DOI: 10.11844/cjcb.2013.08.0128

MoTCTP, A Homolog of Translationally Controlled Tumor Protein, Is Required for Fungal Growth and Conidiation in *Magnaporthe oryzae*

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Abstract Translationally controlled tumor protein (TCTP) is a multifunctional protein abundantly expressed in multicellular organisms. It plays important roles in lots of biological processes, such as cell growth, differentiation and gene expression in animal and plant. In this report, we described the expression, distribution, and function of MoTCTP, a homolog of human TCTP, in the rice blast fungus *Magnaporthe oryzae*. MoTCTP is highly expressed in hyphae, conidia and appressoria. MoTCTP is localized to cytoplasm, mitochondrial outer membrane and cytoplasm nearby vacuoles. Knockout of *MoTCTP* led to reduction in mycelial growth, decrease in conidial production and delay in conidial germination in the fungus, but increase in resistance to hydrogen peroxide stress. The expression of six genes encoding cyclins and cell division control proteins and three genes encoding known conidiogenesis related proteins were downregulated in *MoTCTP* null mutants, but three genes encoding cyclin-dependent kinases were upregulated among fifteen analyzed genes. These results demonstrated the biological function of *MoTCTP* in the control of fungal development and in the response to cellular stresses in rice blast fungus and suggested that cell cycle regulation proteins might be involved in these process regulated by *MoTCTP*.

Key words TCTP; growth; conidiation; *Magnaporthe oryzae*; fungus; cell cycle

稻瘟病菌翻译调节肿瘤蛋白(MoTCTP)参与真菌生长和产孢的调控

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摘要 翻译调节肿瘤蛋白(TCTP)是一种在多细胞生物中高度表达的多功能蛋白, 在动植物的很多生理过程中具有重要的作用: 如细胞生长、分化和基因表达等。该文报道了稻瘟病菌*MoTCTP*基因(人TCTP的同源蛋白基因)在菌丝、孢子和附着胞等不同发育阶段的表达, 在细胞内的分布以及在稻瘟病菌发育过程中的作用。MoTCTP蛋白分布于细胞质、线粒体外膜表面以及液泡周围的细胞质中。敲除*MoTCTP*基因后, 稻瘟病菌的生长减慢、产孢减少、孢子萌发延迟, 但对氧化压力的抗性增加。定量PCR分析了12个细胞分裂周期蛋白和3个产孢相关蛋白在*MoTCTP*敲除

收稿日期: 2013-04-23 接受日期: 2013-05-27
国家自然科学基金(批准号: 30971879)资助的课题
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Received: April 23, 2013 Accepted: May 27, 2013
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网络出版时间: 2013-07-25 14:01 URL: http://www.cnki.net/kcms/detail/31.2035.Q.20130725.1401.001.html

突变体中的表达量变化, 发现6个细胞分裂周期蛋白和3个产孢相关蛋白的表达量下降、3个细胞分裂周期蛋白的表达量上升。这些结果说明, *MoTCTP*基因在稻瘟病菌发育和抗胁迫过程中具有重要的功能, 并且这些功能可能与细胞周期蛋白的表达调控有关。

关键词 翻译调节肿瘤蛋白; 生长; 产孢; 稻瘟病菌; 细胞周期蛋白

Translationally controlled tumor protein (TCTP) is an evolutionarily highly conserved protein and initially identified as a factor involved in cell growth from human tumor cells[1-2]. Subsequent researches in animal and human cells reveals that TCTP is a multifunctional protein, which functions in cell growth, development, differentiation, apoptosis, cellular stress responses, and a lot of cell physiological events in cancer by regulating cell cycle, expression regulation, protein synthesis regulation and cytoskeleton networks[2-8]. In plants, TCTP is associated with long-distance movement of phloem proteins[9] and cell division in the root cap[10]. *Arabidopsis thaliana* AtTCTP regulates mitotic growth positively through affecting the duration time of the cell cycle[11]. And silencing of TCTP leads to slowed vegetative growth, reduced lateral root formation and impaired root hair development[12].

In yeast, TCTP (TMA19 or MMI1) binds to ribosomes[13], and localizes to the cytoplasm and relocates to the mitochondrial outer surface upon mild oxidative stress, replicative ageing or mutation of cdc48[14]. Deletion of *MMI1* increases the resistance to hydrogen peroxide stress, and confers sensitivity to benomyl in *Saccharomyces cerevisiae*[14]. MMI1 might interact with microtubules and mitochondria for correct localization of mitochondria during cell division[14]. The protein structure of TCTPs in *Saccharomyces pombe* and human shows that TCTPs contain α helix domains and β sheet domains, which bind to microtubules and calcium ions[1,15-16], Rab GTPase[17], polo kinase[18], Na^+/K^+ ATPase[19] and MCL/BCL-xL[20].

