, we isolated and characterized a novel Ras small monomeric GTPase Rsr1 gene, designated *PmRsr1*, from yeast-form *Penicillium marneffei*. The full-length *PmRsr1* cDNA sequence is 1,866 bp in size, and contains an open reading frame of 642 bp encoding 213 amino acids. The predicted molecular mass of PmRsr1 is 24.41 kDa with an estimated theoretical isoelectric point of 9.21. The deduced amino acid sequence of PmRsr1 shows 87% identity with that of *Aspergillus fumigatus* and *A. clavatus*. Eight exons and seven introns are identified within the 2,102 bp *PmRsr1* genomic DNA sequence of *P. marneffei*. The open reading frame was subcloned into the pcDNA6-myc-His B expression vector, and the recombinant plasmid was transfected into Vero cell line. The expressed fusion protein was analyzed by SDS-PAGE and western blotting. Differential expression of the *PmRsr1* was demonstrated by real-time RT-PCR. The expression of *PmRsr1* was the highest in the yeast phase comparing with that in the mycelia and conidia phases.

Keywords *Penicillium marneffei* · Ras-related gene · *PmRsr1* · Gene cloning · Differential expression

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Abbreviations

SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
RT	Reverse transcriptase
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
MMLV	Moloney murine leukaemia virus
HRP	Horseradish peroxidase

Introduction

Penicillium marneffei is an opportunistic human pathogen endemic in Southeast Asia and is the only known *Penicillium* species which exhibits temperature-dependent dimorphism [1–3]. At 25°C, *P. marneffei* grows as septate, branched hyphae bearing typical penicilliate structures of *Penicillium* species. At 37°C, it forms uninucleate yeast cells which divide by fission. The yeast form of *P. marneffei* is its pathogenic form, predominates intracellularly during the host infection [4]. Dimorphic switching requires fungal cells to undergo changes in polarized growth in response to environmental stimuli [5]. The molecular mechanisms of controlling dimorphic switch and polarized growth in *P. marneffei* remain unknown.

Polarity establishment allows the development of a wide variety of cell morphologies and the differentiation of distinct cell types. Selection of a site for polarization requires a group of proteins including a Ras-like GTPase and its regulators [6]. Rsr1 (also known as Bud1), a GTPase in the Ras superfamily, contributes to cell morphology and virulence in several species of fungi [7]. In the budding yeast *Saccharomyces cerevisiae*, deletion of *ScRSR1* causes a randomization of bud position [8].

The Rsr1p is hypothesized to affect cell polarity via a direct interaction with Cdc42p [9]. The Rsr1 of the hemiascomycetous *Ashbya gossypii* plays a role in stabilizing polarisome components at the actively growing tip. Cells lacking AgRsr1p have slowed hyphal elongation due to frequent phases of pausing of growth at the hyphal tip [10]. In *Candida albicans*, the *CaRSR1* gene is involved in bud site selection, yeast phase cell morphogenesis, germ tube emergence and hyphal growth. Mutants in *CaRSR1* exhibit low virulence and the strains are unable to invade agar [11, 12]. In addition, Rsr1 plays a key role in hyphal tip orientation in response to the external environmental cues. The deletion of *CaRSR1* leads to reduce the hyphal infiltration of kidney tissue during systemic infection. Rsr1 is thought to be an internal polarity landmark, which contributes to the pathogenesis of *C. albicans* infections by influencing hyphal tip responses triggered by interaction with host tissues [13]. We have previously reported differential expressed genes between the mycelia and the yeast phases of *P. marneffei* using suppression subtractive hybridization. The expressed sequence tag of clone Y552 is homologous to the RAS small monomeric GTPase Rsr1 from *A. fumigatus* and increases obviously in the yeast form of *P. marneffei* [14].

In this study, we described the molecular cloning of a Ras-related gene from the yeast-form *P. marneffei*, designated *PmRsr1*. We performed some necessary function analysis of the *PmRsr1* gene as well as detecting the differential gene expression in the life cycle of *P. marneffei*. These will establish the primary foundation of understanding the function of *PmRsr1* gene. The cloning and characterization of *PmRsr1* in the pathogenic fungus was first reported.

Materials and methods

Fungal strain, media and growth conditions

The *P. marneffei* strain SUMS0152 used in this study was preserved at the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan (IFM52703).

