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MoTlg2, a t-SNARE component is important for formation of the Spitzenkörper and polar deposition of chitin in *Magnaporthe oryzae*

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ABSTRACT

T-SNAREs are a family of conserved proteins involved in intracellular transport of membrane-coated cargo among subcellular compartments. In this study, we identified a putative t-SNARE gene, *MoTLG2*, in *Magnaporthe oryzae* via insertion mutagenesis. Deletion of *MoTLG2* resulted in slower vegetative growth and less conidiation relative to the wild-type strain, but the *ΔMotlg2* null mutant was as virulent as the wild-type strain. MoTlg2 has 30% overall amino acid identity with *Saccharomyces cerevisiae* Tlg2, and rescued the defect of monensin de-sensitivity in the yeast strain where *TLG2* had been deleted. More importantly, apical regions of the hyphae of the *ΔMotlg2* null mutant were only weakly stained by FM4-64, which was reported as an excellent vesicle tracer, suggesting that the Spitzenkörper was not well formed in the *ΔMotlg2* null mutant. In addition, more uneven lateral deposition of chitin was observed in the cell wall of vegetative hyphae of the *ΔMotlg2* null mutant. Taken together, this study shows that the t-SNARE Tlg2 is important for both vegetative hyphal growth and conidiation, but dispensable for plant infection in filamentous fungi, and suggests that Tlg2 is important for formation of the Spitzenkörper and polar distribution of chitin.

Introduction

SNAREs are a large protein superfamily that mediates membrane fusion in almost all trafficking steps of the secretory and endocytic pathways [1]. Based on localization in 'donor' or 'acceptor' compartments, SNAREs can be divided into two major subgroups, v-SNAREs (vesicle-membrane SNAREs) that are associated with vesicles, and t-SNAREs (target-membrane SNAREs) that are associated with target compartments [2]. v-SNAREs usually consist of a tail-anchored SNARE having one SNARE motif, and t-SNAREs usually have additional two or three polypeptides [3]. A heterodimeric t-SNARE is usually comprised of a member of the syntaxin subfamily that contributes one SNARE motif as the t-SNARE heavy chain, and a member of the SNAP-25 subfamily that contributes two SNARE motifs as two t-SNARE light chains. When membrane fusion occurs, interactions between v-SNARE and t-SNARE lead to the vesicle and target compartment joining together and forming a *trans*-SNARE complex called SNAREpin, where the four twisted parallel SNARE motifs bundle together. Formation of this bundle is thought to provide energy to catalyze the fusion of vesicles within the target membranes [1,4–7].

Hyphal tip growth is a major distinguishing feature of filamentous fungi. There are a number of processes that are well coordinated to create the tapered morphology of hyphal tips. Among them are highly polarized apical synthesis and secretion, which require the SNAREs to cause the fusion of vesicles carrying cell wall components [8]. The Spitzenkörper, a structure commonly detected in growing hyphal apices and marked with an aggregate of vesicles, is supposed to act as a vesicle supply center for generating the exocytosis gradient in filamentous fungi [9]. In *Aspergillus oryzae*, SNAREs destined to be recycled to the tip by endocytosis are mostly internalized from the subapical tip region of plasma membranes to the endocytic recycling compartment, and thereafter are transported to the Spitzenkörper to complete the recycling of certain components required for hyphal tip growth [10]. However, not all

Abbreviations: ATMT, *Agrobacterium tumefaciens* mediated-transformation; BIC, biotrophic interfacial complex; bp, base pair(s); CFW, calcofluor white; CM, complete media; HAP, high affinity purification; HBSS, Hanks' balanced salt solution; *hph*, hygromycin phosphotransferase gene; kb, kilobase pair(s); ORF, open reading frame; OTA, oatmeal tomato agar; SC, synthetic complete media; SNARE, soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptors; TAIL-PCR, thermal asymmetric inter-laced polymerase chain reaction; UDM, uracil-deficient media.

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tip-growing cells contain the Spitzenkörper in their apices [11], and the relationship between the Spitzenkörper and SNAREs is still not clear.

Over the past decades, SNAREs have been widely studied in different eukaryotic organisms, and more than 60 SNAREs in yeast and mammalian cells and 21 putative SNAREs in *A. oryzae* have been characterized [1,7,12,13]. In the pathogenic basidiomycetous fungus *Ustilago maydis*, a t-SNARE Yup1, has been found to mediate endocytic recycling through early endosomes, and is required for hyphal morphogenesis and pathogenesis [14,15]. Although increasing numbers of studies focus on SNAREs, the role of these proteins in asexual development and pathogenesis in filamentous fungi remains to be investigated.

The haploid ascomycetous fungus *Magnaporthe oryzae* is the causal agent of rice blast that occurs throughout the world and has been developed as a model system to investigate interactions between pathogens and host plants [16–19]. Whole genome sequencing revealed multiple SNAREs in the *M. oryzae* genome. Two SNARE genes from *M. oryzae*, *MoSEC22* and *MoVAM7*, played important roles in growth, differentiation, and virulence [20,21]. Another t-SNARE gene *MoSSO1* was also recently reported to be required for full virulence and normal development of the BIC for accumulation of effectors [22]. However, the roles of other putative SNARE genes in *M. oryzae* have not been reported. In this study, we identified a t-SNARE gene *MoTLG2* from *M. oryzae*. This gene was important for vegetative hyphal growth and conidiation in *M. oryzae* and could substitute for *TLG2* to restore monensin resistance in deletion mutants of *Saccharomyces cerevisiae*. In addition, we found that deletion of *MoTLG2* from *M. oryzae* affected formation of the Spitzenkörper and distribution of chitin among hyphal compartments.