Little was known about TCTP protein's function in filamentous fungi. Only two reports were published recently. They were about *Cladosporium herbarum* and *Alternaria alternata* TCTPs, respectively, which were cross-reactive fungal allergens[21-22]. However, the functions of TCTPs in filamentous fungi are still unclear. Here we showed that *MoTCTP* was abundantly expressed in mycelia, conidia and appressoria in *Magnaporthe oryzae*, and filamentous fungal TCTPs shared high sequence identity with each other as well as with human TCTP. The deletion of *MoTCTP* led to the changes of cell cycle-related genes and conidiogenesis-related genes at mRNA expression level in mycelia, which resulted in the defects in fungal growth, conidiation and germination consequently.

1 Materials and Methods

1.1 Fungal strains, media and culture conditions

M. oryzae strain Guy11 was used as a wild type in this study. The wild type and its *MoTCTP* null mutants or ectopic transformants were grown at 25 °C under a 14-h light and 10-h dark cycle using fluorescent lights on complete medium (CM)[23] or under dark on defined complex medium (yeast nitrogen base without amino acids, 1.7 g/L; asparagine, 2 g/L; NH_4NO_3, 1 g/L; glucose, 10 g/L; pH to 6.0 with Na_2HPO_4).

1.2 Nucleic acid manipulation and Real-time PCR

Genomic DNA was isolated from mycelia with 2% CTAB solution and total RNA was isolated from mycelia, conidia or appressoria with Trizol reagent using a standard method[24-25]. Restriction enzyme digestion, agarose gel separation, PCR, RT-PCR and Southern blot were performed following standard procedures[26]. RT-qPCR was conducted following our previous report[24]. The primer sets of PCR for *MoTCTP* DNA manipulation and RT-qPCR for *MoTCTP*, cyclin genes (*CYC1*, *CYC2*, *MoCCNK*, *MoCLB3*, *MoCTK2*, *MoPCL1*, *MoCCL1* and *MoCCNL2*), cell division control protein genes (*MoCDC14*, *MoCDC15*, *MoCDC28* and *MoCDC42*), conidiogenesis related genes (*CON7*, *MoATG5*, *MoTEA4*) and *β-tubulin* were listed in Table 1 and described in the text.