The yeast, conidia, and vegetative hyphal samples were obtained as described [5, 14]. After harvesting, all samples were stored at −80°C immediately, or processed for total RNA isolation directly.

Total RNA and genomic DNA isolation

Approximately 100 mg samples of *P. marneffei* conidia, mycelia and yeast were separately pulverized under liquid nitrogen with a mortar and pestle. Total RNA isolation was carried out according to the manufacturer's protocol using the Trizol Reagent Kit (Invitrogen, Carlsbad, CA) and treated with the RNase-free DNase I kit from TaKaRa to eliminate DNA contamination.

Genomic DNA was isolated from yeast phase colonies following the manufacturer's protocol using the Insta-Gene™ Matrix kit (Bio-Rad, CA).

Cloning of the full-length *PmRsr1* cDNA and its DNA

A 502 bp cDNA fragment of *P. marneffei* yeast phase was obtained from expressed sequence tag clones Y552 (GenBank Accn: EG026135), which was identified to have high similarity with RAS small monomeric GTPase Rsr1 from *A. fumigatus* [14]. To obtain the full length cDNA sequence of the *PmRsr1* gene, 5′-RACE and 3′-RACE were performed with 5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0 kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The total RNA of yeast-form *P. marneffei* was reverse transcribed using an oligo dT primer and MMLV-RT. The reverse transcription products were amplified by PCR, using the primers (5RACE-R180, 5RACE-R112 and 3RACE-F163) designed according to the Y552 sequence. Amplified cDNA fragments were subcloned into a pMD20-T vector (TaKaRa, Tokyo, Japan) and sequenced. Sequences information from the Y552, 5′-RACE and 3′-RACE experiments as well as the open reading frame regions (ORF) were assembled using Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI). Full-length cDNA of the *PmRsr1* gene containing the ORF was obtained by PCR using the PmRsr1-F1 and PmRsr1-R1 as primers.

To determine the nucleotide sequence of the genomic DNA corresponding to the *PmRsr1* cDNA, the genomic DNA was amplified by PCR using the primers PmRsr1-F1 and PmRsr1-R1, and then the PCR products were sequenced. The sequences of all the primers used in this study were listed in Table 1.

Table 1 Sequence of primers in this study

Primer	Sequence
5RACE-R180	5′-ATGGAGAAGACGAGGAGGAA-3′
5RACE-R112	5′-TGTGCCAGCAGTATCAAGAAT-3′
3RACE-F163	5′-CCTCCTCGTCTTCTCCATTACCA-3′
PmRsr1-F1	5′-GAAACGAAACATTGGAAAGC-3′
PmRsr1-R1	5′-TCCTTCGGGTTTCTTCC-3′
Y552-F1	5′-AAGGCTTCCTCCTCGTCTTC-3′
Y552-R1	5′-TAATCTGCCGACACAAATCG-3′
Tub02	5′-GGTAACAACTGGGCTAAGGG-3′
Tub04	5′-AACGACGGAGAAGGTGGC-3′

Bioinformatics and phylogenetic analysis of *PmRsr1*

Nucleotide sequences and deduced amino acid sequences of the cloned Rsr1 gene were analyzed. The nucleotide sequences were analyzed using Sequencher 4.8 software and BLAST network service of National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI). The ORF prediction was performed using the ORF Finder Tool at the NCBI server (http://www.ncbi.nlm.nih.gov/projects/gorf/). The promoter sequence was performed using Neural Network Promoter Prediction Analysis Servicer. The sequences between the genomic DNA and the cDNA were aligned by Spidey software. The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (http://expasy.org/tools/). The Rsr1p protein sequences of yeast-form *P. marneffei* and of 11 other species were aligned for phylogenetic analysis using the neighbour-joining (NJ) methods from MEGA version 4.1 [15].

