Characterization of a G protein α subunit encoded gene from the dimorphic fungus-*Tremella fuciformis*

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Abstract  *Tremella fuciformis* is a dimorphic fungus which can undertake the reversible transition between yeast and pseudohypha forms. G protein α subunit (Gα) carries different signals to regulate a variety of biological processes in eukaryotes, including fungal dimorphism. In this study, a novel Gα subunit encoded gene, *TrGpa1*, was firstly cloned from *T. fuciformis*. The *TrGpa1* open reading frame has 1059 nucleotides, and encodes a protein which belongs to the group I of Gα superfamily. Furthermore, the role of *TrGpa1* in the *T. fuciformis* dimorphism was analysed by gene overexpression and knockdown. Stable integration of the target gene into the genome was confirmed by PCR and Southern blot hybridization. Transformants with the highest and lowest *TrGpa1* expression levels were selected via quantitative real-time PCR analysis and Western blot. Each transformant was compared with the wild-type strain about the morphological change under different environmental factors, including pH values, temperature, cultivation time, inoculum size, and quorum-sensing molecules (farnesol and tyrosol). Comparing with the wild-type strain, the overexpression transformant always had higher ratios of pseudohyphae, while the knockdown transformant had less proportions of pseudohyphae. Therefore, the *TrGpa1* is involved in the dimorphism of *T. fuciformis* and plays a positive role in promoting pseudohyphal growth.

Keywords  *Tremella fuciformis* · Dimorphism · G protein α subunit · Gene function · Morphological change

Introduction

The heterotrimeric guanine nucleotide-binding proteins (G proteins), universal signaling proteins in eukaryotes, carry different signals from the receptors to various effectors, then regulate a variety of biological processes (Kang et al. 2011). The G proteins are highly conserved, and consist of Gα, Gβ and Gγ subunits. In the inactive state, these three subunits are tightly associated together. Once the Gα subunit was
activated by G protein-coupled receptors (GPCRs), G protein dissociates to form Ga and Gβ-Gγ dimeric subunits. Each of them can interact with downstream effectors, which subsequently trigger a series of intracellular responses (Perez-Sanchez et al. 2010; Valle-Maldonado et al. 2015).

Fungal dimorphism is an intriguing morphological transition, which undertakes a morphological interconversion between the yeast form and the mycelial/pseudoohypha form (Nickerson and Atkin 2017). This switch promotes the disease progression in the pathogenic filamentous species, which is necessary for the invasion to hosts and the expression of virulence factors (Wilson et al. 2010; Boyce and Andrianopoulos 2015). Lots of researches showed Ga subunits had linkage with morphological transition in dimorphic fungi. For Ustilago maydis, the yeast colonies of capl-defective cells failed to form filamentous colonies, which resulted in a significantly decreased pathogenicity (Takah and Gold 2010). In Sporothrix schenckii, Ga subunits SSG-1 and SSG-2 are involved in the dimorphism and pathogenicity (Pérez-Sánchez et al. 2010; Yemelin et al. 2017). The ΔMgGpa3 mutant of Mycosphaerella graminicola showed more pronounced yeast-like growth accompanied with hampered filamentation, which suppressed the transition from yeast-like form to filamentous form (Orton et al. 2011). As to Mucor circinelloides, the gpa3 expression levels was decreased during the dimorphic transition from mycelium to yeast cell (Valle-Maldonado et al. 2015). In Candida albicans, the Gpa2 played an important role in the yeast-hypha dimorphic transition during the response of C. albicans to some environmental inducers (Wilson et al. 2010). The Δgpa2 mutant strains of Saccharomyces cerevisiae had a defect in pseudohyphal growth, while constitutive overexpression of gpa2 stimulated filamentation of the mutant (Kayikci and Magwene 2018).