Table 1 Primers used in this experiment

Primer	Sequence(5′→3′)	Usage
upF	TCT CGA GAA GGG GTG GCA AAT CCG ACA T	MoTCTP, PCR
upR	TGT CGA CTG CCC CGC CAC CAG TTG CTA C	MoTCTP, PCR
dnF	TGA TAT CAC ATT TGG GTC TCG GGC ATC TG	MoTCTP, PCR
dnR	TGG ATC CAC GGC AAA CGG GCT CAA CGA AAA G	MoTCTP, PCR
CKF	CTT GGC ATT CGT TGG TTT GTA G	MoTCTP, PCR
CKR	TTT CCT TCC GTG TGG GTA TAT G	MoTCTP, PCR
HBF	TAG AAT TCT TGT ATA GGG CGC CGT AAG CGT AA	MoTCTP, PCR
HBR	TAC TGC AGT ACG GCA TTG CAG ACA AGG TCT CT	MoTCTP, PCR
TCTP4F	TTC TAG AAT GCT TAT TTT CAA GGA TAT TGT	MoTCTP, PCR
TCTP4R	TTC TAG AGC ACT TCA TCT CGG TAA GAC CAT	MoTCTP, PCR
MoTCTP-RTF	ATG CTT ATT TTC AAG GAT ATT G	MoTCTP, RT-PCR
MoTCTP-RTR	TTA GCA CTT CAT CTC GGT AAG	MoTCTP, RT-PCR
Tubulin-RTF	TTC CGC CTG TCA CCG TTC C	β-tubulin, RT-PCR
Tubulin-RTR	GGG CCT CCT CCT CGT ACT CCT CTT	β-tubulin, RT-PCR
qTubulin-F	ATT GTT CAC CTT CAG ACC GG	β-tubulin, RT-qPCR
qTubulin-R	TTG AAG TAG ACG CTC ATA CGC	β-tubulin, RT-qPCR
MoTCTP-qRTF	CTG CTC AAC TAC AGG GAA GAT G	MoTCTP, RT-qPCR
MoTCTP-qRTR	ATG CCC GAG ACC CAA ATG	MoTCTP, RT-qPCR
MoCTK2-qRTF	CTA CGA CTC CAG ATG ACA AGA TG	MoCTK2, RT-qPCR
MoCTK2-qRTR	GAT CAA GTA CTT TTG CGG GTG	MoCTK2, RT-qPCR
MoCDC14-qRTF	AGC AAG ATC CTC TTT ATG CGG	MOCDC14, RT-qPCR
MoCDC14-qRTR	CAA ATG TGC GAG TGT TCT GG	MOCDC14, RT-qPCR
qCDC42f	AAG AAC TCG GTG CGG TTA AG	CDC42, RT-qPCR
qCDC42r	TTG GAT TTC CTC TTG GGT GTC	CDC42, RT-qPCR
qCDC15f	CCT CGG ACT GCC TAA ACA TC	CDC15, RT-qPCR
qCDC15r	GAG AAC CTG CGT CAT ATA CAC C	CDC15, RT-qPCR
CYC1-qRTF	AGC CCG TTG TAA ACC TCA TG	CYC1, RT-qPCR
CYC1-qRTR	GGT CAA TGC CAA AGA GAT AAC C	CYC1, RT-qPCR
CYC2-qRTF	GAT TCT TGA GAA GGG TGA CTG G	CYC2, RT-qPCR
CYC2-qRTR	CTT GAT GGT GCT TCT CGG G	CYC2, RT-qPCR
MoPCL1-qRTF	GTA GTC TGT TCC AAC GTC CAG	MOPCL1, RT-qPCR
MoPCL1-qRTR	ACT TAG CGG CCA GAA TCA AAG	MOPCL1, RT-qPCR
MoCCNK-qRTF	CAA ATA ACA TTA TGG GTC GCC G	MoCCNK, RT-qPCR
MoCCNK-qRTR	CAA GAA TAA AGA GGT TGC AGC G	MoCCNK, RT-qPCR
MoCCL1-qRTF	CAG CAC CAT GAT CAA AAC CG	MoCCL1, RT-qPCR
MoCCL1-qRTR	CTC GCA AAG CAG AAA CTC G	MoCCL1, RT-qPCR
MoCLB3-qRTF	TCG GGA ATG AGC TTT CGC	MoCLB3, RT-qPCR
MoCLB3-qRTR	ATG AGG TAG GGA CGC ATA AAC	MoCLB3, RT-qPCR
MoCDC28-qRTF	CCC GGA GAC TCT GAA ATT GAT G	MoCDC28, RT-qPCR
MoCDC28-qRTR	GGC TTG AAG TCA GGG TAG ATG	MoCDC28, RT-qPCR
MoCCNL2-qRTF	TCG ATA CTG GCT TGT GGA TTC	MOCCNL2, RT-qPCR
MoCCNL2-qRTR	GGA AAC TGG AGA AAG GAG GTA G	MOCCNL2, RT-qPCR
MoATG5-qRTF	GGG TCT TTT AGG GTC ATG CAG	MoATG5, RT-qPCR
MoATG5-qRTR	AGC AAG TCA CGT AGT GTT GG	MoATG5, RT-qPCR
MoTEA4-qRTF	AGC ACA TTG AGA CAC CCA C	MoTEA4, RT-qPCR
MoTEA4-qRTR	GGC TAC AGT CTT GGT CTT CC	MoTEA4, RT-qPCR
CON7-qRTF	ACC ATT TAA ACG CGC ATG TG	CON7, RT-qPCR
CON7-qRTR	CGT TCC TCG TCT GCC TTG	CON7, RT-qPCR

1.3 Vector construction and fungal transformation

A gene deletion vector of *MoTCTP* was built according to a previously reported method[24]. A 0.9 Kb downstream flanking sequence fragment and an 1.0 Kb upstream flanking sequence fragment of *MoTCTP*, obtained by PCR using genomic DNA as a template with primer sets upF/upR and dnF/dnR (Table 1), were inserted into the *Xho* I/*Sal* I sites and *Eco*R V/*Bam*H I sites of pBS-HPH1 vector[24] successively. Then, the *MoTCTP* gene deletion construct containing a hygromycin resistance gene cassette (*HPH*) was cut and inserted into the *Xho* I/*Bam*H I sites of pCAMBIA1300 vector. The pCAMBIA1300 vector was transformed into *Agrobacterium tumefaciens* strain AGL1 and then transformed into *M. oryzae* using an *Agrobacterium tumefaciens*-mediated transformation (ATMT) method according to a previous report[27]. The transformants were initially screened on selective CM media containing 200 μg/mL hygromycin and then confirmed by PCR using primer set CKF/CKR (Table 1). Knockout mutants were purified by mono-conidial isolation and confirmed by Southern blot.

For complementation assay, 3.4 Kb DNA fragment covering *MoTCTP* gene amplified from genomic DNA was inserted into *Eco*R I/*Pst* I sites of pKD5 vector containing sulfonylurea resistance gene[28] and then transformed into Δ*Motctp* mutant through ATMT method. The transformants were initially screened on selective defined complex media containing 100 μg/mL chlorimuron-ethyl. The expression of *MoTCTP* in *MoTCTP*-rescued transformants was confirmed by RT-PCR using primer set MoTCTP-RTF/MoTCTP-RTR with *β-tubulin* (primer set, Tubulin-RTF/Tubulin-RTR) (Table 1) as a control.