Differential expression of the *PmRsr1* in three phases

The expression of *PmRsr1* transcript in different phases (conidia, mycelia and yeast cells) were measured by real-time RT-PCR. Total RNA was prepared as described above. cDNA was generated from total RNA with a PrimeScript™ RT-PCR Kit (Perfect Real Time) from TaKaRa using 1–2 μg of total RNA as template. Real-time PCR was performed using the SYBR® Premix Ex Taq™ kit (Takara, Tokyo, Japan) according to the manufacturer's protocol. The amplification conditions were optimized for ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA). The *PmRsr1* gene was amplified with the specific primers Y552-F1 and Y552-R1. β-Tubulin was amplified as the endogenous control using the primers Tub02 and Tub04. Data were analyzed using ABI Prism 7000 SDS Software and using the $\Delta\Delta$Ct method. For multiple comparisons, one-way ANOVA with Tukey's post hoc testing was performed assuming Prism 4.0 (GraphPad Software, San Diego, CA). For statistical analyses, $P < 0.05$ was considered statistically significant.

Cloning *PmRsr1* cDNA into the pcDNA6/myc-His B expression vector and cell transfection

The ORF of the *PmRsr1* gene was amplified by RT-PCR with forward primer 5′-CTT*AAGCTT*ATGCAGCCTCGAC-3′, which contained a *Hin*dIII site before the ATG codon, and reverse primer 5′-CGG*GAATTC*CAAATAATCACACACTGCC-3′, which had an *Eco*RI site after the stop codon. The RT-PCR products were then inserted into pGEM-T by A-T cloning (Promega). After we confirmed that there was no alternation by sequence analysis, the *PmRsr1* ORF was excised from pGEM-T with *Eco*RI and *Hin*dIII and ligated into pcDNA6/myc-His B expression vector (Invitrogen, Carlsbad, CA) to form pcDNA6/myc-His B-Rsr1. Transient transfection of Vero cells with pcDNA6/myc-His B-Rsr1 was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

SDS-PAGE and western blotting analysis

Protein was extracted with PIERCE M-PER Mammalian Protein Extraction Reagent (Pierce). Fifteen percent SDS polyacrylamide gels were normally used to analyze the recombinant protein using Lane Marker Reducing Sample Buffer (Pierce). Western blotting was performed following the manufacturer's protocol using the WeaternBreeze Chemiluminescent Kit (Invitrogen, Carlsbad, CA). Briefly, the membrane was blocked in 10 ml blocking solution for 30 min and incubated with the rabbit-anti-His-Tag antibody (Sigma; diluted 1:500) for 2 h at room temperature. It was then incubated with HRP-conjugated horse anti-rabbit IgG antibody (Sigma; diluted 1:2000) for 1 h at room temperature. Immunopositive bands were stained using Chemiluminescent Substrate in the kit.

Accession number

The full length of cDNA sequence and genomic DNA sequence of the *PmRsr1* gene were submitted to the GenBank database under the accession number EU681274 and EU681275 respectively.

Results and discussion

Cloning the full-length of *PmRsr1*

The expressed sequence tag of clone Y552 obtained from suppression subtractive hybridization library showed 85% homology with RAS small monomeric GTPase Rsr1 of *A. fumigatus* using NCBI tblastn service. The high degree of similarity indicated that the cloned fragment contained a portion of the putative *Rsr1* coding region and that the 5′ and 3′ ends of the coding region were missing. This partial sequence data can be used to clone full-length cDNA using RACE technique. Based on the 502 bp partial sequence, the gene specific outer and inner primers were used along with TaKaRa's adaptor primers to perform RACE of *PmRsr1* cDNA.

In this study, a full-length cDNA clone of *PmRsr1* was obtained from yeast-form *P. marneffei*. The *PmRsr1* cDNA is 1,866 bp in length, and consists of a 761 bp 5′-untranslated region (UTR), a 642 bp ORF, and a 463 bp 3′-UTR (Fig. 1). The ORF of *PmRsr1* cDNA predicts a

