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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS

Rhf1 gene is involved in the fruiting body production of *Cordyceps militaris* fungus

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Abstract *Cordyceps militaris* is an important medicinal fungus. Commercialization of this fungus needs to improve the fruiting body production by molecular engineering. An improved *Agrobacterium tumefaciens*-mediated transformation (ATMT) method was used to select an insertional mutant (g38) which exhibited fast stromatal differentiation and increased yield. The *Rhf1* gene encoding filamentation protein was destroyed by a single T-DNA and no *Rhf1* transcription was detected in mutant g38. To verify the function of the *Rhf1* gene, RNA interference plasmid and overexpression vector of the *Rhf1* gene were constructed and transferred to the wild-type JM4 by ATMT. Fast stromatal differentiation and larger fruiting bodies were found in the RNAi-*Rhf1* mutants (JM-i*Rhf1*). In the overexpression mutants (JM-OE*Rhf1*), neither stromata nor fruiting bodies appeared. The rescued strain (38-OE*Rhf1*) showed similar growth characteristics as JM4. These results indicated that the *Rhf1* gene was involved in the stromatal differentiation and the shape formation of fruiting bodies.

Keywords *Cordyceps militaris* · Filamentation protein · Stromatal differentiation · Fruiting body

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Introduction

Cordyceps militaris (Cordycipitaceae, Hypocreales, Sordariomycetes, and Ascomycota) [44] is an entomopathogenic fungus with various biological and pharmacological activities, such as multitude immunomodulation, antitumor, anti-inflammation, and antioxidation [3, 5, 17, 21, 22, 25], similar to another medicinal fungus *Ophiocordyceps sinensis* [10, 42, 45]. *C. militaris* extracts showed antidiabetic activity for diabetic Sprague–Dawley rats, displaying strongly reduced blood glucose levels and total cholesterol and triglycerides concentrations in serum [12]. Three micro-molecular compounds with antioxidant and HIV-1 protease-inhibiting activities from fruiting bodies of *C. militaris* were isolated [23]. *C. militaris* extracts were also helpful for the treatment of metabolic disorder obesity [26].

Due to the great demand for *C. militaris* products in the market, methods for the commercial production of fruiting bodies of this fungus have been established in artificial media [48, 62] or with several insects, including higher heteroceriid lepidopterans and unidentified hymenopteran species [27]. Current industrial and commercial development of *C. militaris* focus on increasing the yields of fruiting bodies and the concentrations of bioactive compounds [18, 42] through in vitro and in vivo culture methods and genetic engineering [32, 35].

In practice, strain degeneration, medium components and culture parameters [4, 9, 32, 46, 47], nutrition and mating behavior [7, 65] influence the industrial production of fruiting bodies. Different medium components, including rice, bean powder, corn grain, and wheat grain, show significant effects on the production of *C. militaris* fruiting bodies [32, 35, 46, 47]. Light is the most important factor affecting stromatal differentiation. Better fruiting body growth and bioactive complements were obtained under

12:12-h light/dark cycle in rice medium [2]. Higher yields of exopolysaccharide from *C. militaris* were obtained by UV mutagenesis [33]. The other factors, such as static culture or shake culture [8], light wave length [11], heavy metals and surfactants [9], salinity [55], culture temperature, humidity, air exchange, and plant hormones [1, 9, 11], were also important for *C. militaris* production and bioactive compound synthesis.

Agrobacterium tumefaciens-mediated transformation [62], a reference gene validated under different experimental conditions [30], and the genome sequencing of *C. militaris* for better understanding of *Cordyceps* biology [60], may provide an ideal basis for genetic and molecular studies of this fungus. Fruiting body production can also be improved by genetic methods. *C. militaris* is a bipolar heterothallic fungus [41, 60], with two different mating-type (MAT) genes (*MAT1-1:MAT1-2*) to regulate sexual reproduction [7]. Cross-mating between the opposite mating types produced over fivefold well-developed fruiting bodies than self- or cross-mating between strains within the same mating type [56]. From the genome analysis, it was estimated that the Zn2Cys6-type transcription factors and MAPK pathway were induced during fruiting, but not the PKA pathway, and more than 63 % of the total 9684 genes were expressed during both mycelial growth and fruiting body formation [49, 60]. Some *C. militaris* genes have been cloned in recent years, including the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene [16], the Cu/Zn superoxide dismutase gene (CmSOD) [64], *coxI* with putative LAGLIDADG endonuclease [63], the mating-type genes (MAT1-1-1 and MAT1-1-2) [7], geranylgeranyl diphosphate synthases [28], SSU rDNA group I introns [29], and the blue-light receptor gene Cmwc-1 [52]. The transcriptome and proteome for fruiting body production have been reported [53, 61]. However, the specific genes involved in fruiting body formation remain unknown.

RNA interference (RNAi) or overexpression efficiently regulates the expression of targeted genes in fungus [13, 36]. The RNAi method was developed in *Trichoderma reesei* using opposite dual promoters [19]. Protein kinase C in *Penicillium digitatum* under the CYP51 promoter region was overexpressed to suppress calcineurin-associated defects in *Aspergillus nidulans* [6]. Overexpression of the *Metarhizium robertsii* HSP25 gene increased fungal thermotolerance and survival in soil [31]. Expressed glutathione peroxidase could recover the fruiting body growth of the degenerated strain of *C. militaris* [50].

In this study, the ATMT method was used to identify the *Rhf1* gene involved in in vitro and in vivo fruiting body production from the *C. militaris* mutant library and to verify the functions of the selected gene by RNAi and gene overexpression.

Materials and methods

Fungal and bacterial strains

C. militaris wild-type JM4 strain and transformants, including g38, JM-i*Rhf1*, JM-RNAi, JM-OE*Rhf1*, JM-OE, 38-OE*Rhf1,* and 38-OE strains (Table 1), generated by ATMT were maintained/cultured in Guangdong

Table 1 Fungal stains and plasmids used in this study

Name	Strain or plasmid	Feature
JM4	Strain	Wild-type *C. militaris*
g38	Strain	*C. militaris* mutant with disrupted the *Rhf1* gene by T-DNA
pKHt	Plasmid	The binary vector applied for ATMT in *C. militaris* for insertional mutagenesis [37]
RNAi	Plasmid	RNAi vector with the opposite dual promoters (the *gpd* and *trpC* promoters of *Aspergillus nidulans*) for dsRNA production
i*Rhf1*	Plasmid	Vector for RNAi knockdown of the *Rhf1* gene
OE	Plasmid	Overexpression vector with the *gpd* promoter of *A. nidulans* for exogenous expression of the *Rhf1* gene
OE*Rhf1*	Plasmid	Vector for the exogenous *Rhf1* mRNA expression
JM-RNAi	Strain	Negative control strain; RNAi vector was transferred into JM4
JM-i*Rhf1*	Strain	Transformants of the *Rhf1* gene knockdown in JM4 by RNAi
JM-i*Rhf1*-1	Strain	One of the JM-i*Rhf1* transformants
JM-OE	Strain	Negative control strain; OE vector was transferred into JM4
JM-OE*Rhf1*	Strain	Transformants with overexpression of the *Rhf1* gene in JM4
JM-OE*Rhf1*-43	Strain	One of the JM-OE*Rhf1* transformants
38-OE	Strain	Negative control strain; OE vector was transferred into g38
38-OE*Rhf1*	Strain	Transformants with rescue of the *Rhf1* gene in g38
38-OE*Rhf1*-1	Strain	One of the 38-OE*Rhf1* transformants

Entomological Institute, Guangdong, China, as previously described [63]. Potato dextrose agar supplemented with 10 % peptone (PPDA) was prepared for culturing *C. militaris*. *A. tumefaciens* strain AGL-1 (provided by Prof. Zide Zhang from South China Agriculture University, China) and *Escherichia coli* strain DH5a (Tiangen, China) were grown on Luria–Bertani medium at 28 and 37 °C, respectively.