Tremella fuciformis, or white jelly mushroom, is a typical dimorphic fungus having the yeast-hypha and yeast-pseudoohypha transition triggered by environmental cues (Hou et al. 2011). Previous studies about the T. fuciformis dimorphism mainly focused on the environmental factors which affected its dimorphism and cell wall polysaccharides changes during dimorphic transition (Zhu et al. 2016). Little is known about the functions of signaling proteins during the dimorphic change of T. fuciformis. In the present study, a Ga subunit gene (TrGpa1) which was cloned and characterized the contributions to the T. fuciformis dimorphism. The gene overexpression and knockdown vectors were constructed to evaluate its roles in the dimorphic transition in response to the environmental inducers.

Materials and methods

Strains and culture conditions

The T. fuciformis haploid yeast-like cell Y32, was maintained in the Laboratory of Food Microbiology, Huazhong Agricultural University, and subcultured on potato dextrose agar (Difco, Detroit, MI, USA) slants. The LM medium (20 g L−1 glucose, 1.32 g L−1 (NH4)2SO₄, 0.25 g L−1 MgSO₄·7H₂O, 0.5 g L−1 KH₂PO₄·3H₂O, 0.2 mg L−1 vitamin B₁, 2 mg L−1 ZnSO₄·7H₂O, and 0.5 g L−1 CaCl₂·2H₂O) was designed for strain culturing. The strains were incubated at 25 °C using an orbital shaker (Fuma, Shanghai, China) at 150 rpm. Escherichia coli DH5α (Takara, Dalian, China) was used as a host for vector cloning and propagation. The Agrobacterium tumefa-

Full-length gene cloning and bioinformatical analysis

Gα subunit gene, named as TrGpa1, and the DNA (GenBank accession no. MH091706) and cDNA (GenBank accession no. MH101517) were acquired in our previous work (data not published). The qRT-PCR analysis of TrGpa1 was performed in the yeast form and mycelial form of T. fuciformis, and results showed TrGpa1 was differentially expressed, which indicates this gene may be involved in the dimorphic changes (data not shown). Total RNA and DNA were extracted from Y32 strain using the RNAiso™ plus (Takara, Dalian, China) and the cetyltrimethylammonium bromide (CTAB) method (Yin et al. 2015), respectively. The TrGpa1 was cloned by PCR with the
specific primers listed on Table 1. The amplification procedures were carried out as follows: an initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C denaturation for 30 s, 60 °C annealing for 30 s, 72 °C elongation for 90 s; and a final extension at 72 °C for 10 min.

Bioinformation analysis of TrGpa1 was performed by following steps. The amino acid sequence was deduced by Translate tool (http://web.expasy.org/translate/). The theoretical isoelectric point and molecular weight were predicted using Compute pI/Mw (http://expasy.org/tools/protparam.html). The conserved domains were identified from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Sequence similarity was analysed using Basic Local Alignment Search Tool (BLAST) at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multi-sequence alignment was generated using ClustalW (http://www.clustal.org/). The phylogenetic tree was constructed using neighbor joining method implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 6 program.

The overexpression and knockdown vectors of TrGpa1 were constructed according to the vector pGEH-GH (Zhu et al. 2017) based on pCAMBIA 1302 backbone (Cambia, Brisbane, Australia). The TrGpa1 amplified using the primers with MluI and AsuII restriction sites were digested and introduced into pGEH-GH to generate the overexpression vector pTrGpa1-OE (Fig. 1a). The knockdown vector (pTrGpa1-hp) was generated by the ligation of a 439 bp fragment (a 325 bp fragment and a 124 bp spacer fragment, flanking MluI and BlII restriction sites) and a 325 bp (flanking BlII and AsuII restriction sites) reverse complementary fragments. The plasmid was expected to encode a hairpin RNA included two 325 bp complementary regions separated by a 124 bp spacer fragment (Fig. 1b). The overexpression and knockdown elements were under control of T. fuciformis endogenous constitutive promoter—glycer-aldehyde-3-phosphate dehydrogenase gene (gpd) promoter.