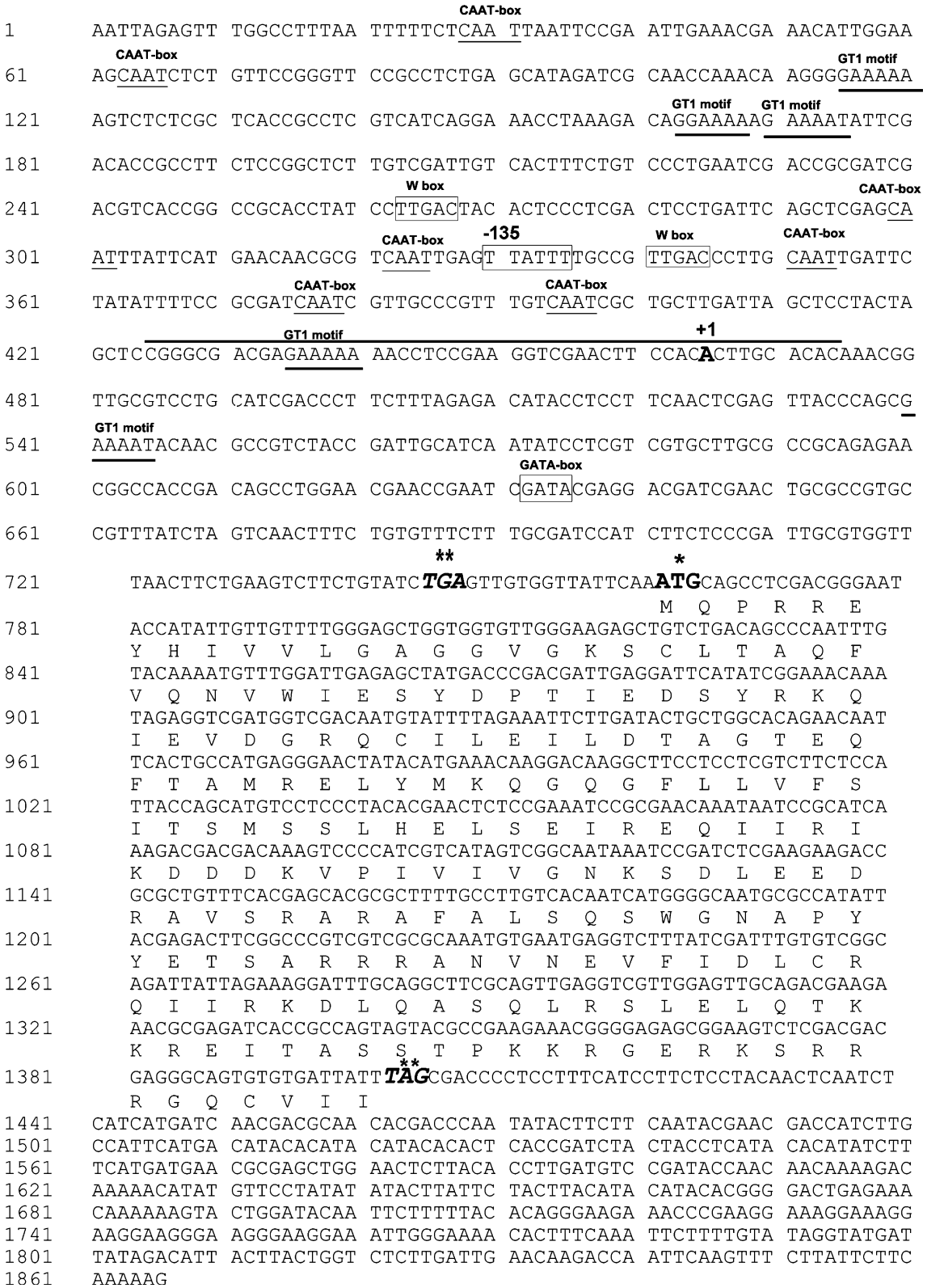

Fig. 1 Nucleotide sequence of the *PmRsr1* and the predicted amino acid sequence of the *PmRsr1* product. The cDNA sequence of *PmRsr1* is listed in the top lines. Deduced amino acid sequence of ORF is shown by the single-letter amino acid codes. Start codon (ATG) and stop codons (TGA/TAG) are indicated by * and **, respectively. ''+1'' indicates the predicated transcription start site. The promotor sequence which scores 0.98 is *overlined*. The CAAT-box and GT1 motif are indicated by *thin overlines* and *thick overlines*, respectively. The TATA-box, GATA-box and W boxes are indicated by *boxes*

protein of 213 amino acids. An inframe translational stop codon TGA is found 15 bp upstream of the ATG. This indicates that the ORF contained the complete protein-coding region. Though no poly (A) tail was observed for this full-length cDNA, a putative poly (A) addition signal (ATGATCAACG/ATGATGAACG) was found 44/161 bases downstream of the stop codon using the GeneBuilder network service for POLY-A site prediction.