Fungal cultures

Liquid, solid and rice media for *C. militaris* and the transformants (Table 1) were prepared according to the described method [62]. The culture conditions for mycelia in liquid or solid PPDA and for fruiting body in an artificial rice medium were described previously [63]. JM4 and the transformants were cultured on solid PPDA at 23 °C for 7 days. The mycelia from PPDA were transferred to a 250-ml flask containing 50 ml liquid medium (LM) [200 g potato extract, 20 g glucose, 2 g KH_2PO_4, 1 g $MgSO_4$, 1 g ammonium citrate ($C_6H_5O_7$ $(NH_4)_3$), 5 g peptone, 20 mg vitamin B_1, and 1000 ml distilled water]. The flask was then incubated at 22 °C on a 150-rpm shaker for 7 days. For the fruiting body production of this fungus, a fungal culture of LM was diluted three times with sterile water, and 15 ml of the diluted culture as inoculum was added into the sterilized rice medium containing 20 g rice, 0.5 g powder of silkworm pupae, and 25 ml nutrient solution (20 g glucose, 2 g KH_2PO_4, 1 g $MgSO_4$, 1 g ammonium citrate, 5 g peptone, 20 mg vitamin B_1, and 1000 ml distilled water) in a glass bottle (diameter = 60 mm, height = 90 mm). After dark cultivation at 20 °C for 7 days, followed by illuminated cultivation for 5 days, the cultures in the bottles were kept for aerobic growth at 20 °C. Illumination (12 h) and aeration were applied to induce color change in the mycelia and stimulated the stromata and fruiting body formation. The whole growing period for fruiting body production lasted 6–8 weeks.

Fungal transformation

Transformation of *C. militaris* JM4 strain was conducted by the ATMT method as described previously [62]. The binary vector pKHt, carrying a selectable marker coding for hygromycin B phosphotransferase gene (*hyg*) under the control of the *A. nidulans trpC* promoter (*PtrpC*), was used for fungal transformation to produce the mutant library [37]. All primers described in this work are listed in Table 2.

C. militaris transformants were maintained/cultured in the same way as JM4. The phenotypic characteristics of the transformants, such as the stromata stimulation and fruiting body formation on the artificial medium, were observed and recorded during the growth period by an EOS 600D camera (Canon, Japan). The fresh weight (FW) of the fruiting bodies in each bottle was calculated [38].

A mutant named g38 from approximately 800 transformants was isolated based on its colonical and growth characteristics: fast stromatal differentiation, increased dry weight of mycelia and fruiting bodies, compared with JM4.

Screening of transformed hypha was achieved by removing small samples from the solid PPDA plates. Aerial mycelia were fully dispersed in sterile water for fluorescence analysis [20, 39]. Fluorescence microscopy was performed with a Nikon 80i fluorescence microscope (Nikon, Japan), and images were captured using a Nikon DS-Fi1 camera (Nikon, Japan).

Molecular analysis of the transformants

Isolation of nucleic acids

The total fungal genomic DNA (gDNA) and the RNA of all *C. militaris* strains were, respectively, prepared by the E.Z.N.A.™ HP Fungal DNA Kit (Omega, USA) and the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions [63]. The mycelia from liquid PPDA at 22 °C in a shaking platform (120 rpm) for 7 days were harvested, washed twice with sterile distilled water and used for gDNA or total RNA isolation [50]. The quality and concentration of gDNA and RNA were analyzed by agarose gel electrophoresis and spectrophotometry, respectively. The total RNA was treated with DNaseI(RNase-free) (TaKaRa, Japan) to remove possible gDNA. cDNA was synthesized with a PrimeScript™ 1st Strand cDNA Synthesis Kit with eraser (TaKaRa, Japan). Both DNA and RNA were stored at −80 °C for further use.

TAIL-PCR of T-DNA flanking sequences and full-length genes

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) [15, 37] was used to obtain genomic DNA flanking of the inserted T-DNA from mutant g38. The right border primers (RB1, 2, and 3) and the left border primers (LB1, 2, and 3) listed in Table 2 were used, as previously described [37]. The tertiary TAIL-PCR products were ligated into the pMD19-T vector (TaKaRa, China) and sequenced by Invitrogen, Shanghai. *Rhf1* gene inserted by T-DNA in mutant g38, T-DNA insertion site [15] and gene loss in the genome were detected. To obtain the full-length *Rhf1* sequence, 3 specific nested primers *Rhf1*-G1, *Rhf1*-G2, and *Rhf1*-G3 (Table 2) were designed, and the special TAIL-PCR (or genome walking) with the arbitrary degenerate primer AD1 was performed to obtain the 3′ end of *Rhf1*. The PCR products were sequenced and confirmed as above.

Table 2 List of primers used for TAIL-PCR, PCR, cloning and vector construction

Primer name	Nucleotide sequences (5′-3′)	Feature
PtrpC-EcoF	CCGAATTCTGATATTGAAGGAGCATTTTTTG	The primers for the *gfp* gene under *PtrpC* and *lacZ* promoter at the *Eco*R I restriction sites for the pKHt-*gfp* vector
gfp-R	CCGAATTCTTATTTGTATAGTCATGCAT	
AD1	WAGTGNAGWANCANAGA	The arbitrary degenerate primer for all TAIL-PCR [37]
LB1	AGGGTTCCTATAGGGTTTCGCTCATG	Primers on the right border (RB) and left border (LB) of T-DNA for TAIL-PCR [37]
LB2	CATGTGTTGAGCATATAAGAAACCCT	
LB3	CGAATTAATTCGGCGTTAATTCAGT	
RB1	GGCACTGGCCGTCGTTTTACAACG	
RB2	AACGTCGTGACTGGGAAAACCCTG	
RB3	CCCTTCCCAACAGTTGCGCAG	
Rhf1-G1	CAACCCAACCAGCCACGCACCCTCC	Special primers of the *Rhf1* gene for genome walking
Rhf1-G2	GAGAAATCGCGGGCGCTGTCGAGGC	
Rhf1-G3	GAGACTCCGCGACCACCGACATCCAG	
Rhf1-F	GCTCTAGAATGAGGTACACTGGGCAACAGC	Primers with *Xba* I restriction site for the full-length *Rhf1*
Rhf1-R	CCCTCTAGATTAAACAATGTAGCCGTTGGCAG	
LRh-F1	AGGTACACTGGGCAACAGCACATGAAGAATT CCGAAAAATTAGTTACACAAAAGCCTG	Fusion PCR primers of *Rhf1*-1 for the first part cds of *Rhf1*
LRh-R1	GCTAACTCCCTTAGGTGCAGGTCCGTGACAGCAG CGAG	
LRh-F2	CTGCTGTCACGGACCTGCACCTAAGGGAGTTAGC CCAGC	Fusion PCR primers of *Rhf1*-2 for the second part cds of *Rhf1*
LRh-R2	TTGCTTTGGGTGACGACTGCACGCAATGGCATCT GCAAG	
LRh-F3	AGATGCCATTGCGTGCATGTGTCACCCAAAGCA AGCAAG	Fusion PCR primers of *Rhf1*-3 for the third part cds of *Rhf1*
LRh-R3	GAGGCGCTGTCAGTTAGCGTCATGTTTGCGCAGAGA	
Rhf1-F2	CTGACAGCGCCTCCCTCCAGACCCCTCTAGAT-TAAACAATGT	PCR primers of *Rhf1*-4 in the RT-PCR reaction for the fourth part cds of *Rhf1*
Rhf1-R	AGCCGTTGGCAG	
q*Rhf1*-G1	CAACCCAACCAGCCACGCACCCTCC	qRT-PCR primers of the *Rhf1* (217 bp) in *C. militaris*
q*Rhf1*-G1R	GGAGTCTCGTCTGTTACCTGAATGG	
qGF1	TTGGCATCAACGGCTTCGG	qRT-PCR primers of the reference gene (278 bp) of glyceraldehyde-3-phosphate dehydrogenase (Cm-*gpd*)
qGR1	GGACTCGACAACGTACTCGG	
Pgpd-F	GGGAAGCTTCAATTCCCTTGTATCTCTACAC	Primers of glyceraldehyde-3-phosphate dehydrogenase gene promoter in *A. nidulans* with *Hin*d III and *Xba* I restriction sites and with the change of first base G to C
Pgpd-R	GGGTCTAGAGGGAAAAGAAAGAGAAAAGAAAAG	
iF5-F	GGGGTACCCTTGGTTTACTCTCAATTGCCCG	Primer with *Kpn* I for the 633 bp segment of *Rhf1* with primer *Rhf1*-R for RNAi
PtrpC-F	GGGCTGCAGGGTACCTGATATTGAAGGAGCATTTTTTG	Primers of the *A. nidulans trpC* promoter with *Pst* I and *Kpn* I restriction sites in the upstream primer and *Pst* I in the downstream primer
PtrpC-R	GGGCTGCAGTTGGATGCTTGGGTAGAATAGG	
TtrpC–F	GGGCTGCAGGGTACCGATCCACTTAACGTTACT-GAAATGGG	Primers of the *A. nidulans trpC* terminator with *Pst* I and *Kpn* I restriction sites in the upstream primer and *Pst* I in downstream primer
TtrpC –R	CTGCAGCTAGAAAGAAGGATTACCTCTA	
Phyg-F	CGGAATTC TGATATTGAAGGAGCATTTTTTG	Primers for *Phyg* (modified hygromycin B resistance (*hph*) gene under *A. nidulans trpC* promoter) [37] with *Eco*R I and *Hin*d III
Phyg-R	CCCAAGCTTCTATTCCTTTGCCCTCGGA	