All vectors were transformed into the A. tumefaciens strain EHA105 component cells. Agrobacterium-mediated transformation of T. fuciformis Y32

### Table 1 Primers for PCR amplification in this work

| Names    | Sequences (5’ → 3’)                          | Descriptions                                      |
|----------|-----------------------------------------------|---------------------------------------------------|
| TrGpa1-F | CATGGGGTGCACACAGTTCG                         | Primers for TrGpa1                                |
| TrGpa1-R | TCAAAAGCAATCCGCCAATTC                    | Primers for TrGpa1 overexpression                 |
| OE-F     | CCTAGGGATGGGTGCACACAG                       | Amplify a sense fragment for the RNAi vector      |
| OE-R     | TTGAATCAAAGCAATTC                           | Amplify an antisense fragment for the RNAi vector |
| F-F      | ACCGCCTAGAAGACGATATGCTC                      | Detects the eGFP and hygromycin expression        |
| F-R      | CCTAGGGATGGGTGCACACAG                       | TrGpa1 primers for qRT-PCR                        |
| R-F      | CCTAGGGATGGGTGCACACAG                       | Tubulin primers for qRT-PCR                       |
| R-R      | TTGAATCAAAGCAATTC                           | Primers for sequencing                            |
| EH-F     | GCAGAAGAAGCGCATCAAGGTG                      |                                                  |
| EH-R     | CAGGCTCTCGTCAACTCCCC                       |                                                  |
| qGpa1-F  | CCGGCTTTTGGCTCTTCTATT                       |                                                  |
| qGpa1-R  | TAGTTGCTCAGCCGCTTGA                        |                                                  |
| tubulin-F | GATGACCATTTCTGCTCCT                    |                                                  |
| tubulin-R | GTTGACATTTCTGCACAG                      |                                                  |
| M13-F    | CGCCAGGGTTTCCAGTCAGGAC                     |                                                  |
| M13-R    | AGGCGATAACATTTCAGAGG                      |                                                  |

The recognition sequences for restriction enzyme are underlined.
cells were performed according to previous work (Zhu et al. 2017). The transformants were subcultured for five rounds on PDSA, then total DNA were extracted. The existence of enhanced green fluorescent protein gene (egfp)-hygromycin B phosphotransferase gene (hph) fusion gene was assessed by PCR using primers EH-F and EH-R. The integration of genes in the genome was analysed by Southern blot with digoxigenin (Roche Diagnostics, Mannheim, Germany) labeled hph. DNA of Y32 was used as the negative control, and plasmid of pGEH-GH was applied as the positive control. Transformants were measured by using a fluorescence microscope (DM 6000 B, Leica Microsystems, Germany) to analyze the egfp expression. The images were captured under 40 × objective and samples were measured with a green fluorescence filter (546 nm).

Gene expression analysis

Total RNA was extracted and reverse transcribed (TransScript® first-strand cDNA synthesis supermix, Transgen, China) according to the manufacturer’s protocol. Quantitative real-time PCR (qRT-PCR) was performed on the ABI ViiA7 Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer’s protocol (Takara), using β-tubulin as the endogenous control. The primer sequences for the qRT-PCR of TrGpa1 and β-tubulin are listed on Table 1. The qRT-PCR conditions were 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, and 60 °C for 30 s. The expression ratios were calculated according to the $2^{-\Delta\Delta C_t}$ method and each qRT-PCR reaction was carried out in triplicate independently. Data were analysed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple range tests using SPSS 26.0 software.

Transformants selected based on the qRT-PCR assays and Y32 were analysed by Western blot. Cells were lysed in buffer containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO, USA). Then total protein content was measured with BCA Protein Assay Kit according to the manufacturer’s protocol, and 40 µg of proteins were separated on 10% SDS polyacrylamide gels and transferred to PVDF membrane (Thermo Fisher Scientific, USA) using a Semi Dry Blotter (Thermo Fisher Scientific, USA) for 1 h at 20 V. After blocking with TBST buffer (5% dry milk powder in tris-buffered saline and 1% v/v Tween 20) for 1 h, the membrane was incubated with the primary rabbit antibodies against TrGpa1 at 4 °C overnight. After 5 washing steps with TBST buffer, blots were incubated with the secondary antibodies: horseradish peroxidase labeled anti-rabbit IgG in the dilution of 1:2000 in 5% milk/TBST at room temperature for 2 h. The membranes were performed using the SuperSignal ECL Solution for Western blot (Willget Biotech, Shanghai, China). Densitometric evaluation was performed with ImageJ software (National Institute of Health, New York, NY).