Three predicted promoter sequences were found and the sequence of the highest score (0.98) from 425 to 475 bp was choose for the possible promoter sequence of *PmRsr1*. The putative transcription start site of *PmRsr1* was defined as "+1". It is located at 297 bp upstream from the start codon ATG. The most probable TATA box is located at −135, which is observed in the majority of yeast genes [16]. There are about 15 CAAT boxes in the 5′-UTR of *PmRsr1*, which are critical for eukaryotic transcription initiation. Some of the cis-elements were marked in Fig. 1.

The *PmRsr1* genomic DNA is 2,102 bp in length. The aligned results revealed that there are eight exons and seven introns between the sequences of the genomic DNA and the cDNA. Like most fungal genes, the *PmRsr1* has relatively short introns. Most of their 5′ and 3′ ends conformed to the basic consensus, GT/AG, for the eukaryotic splice donor and acceptor site, except for the intron 7. Since fungal introns are short and have polypyrimidine tracts primarily in the region between the 5′ splice site and the branch point, the splicing mechanisms of fungi may differ from the generally accepted splicing mechanisms described for metazoans [17, 18], and the automated annotations of euascomycete genomes may incorrectly identify intron boundaries for all of the confirmed [19].

Homology and phylogenetic analysis of PmRsr1

The ORF encoded a peptide of 213 residues. PmRsr1 protein had a predicted molecular mass of 24,412 Da and a theoretical isoelectric point of 9.21. Multi-alignment analysis by Clustal W indicated that PmRsr1 was with highly identity to Rsr1 reported in other species, sharing a similarity of 94% identity to *A. oryzae* (EMBL Accn: Q2U452), and 89% identity to *A. niger* (EMBL Accn: A2QN33), 87% identity to both *A. fumigatus* (EMBL Accn: B0Y446) and *A. clavatus* (EMBL Accn: A1CAG5), 62% identity to *C. albicans* (EMBL Accn: P52498), and 57% identity to *S. cerevisiae* (EMBL Accn: P13856) (Fig. 2). PmRsr1 contains several sequence motifs similar to those found in the small GTPases (G1 to G5) [20, 21]. Figure 2 shows the consensus sequences for these motifs, which are known to be involved in GDP/GTP binding and GTP hydrolysis. The predicted G1 region (also called the P-loop, consensus GXXXXGK(S/T). X, any amino acid), the phosphate binding motif, begins at position 13–20 in the amino acid sequence of PmRsr1. The XTX and DXXG Mg^{2+} binding motifs (G2 and G3) are found at positions 38–40 and 60–63. The (N/T) KXD and EXSAX guanine binding motifs (G4 and G5) are also found at positions 119–122 and 148–152 respectively. There were at least three Casein kinase II phosphorylation sites (residues 38–41; 92–95; 121–124) and one site for N-glycosylation (residues 119–122) in PmRsr1, which is important for transformation. The deduced C-terminus motif of PmRsr1, CAAX (C is Cys, A is aliphatic, and X is a variety of amino acids), is the motif of Ras protein families which is modified by a geranylgeranyl moiety. The lipid modifications are necessary for their binding to membranes and regulators and for their activation of downstream effectors (Fig. 2).

Two groups were clearly generated in the phylogenetic tree (Fig. 3). Molecular and morphological phylogenetic analyses showed that *P. marneffei* is in closely related genera with *A. fumigatus* [2, 22]. The PmRsr1 identified in this study appeared most closely related to sequences from *Aspergillus* spp., especially *A. niger*, *A. clavatus* and *A. fumigatus*, whereas the *A. oryzae* clustered in a separate branch. Galagan et al. [23] compared the genome sequence of three *Aspergilla*, the results showed *A. oryzae* being 31% bigger than *A. fumigatus* and 24% bigger than *A. nidulans*. This may explain why *A. oryzae* and *P. marneffei* are not in the same branch. The result also suggested that the evolutionary relationship of *Rsr1* in *P. marneffei* might be different from that in *A. oryzae*.

Expression and purification of PmRsr1 protein and western blotting analysis

For the convenience of protein purification and identification, ORF of *PmRsr1* was expressed in Vero cells as a fusion protein with an N-terminal His tag. The induced and non-induced samples were analyzed by 15% SDS-PAGE (Fig. 4). A band (about 30 kDa) corresponding to PmRsr1 protein was observed in the induced sample when compared to the sample without induction. PmRsr1 recombinant protein had predicted 24.4 kDa molecules from sequence data, and the myc-his epitope tag addressed about 6.0 kDa. The results of western blotting analysis showed that an intense band was detected in the culture media compared with the inner control, which clearly demonstrating that the protein was successfully expressed (Fig. 4).