Underlined sequences indicate restriction endonuclease sites

Southern blot

To determine the copy numbers of *Rhf1* in JM4 and the T-DNA insertions in mutant g38, Southern hybridization was performed. Approximately 10^5 conidia of the indicated strains were inoculated in liquid PPDA at 22–25 °C on a 120 rpm shaker for 7 days in dark. Mycelium pellets of JM4 and mutant g38 were washed twice with sterile

distilled water and gDNA was isolated. The gDNA (5 μg) of JM4 was digested with *Xba* I, *Sal* I,and *Bgl* II restriction enzymes. The resulting fragments were transferred onto a nylon membrane after fractionation by 1 % agarose gel electrophoresis [51]. Probe DNA was prepared with 1488 bp DIG-labeled *Rhf1*G1 fragments (amplified with primers *Rhf1*-G1 and *Rhf1*-R from the gDNA) for the determination of the copy number of the *Rhf1* gene. The gDNA (5 μg) of g38 was digested with *Bgl* II and *Hin*d III restriction enzymes and was probed with 969 bp DIG-labeled *lacZ-gfp* fragments. Probe labeling and hybridization were performed with DIG High Prime DNA labeling and detection starter kit I (Roche, Germany) [58].

Real-time transcript quantification PCR (qRT-PCR)

Total RNA from JM4 and all transformants was isolated and quantified as described above. After sample purification and reverse transcription, the 1st cDNA was synthesized by qPCR with primers of *Rhf1* (primer q*Rhf1*-G1 and q*Rhf1*-G1R) and GAPDH (primer qGF1 and qGR1) (Table 2). *C. militaris* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sequence ID: ref|XM_006669697.1|) was used as a Ref. [30]. The efficiency of each primer set was first validated by constructing a standard curve through five serial dilutions. PCR reactions were performed in an Mx3000P™ Real-Time PCR System (Stratagene, USA), using SYBR Green (SYBR®Premix Ex Taq™ II, TaKaRa, Japan). The PCR program and data analysis were performed by the described protocols [57, 59]. *Rhf1* mRNA levels were calculated relative to GAPDH expression using the Mx3000P™ Software (version 4.1) (Agilent, USA). The qPCR products were analyzed by 1.2 % agarose electrophoresis (data not shown).

Plasmids and transformants for RNAi and overexpression

pKHt-gfp

The vector pKHt provided a backbone for the construction of pKHt-*gfp*. A fragment (*PtrpC-lacZ-gfp*) of green fluorescent protein gene (*gfp*) under *PtrpC* (*A. nidulans trpC* promoter) and *lacZ* gene were amplified from pMD19-T-*PtrpC-lacZ-gfp*. The purified fragments of *PtrpC-lacZ-gfp* and pKHt plasmid were digested with *Eco*R I and were purified with a gel extraction kit (Qiagen, Germany). After treating with CIAP (TaRaka, Japan), the *Eco*R I-digested pKHt was ligated with *PtrpC-lacZ-gfp* to produce pKHt-*gfp* vector.

RNA interference plasmid (RNAi) and overexpression plasmid (OE)

RNA interference plasmid (with the dual reverse *gpd* and *trpC* promoter of *A. nidulans*) and overexpression plasmid (with the *gpd* promoter of *A. nidulans*) were constructed. The fragments, including *Pgpd* (the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene with the *Hin*d III and *Xba* I restriction sites) and *TtrpC* (the terminator of the *trpC* gene), were amplified from *A. nidulansP-trpC* (the promoter of the *trpC* gene) and *phyg* (the modified hygromycin B resistance gene under the *A. nidulans trpC* promoter) with the *Eco*R I and *Hin*d III restriction sites were amplified by PCR from the pKHt vector.

PtrpC and *TtrpC* were digested with *Pst*I and subsequently ligated into the vector pKHt after handling with CIAP (TaKaRa, Japan). The *Kpn* I site (in the primer of *TtrpC*-F/the primer of *PtrpC*-R) was introduced in the upstream of *TtrpC*/downstream of *PtrpC* following the *Pst* I site. The correct ligation clones (*Kpn* I site close to the right border (RB) of the pKHt vector) were identified by double enzyme digestion with *Eco*R I and *Kpn* I and were named pKHt-*T* and pKHt-*P*, respectively.

The purified *Pgpd* fragment, after double-digesting with *Hin*d III and *Xba* I, was subcloned in the pMD™19-T-*gfp* vector (TaKaRa, Japan) with the same processing, and then pMD™19-*Pgpd* was digested with *Hin*d III and *Kpn* I to obtain *Pgpd* fragments composed of restriction sites of *Kpn* I, *Xma* I, *Sma* I, *Bam*H I, and *Xba* I in the downstream. *Pgpd* digested with *Hin*d III and *Kpn* I was ligated into pKHt-*P* and pKHt-*T* to produce pKHt-*PP* and pKHt-*TP* vectors. The fragments containing *Pgpd*, *TtrpC*/*PtrpC*, and 2.2 kb segments of the ColE1 origin replication and the chloramphenicol resistant gene were isolated by digesting pKHt-*PP* or pKHt-*TP* with *Eco*R I and *Hin*d III. These two fragments were ligated to the *Phyg* digested with *Eco*R I and *Hin*d III to obtain the overexpression vector (OE) or RNA interference vector (RNAi).

iRhf1 plasmid

Fragment iF5 (the last 633 bp of *Rhf1* cDNA) was amplified and cloned into RNAi vector digested with *Xba* I and *Kpn* I to construct an RNAi vector for the *Rhf1* gene (i*Rhf1*).