Phenotypic analysis

The sub-cultured cells in LM medium were aseptically collected by centrifugation at 5000 × g for 5 min, washed three times and distributed in 50 mL of medium to obtain final concentration of $10^5$
cells·mL⁻¹. Except the given situations, the 50 mL of LM medium containing cells were incubated at 25 °C on an orbital shaker at pH 7 for 3 to 5 d. The pH of the medium was adjusted by the addition of dibasic phosphate-citric acid buffer to the desired pH value.

Transformants and Y32 were cultured under different conditions to test the morphological changes in transformants and Y32. 20 °C, 25 °C, 28 °C, 30 °C, 37 °C were chosen as the temperature parameters and 3, 4, 5, 6, 7, 8 as the pH parameters. The cells were incubated for 2 to 9 d for the culture time parameters. Each LM medium containing different concentrations of cells (10³, 10⁴, 10⁵, 10⁶, and 10⁷ cells·mL⁻¹) was cultured respectively. Quorum sensing molecules (QSMs) including farnesol and tyrosol (Sigma, USA) in different concentrations (5, 25, 50, 100, 200 µmol·L⁻¹) were also prepared to observe their effects on morphological changes. Strains supplemented with 1% methanol were the controls for each assay. All experiments were performed in triplicate of each treatment. Data were analysed by ANOVA, followed by Ducan’s multiple range tests.

Samples were observed at 20 × objective by an optical microscope (Leica, Germany). Three or more cells connected at the end of the long axes or in a definite direction or an elongated cell with a daughter cell and an ellipsoidal cell having two branched daughter cells were counted as a pseudohypha. Only differentiated cells were quantified and normalized to 100% (yeast/pseudohypha cells). For each repetition, at least 300 cells were counted under the microscopy.

**Results**

The bioinformation analysis of *TrGpa1*

The DNA sequence of *TrGpa1* is 1436 bp and contains eight introns of 53, 61, 41, 40, 45, 47, 46, and 44 nucleotides, respectively (data not shown). The 5’ and 3’ borders of the eight introns showed the same splicing sites (GT-AG) which are the common sequences for introns of filamentous fungi (Yin et al. 2015). The CDs encoded a protein of 352 amino acid residues. The calculated theoretical isoelectric point of *TrGpa1* was 5.55, and the molecular weight was 40.18 kDa. The conserved domain analysis of the amino acid sequence revealed that *TrGpa1* contained the GTPase domain (G1-G5), an ATP/GTP binding regions (G/AXXXXGKT/S), and the βγ complex interaction site (data not shown).

Multiple sequence alignment of the predicted amino acid sequence of *TrGpa1* with eight known fungal Gα subunits available in NCBI database was performed by ClustalW program (http://clustal.ddbj.nig.ac.jp/). The results revealed that *TrGpa1* had highly identities with other homologues from *Kwoniella heveanensis* (89%, OCF36875.1), *Cryptococcus gattii* (86%, XP_003191999.1), *C. neoformans* (86%, XP_003191999.1), *Schizophyllum commune* (66%, XP_003029155.1), *Laccaria bicolor* (65%, XP_001888946.1), *Lentinula edodes* (65%, AAP13579.1), *Hypsizygus marmoreus* (65%, KYQ31721.1) (Fig. 2).

A phylogenetic tree was constructed based on the multiple sequence alignments with the Gα subunits from other species (Fig. 3). The Gα proteins were divided into four groups according to their evolutionary relationships. It revealed that *TrGpa1* belongs to the group I of mammalian Gα superfamily. The amino acid sequence of *TrGpa1* contained the consensus myristoylation site, indicated as MGXXXS at the N terminus, but did not present a consensus CXXX sequence (pertussis toxin-catalyzed ADP-ribosylation site) at the C terminus (Fig. 2).