Differential expression of *P. marneffei Rsr1* in three phases

The life cycle of *P. marneffei* can be divided into three distinct phases: a multicellular, filamentous vegetative

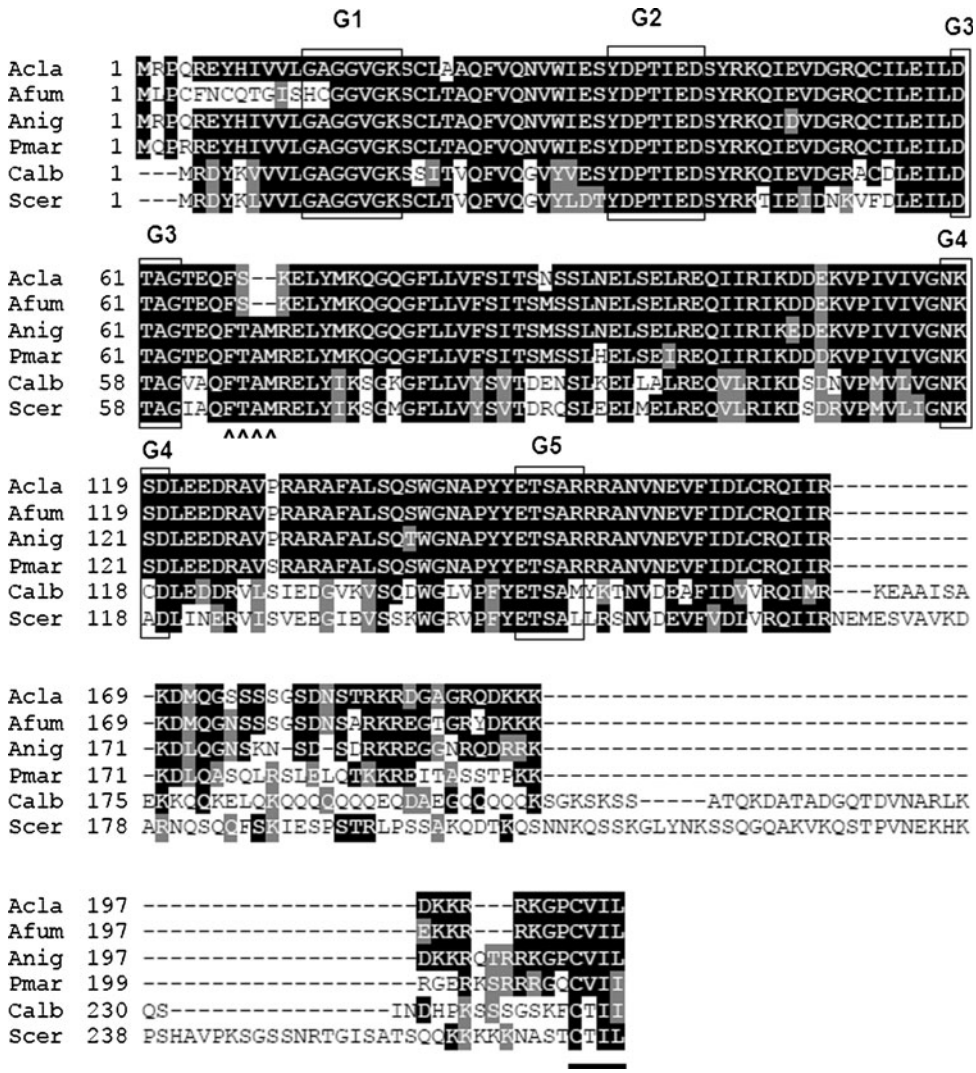

Fig. 2 The alignment of fungal Ras-like GTPase amino acid sequences. Sequences were aligned using the Clustal W and shaded by BOXSHADE. Shade residues indicate ≥75% homology (*black*) or ≥50% homology (*gray*). The GDP/GTP-binding and GTPase activities residues are indicated by boxes (G1–G5). C-terminus signal, CAAX, is shown as a *bold black bar*. Intron 7 boundary where does not match the GT/AG rule is marked (^)