OERhf1 plasmid

The whole open reading frame (ORF) of *Rhf1* was amplified by fusion PCR [54]. In the first round of PCR, 4 fragments of the target gene were separately amplified. The first part (LRh1), including the first exon and the second exon sequences of *Rhf1*, the second part (LRh2), including

the third exon of *Rhf1,* and the third part (LRh3) including the fourth exon of *Rhf1,* were amplified from the gDNA of *C. militaris*, and the last part of *Rhf1* cDNA (LRh4), including all the remaining exons of *Rhf1,* was amplified by reverse transcription PCR (RT-PCR) from the cDNA of *C. militaris*. LRh1 was then connected to the LRh2 fragment to obtain LRh12. LRh3 was connected to LRh4 to obtain LRh34 by fusion PCR. Finally, LRh12 and LRh34 were recombined to produce the full-length *Rhf1* cDNA with primers of *Rhf1*-F and *Rhf1*-R. The full-length *Rhf1* cDNA was inserted into the OE vector by *Xba* I site to produce *Rhf1* overexpression vector (OE*Rhf1*).

RNAi plasmid (RNAi), overexpression plasmid (OE), plasmid i*Rhf1*, and plasmid OE*Rhf1* were transformed into the JM4 strain by ATMT, and the resulting transformants of JM-RNAi, JM-OE, JM-i*Rhf1*, and JM-OE*Rhf1* were obtained. The overexpression vector of *Rhf1* (OE*Rhf1*) was transformed into mutant g38 to produce 38-OE*Rhf1* transformants (the *Rhf1* rescued mutants).

Data analysis

Data were analyzed by SPSS 16.0 software (SPSS Inc., Chicago, USA). Normal one-way analysis was evaluated by an AVOVA test, and the significance between treatments in each experiment was determined by Turkey's multiple tests. The values were expressed as the mean ± SD, and $P < 0.05$ was defined as statistically significant.

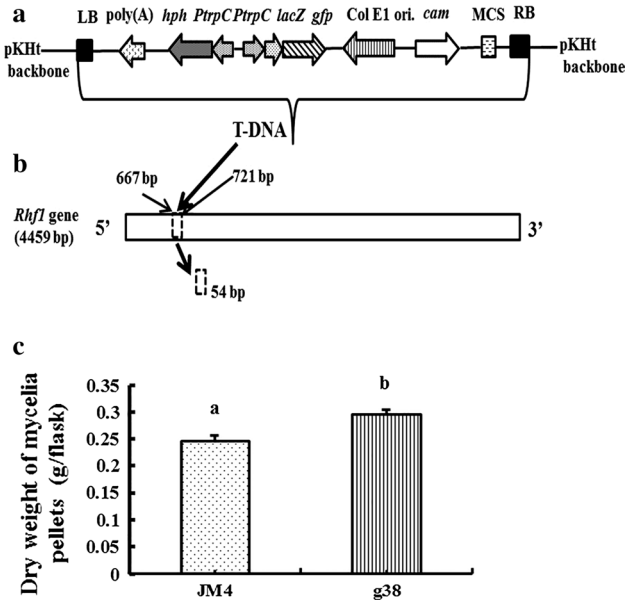

Fig. 1 Screening of transformants. **a** The components of T-DNA in the pKHt-*gfp* vector. The fragment of green fluorescent protein gene (*gfp*) with *PtrpC* and *lacZ* gene was ligated to pKHt at the *Eco*R I site to produce pKHt-*gfp* vector. *Arrows* denote the direction of transcription. **b** *Rhf1* gene in JM4 was inactivated by the inserted T-DNA. A total of 54 bp sequences were lost from the *Rhf1* gene (from 667 bp to 721 bp) in JM4 to generate mutant g38. **c** Dry weight of *C. militaris* mycelium pellets in a 50 ml flask with 20 ml liquid PPDA at 22–25 °C under a shaking platform (120 rpm) for 7 days. Ten replicates were established for each treatment. Data are the mean ± SD. The columns with *different letters* indicate significant differences ($P < 0.05$)

Results

Characterization of mutant g38

Growth of *C. militaris* JM4 was completely inhibited at a hygromycin B concentration of 800 μg/ml. Therefore, this concentration was used for screening *C. militaris* transformants. *C. militaris* transformants were created by co-cultivation of *A. tumefaciens* cells carrying pKHt-*gfp* (Fig. 1a) with the conidia of JM4. Compared with JM4, a mutant called g38 was detected based on its fast stromatal differentiation, higher dry weight of mycelia and fruiting bodies. When 10^5 conidia of JM4 and mutant g38 were cultured in a 50-ml flask with 20 ml liquid PPDA at 22 °C in an 120 rpm shaker for 7 days, the average freeze-dried weight of the mycelium pellets in the flasks was 0.25 g for JM4 and 0.3 g for g38 (Fig. 1c). The biomass of mutant g38 was significantly higher than that of wild-type JM4 at the same day ($P < 0.05$).

For the fruiting body production of *C. militaris* JM4 and mutant g38 on rice medium in glass bottles, g38 completed color change and stromatal formation in 2 weeks, but JM4 was only in a stage with fluffy and orange mycelia (Fig. 2a). After 3 weeks, fruiting bodies (approximately 0.5 cm) appeared from g38, but JM4 had no formation of stromata (Fig. 2b). It took 6 weeks on rice medium to harvest the mature fruiting bodies of mutant g38, but it took 8 weeks for JM4. The fruiting bodies of g38 looked thicker than those of JM4 (Fig. 2c). The average fresh fruiting body weights (20.84 ± 0.51 g/bottle) of g38 from 10 culture bottles were higher than those (17.93 ± 0.43 g/bottle) of JM4 (Fig. 2d). Mutant g38 had excellent cultural traits (quick stromatal formation, larger fruiting body and higher fruiting body yield).

Molecular analysis of mutant g38

TAIL-PCR was used to identify the flanking sequence of the inserted T-DNA in mutant g38. The resulting TAIL-PCR products of LB and RB were approximately 1.2 kb (L3) and 2.7 kb (R3), respectively. A 667-bp fragment in the LB product was identified as the entire 5′end of the filamentation protein (*Rhf1*) by BLASTx, and a 2634-bp fragment in the RB product was identified as the middle section of *Rhf1*. Based on the known