**Stability test of transformants**

Single colonies of *T. fuciformis* transformants were selected randomly and sub-cultured in PDSA for five rounds. To confirm the integration of the overexpression and knockdown fragments, the DNA was extracted from 12 randomly selected transformants, and Y32. The positive amplification of a 500 bp DNA product, suggesting that the egfp-hph fusion gene had been transferred into these transformants (Fig. 4a). Southern blot analysis performed in 10 PCR positive transformants showed that all transformants except one appeared to have copies of the hph gene at random sites, but Y32 showed no hybridization (Fig. 4b). Fluorescence microscopy showed eGFP expressed successfully in the individual transformants (Fig. 5).

**Gene expression analysis of transformants**

The qRT-PCR assays were used to test the mRNA accumulation of *TrGpa1*. For the overexpression transformants (Fig. 6a), the expression level increased...
from 1.5 to 2.5 folds, and the transformant with the highest relative mRNA level was selected and subcultured. In addition, the gene suppression ratio ranged from 52.10 to 68.26% among the knockdown transformants (Fig. 6a). Thus, the transformant having the highest gene suppression ratio was chosen. Then Western blot was subsequently undertaken in Y32 and transformants with the maximum and minimum expression level. As shown in Fig. 6b, TrGpa1 in the overexpression transformants was expressed at a higher level than Y32, whereas at a lower level in the knockdown transformants, which is in accordance with the qRT-PCR analysis.

TrGpa1 contributes to *T. fuciformis* dimorphism

The dimorphic-related functions of *TrGpa1* were characterized. It was shown that the *TrGpa1* was
required for pseudohyphal differentiation of *T. fuciformis*. When the environmental conditions change, the transition from yeast to pseudohypha in Y32, the overexpression and knockdown transformants were influenced by the environmental factors, including pH, temperature, inoculum size, culture time, farnesol and tyrosol concentration (Fig. 7). Comparing with Y32, the overexpression transformant always had higher ratios of pseudohyphae, and the knockdown transformant always had less proportions of pseudohyphae (Fig. 7).

**Discussion**

The dimorphism is a reversible transition and depends upon the environment to which the fungi are exposed (Wang et al. 2020). *T. fuciformis*, an edible jelly mushroom, has the capacity to perform this type of morphogenesis (Zhu et al. 2016). It has been demonstrated that G proteins are key regulators of this morphological transition in many dimorphic fungi (Park et al. 2020). However, little is known about the G proteins of *T. fuciformis* and their functions.

In this work, we have cloned the *TrGpa1*, a gene encoding a Gα subunit from *T. fuciformis*. According to the phylogenetic tree, *TrGpa1*, encoded by *TrGpa1*, belongs to the group I of Gα subfamily containing the characteristic sequence sites (Fig. 3). The site for
myristoylation at the N terminus is important to attachment to the GPCRs in membrane (Li et al. 2019). The amino acid sequence of TrGpa1 did not contain the conserved pertussis toxin site at the C terminus. ADP ribosylation of the Gαi subunits locks the activity of G proteins and prevents the activation by GPCRs (Appleton et al. 2014).

Furthermore, the overexpression and knockdown vectors were constructed for identifying the function of TrGpa1. Since the gene knockout methods are lacking in T. fuciformis, RNA interference (RNAi) was performed for identifying the function of TrGpa1 (Wang et al. 2017). Though it causes only partial gene silencing, RNAi technology provides variable rates of gene suppression transformants. Therefore, it makes it possible to investigate the effects of genes on the phenotypes of interest and the minimum effective inhibition rate. For example, in Magnaporthe oryzae, only a slight decrease in the expression of some calcium signaling related genes caused a complete loss of infection-related morphogenesis and pathogenicity (Lange and Peiter 2020). Nevertheless, strong knockdown of hydrophobin gene Mpg1 did not severely

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**Fig. 5** Detection for fluorescence under bright light (a overexpression transformant; c knockdown transformant) and corresponding UV light (b overexpression transformant; d knockdown transformant). Images were taken with 40 × fields of view, bar = 10 μm