Results

Identification of MoTLG2 from a hyphal growth defective mutant

SX1548, a mutant defective in hyphal growth was isolated from an ATMT mutant library of the wild-type strain P131 that was generated in a previous study [23]. The colony size of this mutant was 88% of the wild type (Table 1), and the colony margin of the mutant was more compact (Fig. 1A). A crossing was made between this mutant (*MAT* 1-1) and a wild-type strain S1528 (*MAT* 1-2). From the crossing, 39 progeny were obtained, including 21 hygromycin-resistant progeny that grew as slowly as the mutant parent, and 18 hygromycin-sensitive progeny that formed wild-type colonies. These results suggested that the mutant had a single T-DNA insertion and indicated that the phenotypic change in the mutant co-segregated with the hygromycin-resistance marker. DNA gel blot analysis of the genomic DNA from SX1548 confirmed that a single copy of T-DNA was integrated into the genome of the mutant (Fig. 1B).

Through TAIL-PCR, the sequences flanking the integrated T-DNA were obtained from SX1548. Sequence analysis revealed that the flanking sequences of the integrated T-DNA were continuous, and the T-DNA was integrated at 550 bp upstream of the translation start site of the predicted gene MGG_06883.6 (named as *MoTLG2* in this study) (Fig. 1C). In the genome of the wild-type P131, it has one copy of *MoTLG2* by DNA gel blot analysis (Fig. S1). The full-length cDNA of *MoTLG2* was isolated, and it contains one 72 bp intron compared to the genomic DNA (Fig. 1C).

MoTLG2 encodes a protein with 357 amino acid residues, which is similar to syntaxin that is thought to facilitate t-SNARE complex formation and mediate fusion of endosome-derived vesicles with the late Golgi [24]. The N-terminus of MoTlg2 was predicted to contain a syntaxin homologous domain and an SNARE domain (known as H3), and an N-terminal regulatory domain (Habc). The C-terminus of MoTlg2 contained an SNARE motif, and the central position (0-layer) of the heptad repeats of the SNARE motif was a glutamine (Gln) residue, indicating that MoTlg2 belongs to the Q-SNARE superfamily [25]. The homologs for MoTlg2 are widely distributed among eukaryotic organisms, including fungi, plants, and animals. A phylogenetic tree was constructed based on MoTlg2 and its homologous proteins (Fig. 2).

To confirm that the phenotype defects in SX1548 were due to disruption of *MoTLG2*, a gene complementation vector pHBP10 containing *MoTLG2* and its 2.5-kb upstream sequence was constructed and introduced into the mutant. All the resulting 25 neomycin-resistant transformants, including TMT4, showed the wild-type colony phenotype (Table 1).

MoTLG2 is important for vegetative hyphal growth and conidiation

To further characterize *MoTLG2*, a gene replacement vector pYZ1 was constructed by replacing the ORF of *MoTLG2* with the hygromycin cassette (Fig. 3A). After linearization by *Not*I, pYZ1 was introduced into wild-type P131. One transformant, DMT1 was confirmed as the *ΔMotlg2* null mutant by PCR with primer pair Z9/Z10 and Z11/Z12 (data not shown) and by DNA gel blot (Fig. 3B). Loss of *MoTLG2* transcript was further confirmed by RT-PCR with the primer pair in1/in2 (Fig. 3C).

Similar to the ATMT mutant SX1548, the *ΔMotlg2* null mutant DMT1 also grew slower. After culture on OTA at 28 °C for 5 days, the colony diameter of DMT1 was 34 mm. Under the same conditions, the wild-type strain P131 grew significantly larger with a 40-mm-diameter colony (Fig. 3D; Table 1). Moreover, the margin of the mutant DMT1 colony was more compact and clearer, and the mycelium was darker (Fig. 3D) than the wild type. While the wild type produced 8×10^7 conidia per plate after 4 days, the *ΔMotlg2*

Table 1
The diameter of colonies (mm) grown on different media for 5 days.

Strains	OTA[a]	CM[b]	CM + monensin[c]	CM + CFW[d]	CM + SDS[e]	CM + CR[f]
P131	40.0 ± 0	38.8 ± 0.5	22.0 ± 0.9	35.3 ± 0.2	21.3 ± 0.6	36.7 ± 0.9
SX1548	32.5 ± 0.5	–[g]	–	–	–	–
TMT4	40.0 ± 0.4	–	–	–	–	–
DMT1	31.0 ± 0.9	29.0 ± 0.5	14.3 ± 0.5	21.7 ± 0.4	12.7 ± 0.6	27.7 ± 0.5
CMT1	40.0 ± 0.3	38.9 ± 0.5	21.7 ± 0.9	35.0 ± 0.5	21.0 ± 0.7	36.5 ± 0.8

[a] OTA = oatmeal tomato media.
[b] CM = complete media.
[c] CM + monensin = complete media with 50 μM monensin.
[d] CM + CFW = complete media with 200 mg/ml calcofluor white.
[e] CM + SDS = complete media with 0.01% sodium dodecyl sulfate.
[f] CM + CR = complete media with 200 mg/ml Cong Red.
[g] The dash "–" represents not tested. The means and standard deviations were calculated from three experiments with four replicates per experiment.