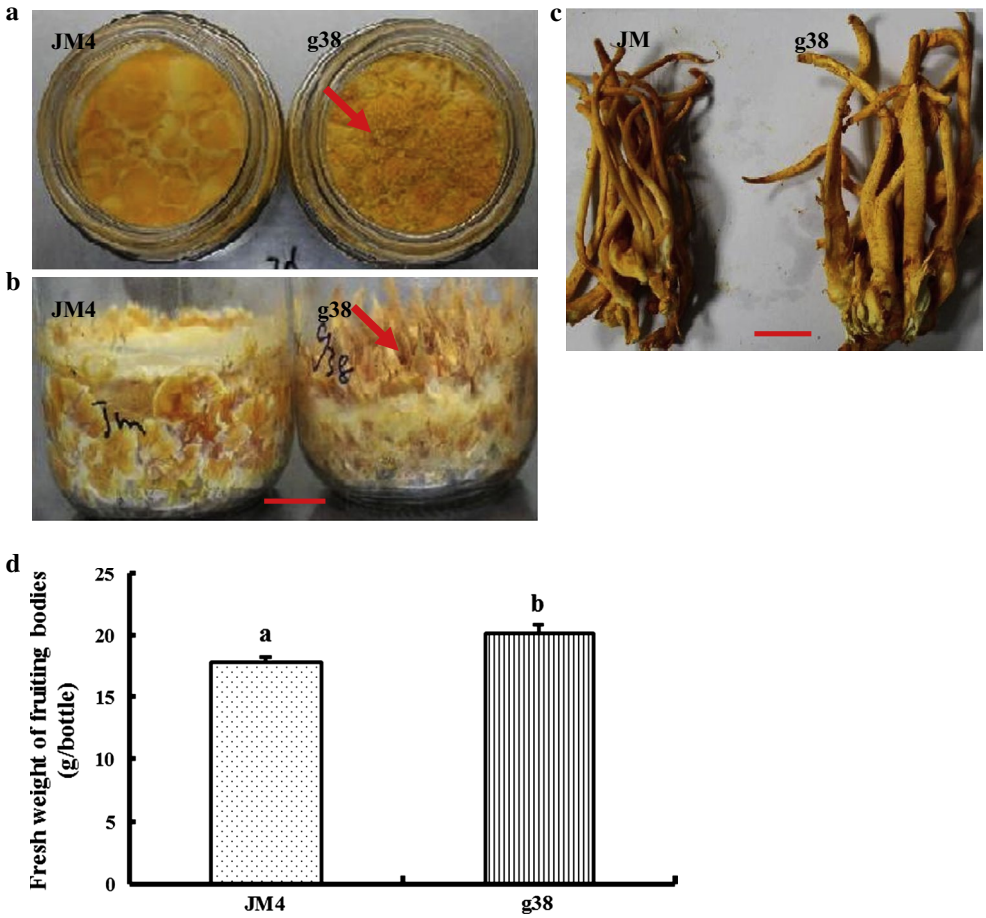

Fig. 2 Stromatal differentiation and fruiting body production of *C. militaris* JM4 and mutant g38. **a** Mutant g38 but not JM4 with stromatal differentiation on rice medium for 2 weeks. *Arrows* denote stromata. **b** Fruiting bodies from mutant g38 on rice medium for 3 weeks. *Bar* = 1 cm. **c** Fruiting bodies from JM4 after 8 weeks and mutant g38 after 6 weeks. *Bar* = 1 cm. **d** Average fresh weight of fruiting bodies from JM4 and g38. Ten replicates were performed for each treatment. The columns with *different letters* were significantly different ($P < 0.05$)

fragment, 3 specific nested primers *Rhf1*-G1, *Rhf1*-G2, and *Rhf1*-G3 (Table 2) for the remaining *Rhf1* were designed, and the specific TAIL-PCR (or genome walking) with the arbitrary degenerate primer AD1 was performed to obtain the 3′ end of *Rhf1*. The resulting product was approximately 1.3 kb (R6), and 1184 bp was identified as the entire 3′end of the *Rhf1* gene by BLASTx. The full-length sequence (4495 bp) of the *Rhf1* gene was amplified from the gDNA of *C. militaris* using primers of *Rhf1*-F and *Rhf1*-R. After alignment analysis of the flanking sequences, a 54-bp fragment (from 667 bp to 721 bp of *Rhf1* gene) was lost in JM4 (Fig. 1b).

Green fluorescence was observed from the aerial mycelia of g38 because of the presence of the pKHt-*gfp* vector but not from the mycelia of JM4 (Fig. 3a). From these results, it was concluded that the full length of the *Rhf1* gene was 4495 bp, and its cDNA was 3969 bp; *Rhf1* gene contained five introns and six exons. T-DNA was inserted into the 667 bp site of the 5′-end of the *Rhf1* gene in the gDNA of g38. The *gfp* gene of the T-DNA was expressed in mutant g38.

qRT-PCR was used to determine the transcriptional level of the *Rhf1* gene in the mycelia of JM4 and g38. No transcriptional level of *Rhf1* gene in mutant g38 was detected at 7 and 14 days. Furthermore, the *Rhf1* transcriptional levels in JM4 were not significantly different from the mycelia at 7 and 14 days, respectively (Fig. 3b).

The copy number of the *Rhf1* gene or T-DNA in JM4 or g38 was examined by Southern blot analysis with a probe of DIG-labeled *Rhf1*G1 and *lacZ-gfp*. A single copy of *Rhf1* gene was detected in the genome of JM4 (Fig. 3c), and a single T-DNA was inserted into the genome to disrupt the *Rhf1* gene (Fig. 3d).