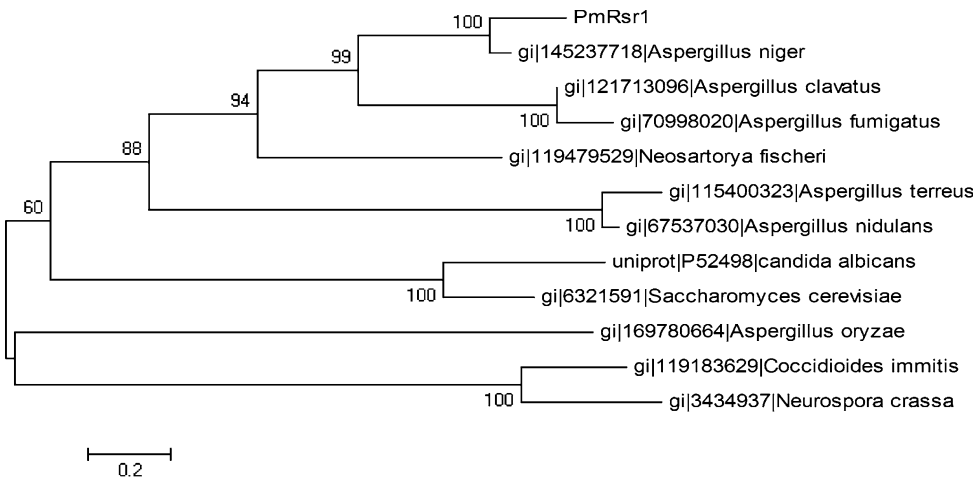

Fig. 3 Phylogenetic relationship among Rsr1 homologues. An NJ tree was generated by MEGA version 4.1 with bootstrap analysis based on 500 replications. Percentage bootstrap values are shown at branch points. Scale bar indicates the number of substitutions per site

form; an asexual reproductive conidia form; and a unicellular yeast-like phenotype. Temperature is the main environmental signal in determining which particular phase is exhibited. Current evidence suggests that particular genes take part in regulating cellular differentiation in *P. marneffei*. Rsr1 is required for the first step in budding and bud site selection, and the localization of Rsr1 is necessary for its function in bud site selection. CAAX box near the

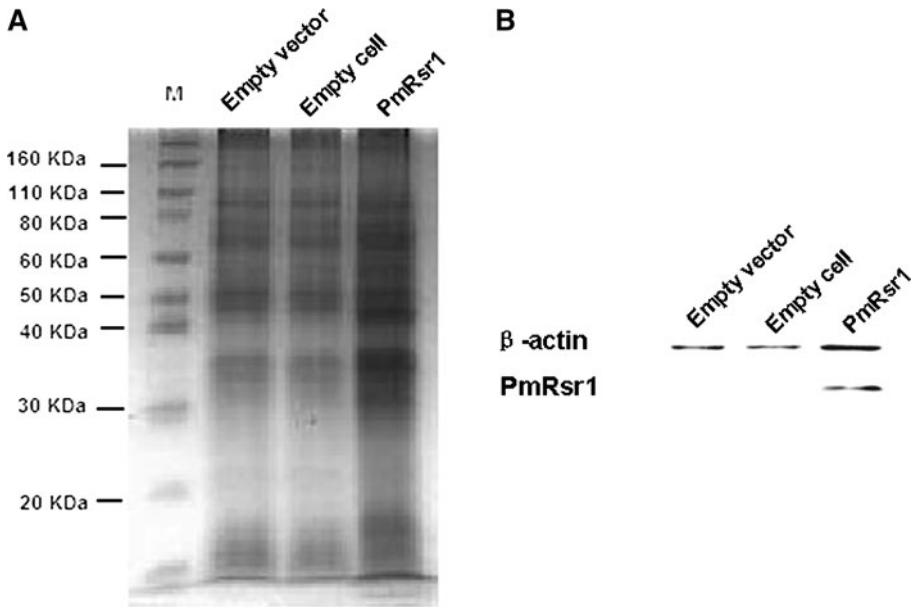

Fig. 4 SDS-PAGE and western blotting analysis of PmRsr1 protein. **a** SDS-polyacrylamide gel electrophoresis. Metal affinity-purified samples of PmRsr1 were run on a 15% polyacrylamide gel. Empty Vero cells and empty vector control plasmids were used as control. The gel was stained with Coomassie Brilliant Blue. M: Novex Sharp protein standards molecular marker (Invitrogen). **b** Immunoblot analysis of PmRsr1. PmRsr1 expression plasmids was positive in lane 3. Empty vector control plasmids were transfected into Vero cells in duplicate. Empty Vero cells were used as control too. *β*-Actin was used as a loading control