**Fig. 6** Gene expression analysis of transformants. a Relative mRNA accumulation levels of TrGpa1 in overexpression (2, 3, 4) and knockdown transformants (5, 6, 7) and Y32 (1). Data were presented as mean values of three replicates with the corresponding standard deviations. b Western blot analysis of Y32 (Lane 1), the overexpression transformant (Lane 2), and the knockdown transformant (Lane 3)
affect its pathogenicity, despite the fact that a knock-
out of this gene presented a drastic reduction in
pathogenicity (Han 2018). Thus, the impacts on the
phenotypes would differ among different genes. Here
we only chose the transformant with the highest or
lowest mRNA accumulation level. The relationship of
gene suppression rate and the dimorphic phenotypes is
worthy to be discussed in our future work.

The characterization of dimorphic related functions
indicated that TrGpa1 played a positive role in the
promotion of pseudohyphal growth. The overexpres-
sion of TrGpa1 enhances the response to different
conditions and promotes the pseudohyphal formation.
Cells lacking TrGpa1 have a defect in pseudohyphal
development in response to specific environmental
cues. For dimorphic fungi, cell morphology is depend-
ing on the inoculation size. There is a general

![Diagram](image_url)
phenomenon for all dimorphic fungi (Wedge et al. 2016), that is when inoculation at $\geq 10^6$ cells mL$^{-1}$, budding yeasts are produced, while pseudohyphae and mycelia are produced following inoculation at $< 10^6$ cells mL$^{-1}$. In the present study, interestingly, the overexpression transformant had high ratios of pseudohyphae, even when the inoculum size was larger than $10^6$ cells mL$^{-1}$. In addition to the inoculation size, extracellular QSMs contribute to T. fuciformis dimorphism. In C. albicans, farnesol is a characterized QSM, which suppresses filamentous formation. Tyr- osol, another QSM produced by C. albicans, stimulates the yeast-to-hypha conversion (Han et al. 2011; Monteiro et al. 2017). In Y32 and the transformants, farnesol and tyrosol played the same roles. Farnesol effectively blocked the transition from yeast to pseudohypha at the concentration of 50 $\mu$mol·L$^{-1}$, while tyrosol stimulated this transition. The pseudohyphae ratios in knockdown transformant were largely decreased with the addition of farnesol, while the pseudohyphae ratios in the overexpression transformant were slightly decreased, which shows the overexpression transformant was less sensitive to the QSMs (Fig. 7).

However, it is still unclear in which signal pathways TrGpa1 is involved. Former studies showed many G$\alpha$ subunits are involved in the cAMP-protein kinase A (PKA) and the mitogen-activated protein kinase (MAPK) pathways (Nogueira et al. 2015; Shwab et al. 2017; Martínez-Soto et al. 2020). Here the exogenous addition of cAMP (10 mmol·L$^{-1}$) had no apparent influences on the yeast-pseudohypha transition, which indicates TrGpa1 may not be involved in the cAMP/PKA pathway (data not shown). Other reagent including dibutyryl cAMP and MAPK inhibitors should be further performed in investigating the TrGpa1 related pathways.

In conclusion, TrGpa1, a gene encoding a G protein $\alpha$ subunit was cloned from the dimorphic fungus T. fuciformis. The TrGpa1, encoded by TrGpa1, belongs to the group I of G$\alpha_i$ superfamily. The function of TrGpa1 was characterized by gene overexpression and knockdown. The results have demonstrated that TrGpa1 is involved in T. fuciformis dimorphism and supports a positive role in the transition from yeast to pseudohyphal growth under different environmental conditions. In our future study, the pathways wherein TrGpa1 is involved and the relationship of TrGpa1 suppression rate and dimorphic phenotypes need to be further investigated.

Author contributions HZ and AM designed the study; LZ and LC collected the samples; HZ and DL performed the laboratory work; HZ performed the data analysis and wrote the manuscript; AM, LZ, and LC reviewed and revised the writing.

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Availability of data and materials The DNA and cDNA sequences of TrGpa1 can be downloaded from the National Center for Biotechnology Information (NCBI), and the GenBank accession numbers are MH091706 and MH101517.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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

Consent for publication All authors read and approved the final version of the manuscript.

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