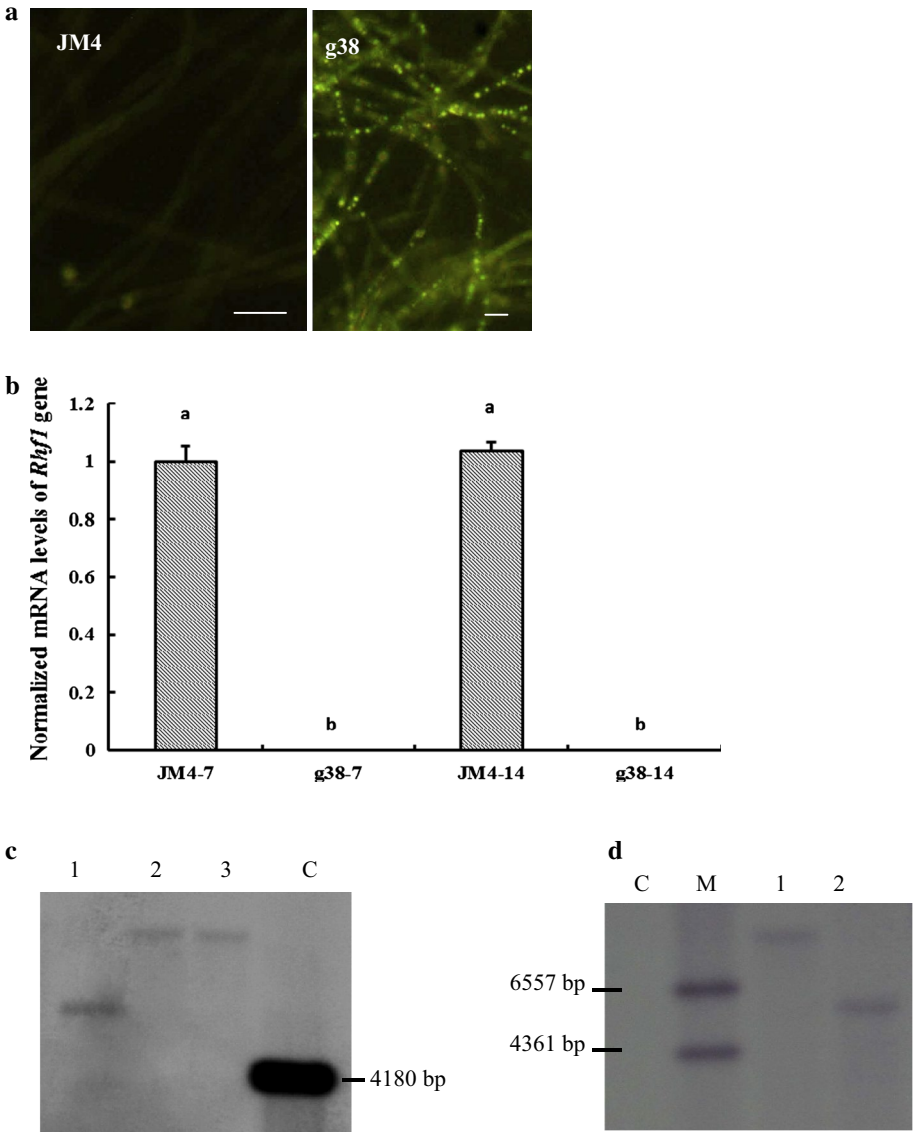

Fig. 3 Molecular identification of *Rhf1* in mutant g38 and wild-type JM4. **a** Detection of green fluorescence from 10-day mycelia of g38 on PPDA plates. *Bar* = 10 μm. **b** qRT-PCR analysis of *Rhf1* mRNA transcription in JM4 and g38 from liquid PPD cultures at 22 °C after 7 (JM4-7 or g38-7) and 14 days (JM4-14 or g38-14). Glyceraldehyde-3-phosphate dehydrogenase (Cm-*gpd*) was used as a reference gene. Data are the mean ± SD. The columns with *different letters* were significantly different ($P < 0.05$). **c** Determination of the *Rhf1* gene copy in JM4 by Southern blot. *Lanes 1–3* Genomic DNA of JM4 digested, respectively, with *Xba* I, *Sal* I and *Bgl* II restriction enzymes and probed with 1488 bp DIG-labeled *Rhf1*G1 fragments; *Lane C* positive control by pMD™19-T-*Rhf1*G1 fragment (4180 bp) digested with *Xba* I restriction enzyme. **d** Determination of T-DNA copy in g38 by southern blot. *Lanes 1–2* Genomic DNA of g38 digested, respectively, with *Bgl* II and *Hin*d III restriction enzyme and probed with 969 bp DIG-labeled *lacZ-gfp* fragments; *Lane C* genomic DNA of JM4 digested with *Hin*d III restriction enzyme; *Lane M* part of the DIG-labeled marker

Knockdown of the *Rhf1* gene in *C. militaris* JM4 by RNA interference (JM-i*Rhf1*)

To verify the *Rhf1* gene function in vivo, *Rhf1* gene was silenced in JM4 by transformation with a construct containing a 663-bp *Rhf1* fragment between two convergent dual promoters (*Pgpd* and *PtrpC*) for dsRNA production of *Rhf1* (Fig. 4a).

The transformants of JM-i*Rhf1* were screened by qRT-PCR. Different expression levels of *Rhf1* gene were detected in the transformants of JM-i*Rhf1*, but the expression of the *Rhf1* gene was not influenced in JM4 by RNAi plasmid only (JM-RNAi) (Fig. 4b). The JM-i*Rhf1*-1 mutant with an approximately 17 % of *Rhf1* mRNA transcription level of JM4 was selected for further study (Fig. 4b).

After dark cultivation in rice medium at 22 °C for 1 week, followed by 12-h illuminated cultivation for another 1 week, stromatal differentiation was observed from mutant g38, and some stomata were found from JM-i*Rhf1*-1 in the culture bottles, whereas no stromatal differentiation was observed from JM4 (Fig. 4c). Normally, 4 weeks are required for stromatal differentiation in JM4 in rice medium (1 week for mycelial growth in the dark and 3 weeks for stromatal differentiation in 12-h illuminated cultivation). *Rhf1* gene can regulate the stromatal differentiation.

Generally, the mature fruit bodies were harvested when they reached approximately 6 cm in this study. It took 6 weeks to harvest the fruiting bodies from g38 on rice medium (1 week for mycelial growth in the dark, 1 week for stromatal differentiation, 4 weeks for fruiting body

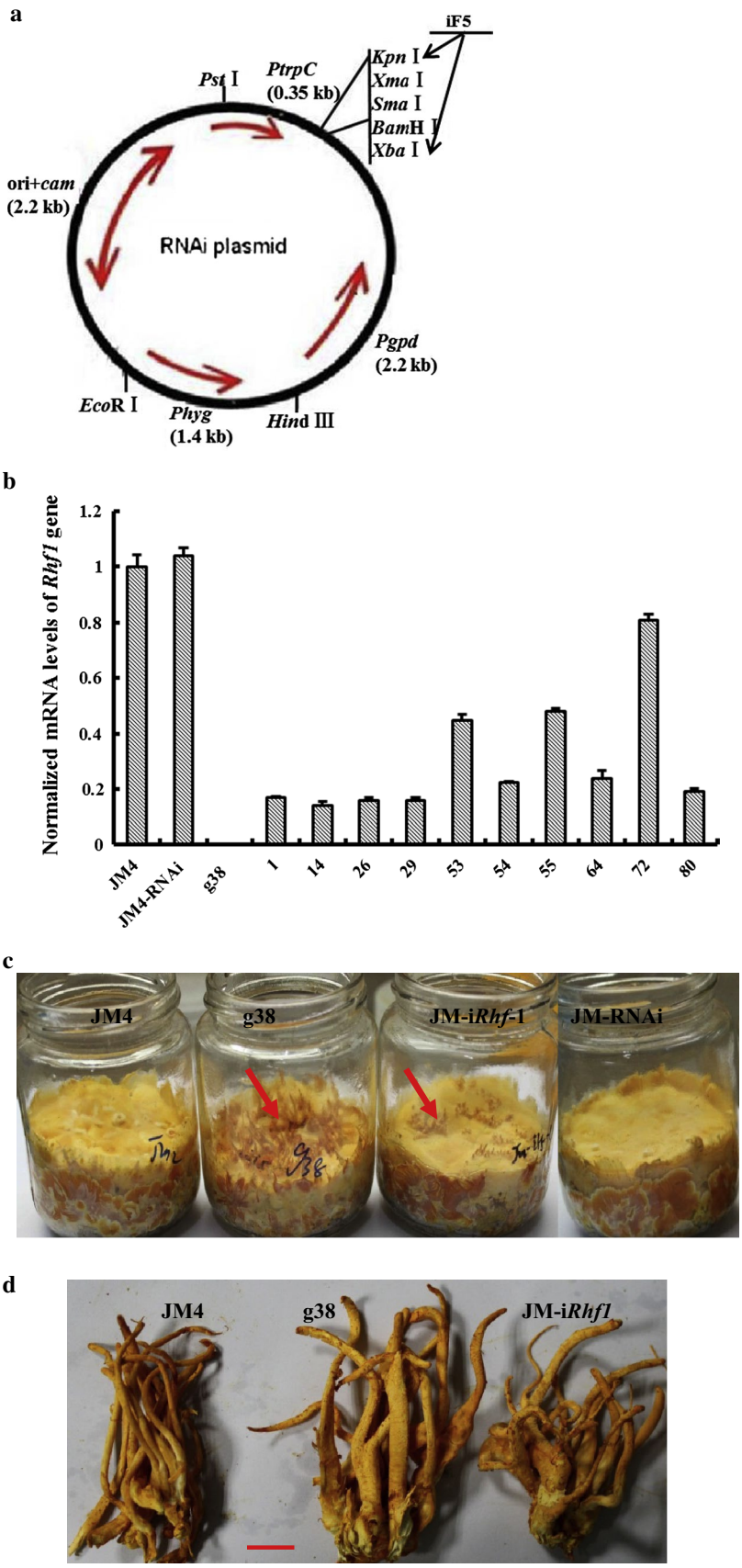

Fig. 4 Knockdown of *Rhf1* gene in *C. militaris* by RNA interference. **a** Maps of RNAi vector for the *Rhf1* gene. The multiple cloning sites include *Kpn* I, *Xma* I, *Sma* I, *Bam*H I and *Xba* I. *Arrows* denote the direction of transcription. **b** Normalized mRNA levels of the *Rhf1* gene in JM-i*Rhf1* transformants by qRT-PCR. Approximately 10^5 conidia of the indicated strains were cultured to a 50 ml flask with 20 ml liquid PPDA at 22–25 °C in a shaking platform (120 rpm) for 7 days. Glyceraldehyde-3-phosphate dehydrogenase (Cm-*gpd*) was used as a reference gene. Data are the mean ± SD. **c** Stromatal differentiation of JM-i*Rhf1* transformants. JM4 and the indicated strains were cultured in rice medium for 2 weeks at 20 °C. *Arrows* denote stromata. **d** Fruiting bodies of JM4 and the indicated strains. JM4 and JM-i*Rhf1* were cultured in rice medium for 8 weeks, and g38 was cultured for 6 weeks. *Bar* = 1 cm

growth), whereas it took 8 weeks from JM4 and JM-i*Rhf1*-1 (1 week for mycelial growth in dark, 3 weeks for stromatal differentiation, 4 weeks for fruiting body growth). Compared with those of JM4, the fruiting body shapes of mutants g38 and JM-i*Rhf1*-1 were larger, but the bases of the fruiting bodies of JM-i*Rhf1*-1 were abnormally large (Fig. 4d). The results indicated that RNAi knockdown of the *Rhf1* gene in JM4 generated mutants similar to g38.