C terminus is important for Rsr1's localization to the plasma membrane and to the polarized growth sites [24]. In addition, *Rsr1* is identified as an allele-specific dosage suppressor of a *cdc42* allele (*cdc42-118*) that is defective in polarity establishment, and an rsr1 deletion mutant is found to be synthetic lethal at 30°C with *cdc42-118* [9]. *Rsr1* is essential in the absence of *Gic1* and *Gic2*, which encode two related targets of Cdc42 that are involved in polarity establishment, the *rsr1 gic1gic2* mutant fails to undergo bud emergence. It is likely that *Rsr1* has a role in polarity establishment [25]. In *P. marneffei*, Boyce et al. [5] reported that RasA (Ras) acted upstream of CflA (CDC42) to regulate germination of spores and polarized growth of both hyphal and yeast cells. In *A. fumigatus*, *rasB* regulates hyphal growth and is necessary for wild-type virulence [26]. Like *Schizosaccharomyces pombe*, *P. marneffei* forms yeast cells by fission, which distinguishes it from the budding yeast *S. cerevisiae* and *C. albicans*. As of yet, no homologous protein to Rsr1 has been found in *S. pombe.* Only one Ras protein, Ras1, is found in *S. pombe*, which regulates the mating and cell morphology pathways [27]. In this study, the alignment results indicated that PmRsr1 had 87% identity with Rsr1 from *A. fumigatus*, 57% identity with *S. cerevisiae*, and all of them had CAAX box, but the functional significance of this similarity in PmRsr1 is not clear. The role of PmRsr1 remains to be answer. Further study is required to better understand the function of PmRsr1 and the interaction between PmRsr1 and Cdc42.

In this study, the differential expressions of *PmRsr1* in different phases (conidia, mycelia and yeast cells) were analyzed by sensitive real-time RT-PCR (Fig. 5). A dissociated curve showing a single peak at the melting temperature expected for that amplicon suggested specific amplification. Expression was determined as fold increased $2^{-\Delta\Delta Ct}$ levels relative to the form with lowest expression (conidia) set to 1. Real-time RT-PCR analysis revealed that *PmRsr1* gene was expressed in three forms of *P. marneffei*, with mRNA levels observed in yeast form (2.15-fold), mycelia form 1.26 fold (Fig. 5). There were significance

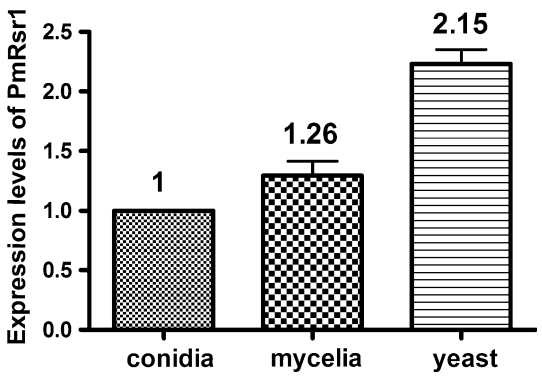

Fig. 5 Real-time RT-PCR analysis of *PmRsr1* mRNA expression levels in difference forms of *P. marneffei*. Data were shown as the $2^{-\Delta\Delta Ct}$ levels calculated relative to the form with the lowest expression (conidia), normalized against endogenous control *β*-tubulin mRNA levels

differences between yeast and mycelia ($P < 0.01$) as well as between yeast and conidia ($P < 0.01$), but no statistical significance between mycelia and conidia ($P > 0.05$). These results suggest that the *PmRsr1* might play an important role in the phase transition of *P. marneffei*. It will be of interest to analyze the relative contributions of *PmRsr1* to the morphology of *P. marneffei* cells in vitro and in vivo. We are carrying out further experiments on generating *PmRsr1*-mutants to study its functions in this important fungal pathogen.

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