Overexpression of the *Rhf1* gene in *C. militaris* (JM-OE*Rhf1*)

The full-length *Rhf1* cDNA was expressed under the *Pgpd* promotor in JM4 (Fig. 5a). JM-OE*Rhf1* transformants (overexpression of *Rhf1* in the wild-type *C. militaris*) were screened by qRT-PCR. The JM-OE*Rhf1* transformants had variable expression levels of *Rhf1* gene, and *Rhf1* expression was not influenced in JM4 by OE plasmid only (JM-OE) (Fig. 5b). JM-OE*Rhf1*-43 had fivefold *Rhf1* mRNA transcription levels compared with JM4 (Fig. 5b) and was selected for the following study. JM-OE*Rhf1*-43 had no differentiated stromata after 3 weeks in rice medium. In contrast, JM4 and JM-OE had some stromata (Fig. 5c). After another 5 weeks, fruiting bodies of JM4 and JM-OE were harvested, whereas JM-OE*Rhf1*-43 had no fruiting bodies (Fig. 5d). Therefore, overexpression of *Rhf1* impaired stromatal differentiation for *C. militaris* and subsequently caused no fruiting body formation.

Complementation of the *Rhf1* gene in mutant g38 (38-OE*Rhf1*)

Rhf1 gene was complemented in mutant g38 by OE*Rhf1*. 38-OE*Rhf1* transformants (rescue of *Rhf1* in mutant g38) were screened by qRT-PCR. Variable expression levels of the *Rhf1* gene were detected in the transformants, and the *Rhf1* expression was not influenced in mutant g38 by OE plasmid only (38-OE) (Fig. 6a).

38-OE*Rhf1*-1 showed fivefold *Rhf1* mRNA transcription levels compared with JM4 (Fig. 6a). After 2 weeks in rice medium, g38 and g38-OE had abundant stromata, but JM4 had no stromata, and 38-OE*Rhf1*-1 had only a few stromata (Fig. 6b). Three weeks were required for 38-OE*Rhf1* to differentiate stromata, but 4 weeks were required for JM4. Therefore, it took 6 weeks for g38 and g38-OE, 7 weeks for 38-OE*Rhf1*, and 8 weeks for JM4 to harvest the mature fruiting bodies. The shapes of the fruiting bodies from g38 and g38-OE were larger than those from JM4. The shapes of 38-OE*Rhf1* were between those of JM4 and g38 (Fig. 6c). These data further indicated that *Rhf1* was critical in stromatal differentiation and fruiting body formation in *C. militaris*.

Discussion

ATMT has been described as an efficient protocol for many filamentous fungi, not only applicable to *C. militaris* [50, 63], but also to several other species, including *M. anisopliae* [43], *A. awamori* [34], *Glomus intraradices* [20], and *Beauveria bassiana* [14]. T-DNA insertion occurred efficiently as a single copy without AS in all transformational processes of *C. militaris* [62]. In the present study, the ATMT method was used to isolate mutant g38 with characteristics of fewer aerial mycelia, rapid stromatal differentiation and higher fruiting body output in less culture time. A single copy of the *Rhf1* gene was disrupted by a single T-DNA insertion in JM4. This gene was identified in the genome sequence of *C. militaris* [60], but its function is unknown. RNAi, overexpression and complementation of *Rhf1* in vivo were performed to verify the *Rhf1* function. The results indicated that the *Rhf1* gene was involved in the fruiting body production of *C. militaris* fungus and that silencing the *Rhf1* gene could improve the formation and yields of fruiting bodies during the commercialization of this medicinal fungus.

C. militaris displayed highly pleomorphic phenotypes when grown in the different culture conditions [52]. Stromatal stimulation and fruiting body yields were affected by many factors, including strains, medium components, inoculated culture methods and parameters [61]. Light was a necessary environmental factor for stromatal formation [52]. In the present study, the *Rhf1* gene is crucial in stromatal differentiation. Generally, 3 weeks are requested for stromatal differentiation in the routine cultivation of *C. militaris*. The present results showed that *Rhf1* knockdown can accelerate the stromatal differentiation from 3 weeks to only 1 week in mutant g38. The decrease of the total culture time of this medicinal fungus would lower the production cost and stimulate the commercial development.

Similar to the fungus *Neurospora crassa* and other fungi, *C. militaris* contained the full components for RNA interference pathways, according to the genome sequencing data [60, 61]. The RNAi of *Rhf1* gene was successfully detected in the JM-i*Rhf1* mutants. Gene overexpression in fungi is an effective strategy to study gene function. The virulence of the insect pathogen *B. bassiana* was increased by expressing a fusion protein with protease and chitinase [14]. A deteriorated *C. militaris* strain was restored to resist oxidative stress and produce fruiting bodies by expressing an antioxidant *gpxA* gene from *A. nidulans* [50]. Exogenous transcription of the *Rhf1* gene was also successfully displayed in the mutants of JM-OE*Rhf1* and 38-OE*Rhf1* in the present study.

GFP protein was introduced into fungi, including *M. anisopliae* [1], *Penicillium nordicum* [40], and *B. bassiana*

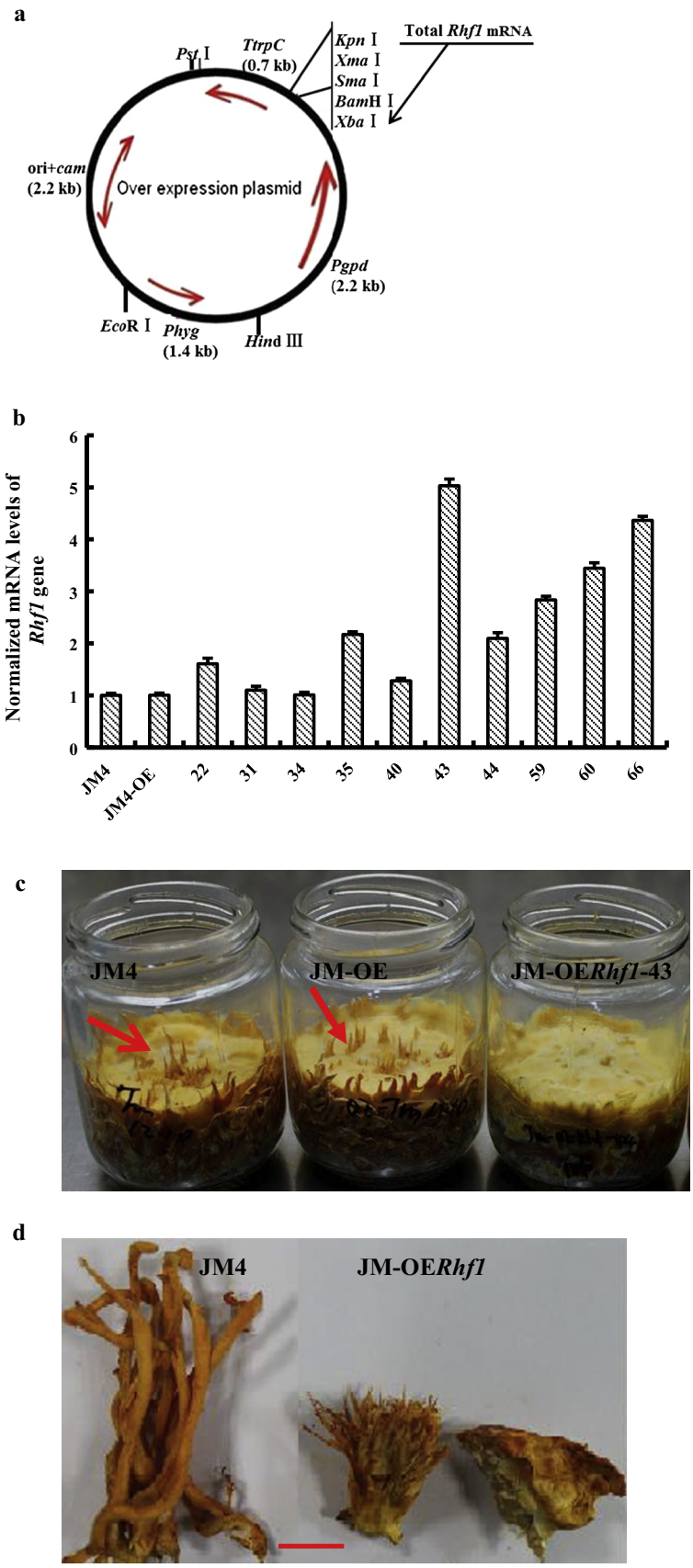

Fig. 5 Overexpression of the *Rhf1* gene in *C. militaris*. **a** Maps of the overexpression vector (OE) for the *Rhf1* gene. Multiple cloning sites are the same as the RNAi vector. *Arrows* denote the transcription directions. **b** Normalized mRNA levels of the *Rhf1* gene in JM-OE*Rhf1* in liquid PPD cultures at 22–25 °C in a shaking platform (120 rpm) for 7 days. Glyceraldehyde-3-phosphate dehydrogenase (Cm-*gpd*) was used as a reference gene. Data are the mean ± SD. **c** Stromatal differentiation of JM-OE*Rhf1* transformants. JM-OE*Rhf1*-43 had no stomata but JM4 and JM-OE had a few stomata after 3 weeks in rice medium. *Arrows* denote stromata. **d** Fruiting bodies of JM-OE*Rhf1* and JM4. *Bar* = 1 cm

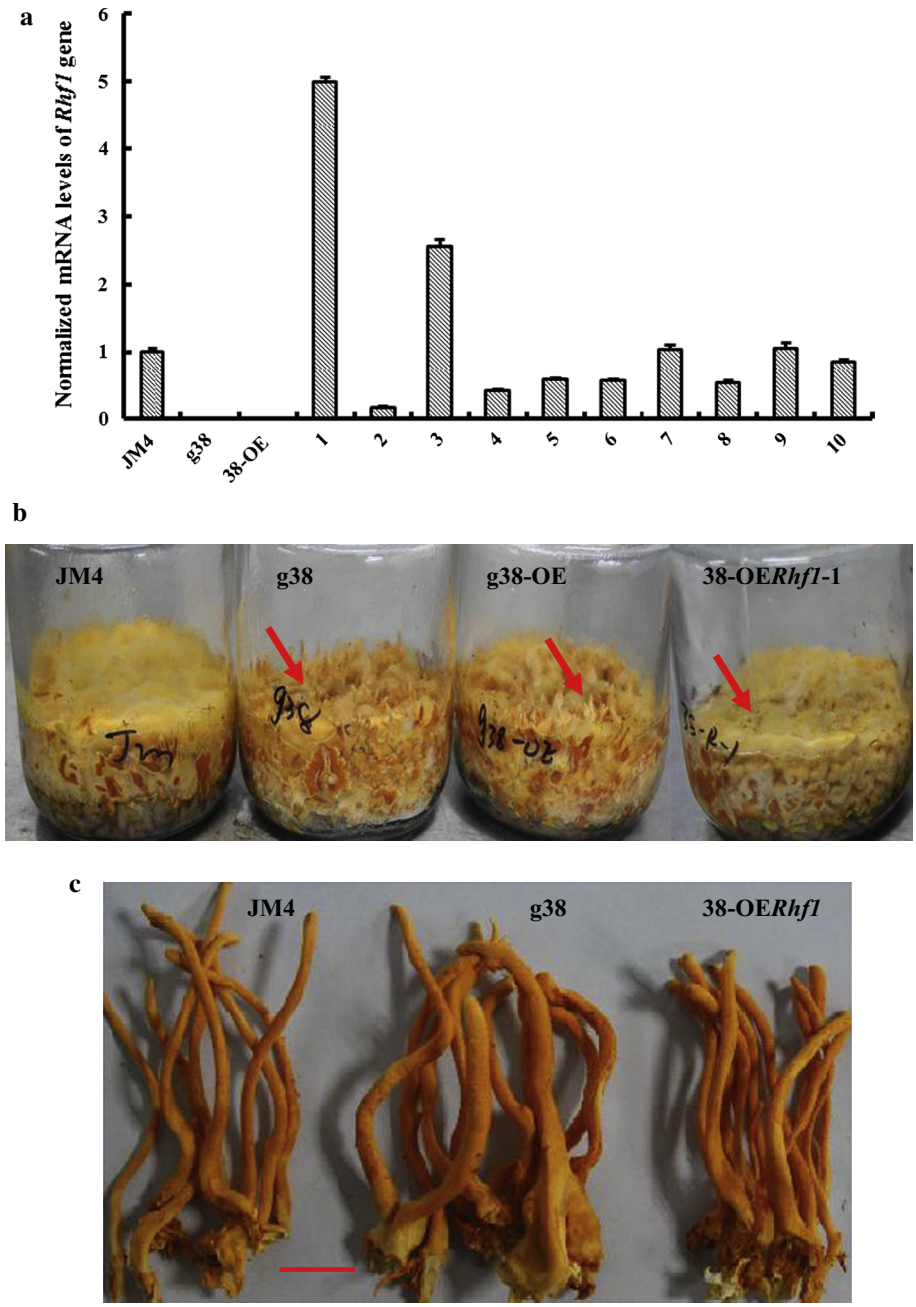

Fig. 6 Complementation of the *Rhf1* gene in mutant g38. **a** Normalized mRNA levels of the *Rhf1* gene in the 38-OE*Rhf1* transformants in liquid PPD cultures at 22–25 °C in a shaking platform (120 rpm) for 7 days. Glyceraldehyde-3-phosphate dehydrogenase (Cm-*gpd*) was used as a reference gene. Data are the mean ± SD. **b** Stromatal differentiation of 38-OE*Rhf1* transformants after 2 weeks in rice medium. *Arrows* denote stromata. **c** Fruiting bodies of 38-OE*Rhf1*, g38 and JM4. *Bar* = 1 cm

[24], as a selection marker. After ligating into the T-DNA, this protein was expressed in the hyphae of *C. militaris* transformants (Fig. 3a). GFP fluorescence was stably expressed after ten continuous batches of the g38 cultures (data not shown). This marker can be used to rapidly screen and to monitor the engineered strains of *C. militaris*.

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Conflict of interest The authors declare that they have no conflict of interest.

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