1.4 Localization of MoTCTP

To test the localization of MoTCTP in a cell, a strain expressed with GFP-MoTCTP fusion protein was built as follows. *MoTCTP* CDS fragment amplified from genomic DNA with primer set TCTP4F/TCTP4R was inserted into the *Xba* I site of pKD5-GFP vector[28]. The GFP-MoTCTP infusion expression vector was transformed into wild type strain through ATMT method. The transformants were screened on selective defined complex media after transformation and then GFP expression was observed under fluorescence microscope.

1.5 Characterization of *MoTCTP* null mutants

CM medium was used to measure the vegetative growth, colony characteristics and conidiation as previously reported[24]. To check the resistance of Δ*Motctp* to hydrogen peroxide stress, 0.1 μmol/L~100 μmol/L H_2O_2 was added to CM medium for 10 days. The experiments were repeated three times with 5 replicates each. Conidial germination and appressorial formation were assayed on plastic coverslips under humid conditions at 25 °C[24]. For conidial germination and appressorial formation assay, more than 300 conidia were examined for each sample with three repeats.

Subcellular localization or co-localization of GFP-MoTCTP fusion protein was observed under fluorescence microscope. For mitochondrial staining, hyphae were cultured with 1 mmol/L Mitotracker red (Invitrogen, USA) for 40 minutes. For vacuole staining, hyphae were incubated with 7.5 μmol/L FM4-64 (Invitrogen, USA) for 2.5 h. And for DNA staining, hyphae were stained with 5 μg/mL DAPI (Beyotime, China) for 15 minutes.

1.6 Plant infection assays

The virulence test on barley (*Hordeum vulgare*) seedlings was performed as described previously[24]. For barley leaf explants inoculation, 5 mm mycelium blocks of Guy11 and transformants were inoculated on leaf, and then cultured in a wet box at 25 °C for 4 days. Disease lesions were recorded 4 days for barley after inoculation. The experiments were repeated three times independently.

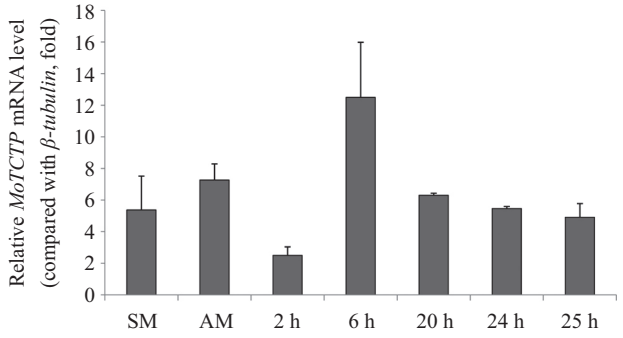
2 Results

2.1 MoTCTP is an abundantly expressed gene in *M. oryzae* and shares high homologous TCTP domain sequence with human TCTP

The gene expression profiles in aerial mycelia and appressoria of *M. oryzae* were analyzed using the Illumina sequencing platform (data not shown)

and putative *MGG_06249.7* gene was found to be listed as the 24th most abundantly expressed genes with 1571 TPM (number of transcripts per million clean tags) in aerial mycelia. After blast search on NCBI, MGG_06249.7 was identified as a homolog of human translationally controlled tumor protein domain (TCTP, pfam00838), and such was named as MoTCTP in present work. The cDNA sequence of MoTCTP was cloned from cDNA library of *M. oryzae*[25] and submitted to NCBI (GenBank KC834738). The cDNA confirmed MoTCTP protein contained 170 amino acids with 39% identity to human TCTP protein, and exhibited much higher similarities to its fungal homologues, e.g., 52% identity to *S. cerevisiae* TMA19, 61% identity to FoTCTP in *Fusarium oxysporum*, 59% identity to GzTCTP in *Gibberella zeae*/NcTCTP in *Neurospora crassa*, and 56% identity to AaTCTP in *Alternaria alternate*.

MoTCTP mRNA levels during vegetative growth, conidiation, conidial germination, and appressorium formation were analyzed using quantitative Real-time PCR (RT-qPCR) to compare transcriptional changes of *MoTCTP* during the fungal development. The results showed that *MoTCTP* is abundantly expressed in *M. oryzae,* even more than *β-tubulin*, a highly expressed house-keeping gene, in all checked developmental stages (Fig.1). The relative abundance of *MoTCTP* transcripts to those of *β-tubulin* were 5.4-fold (substrate mycelia) and 7.3-fold (aerial conidium-produced mycelia), 2.5-fold (germinated conidia) and 12.5-fold to 4.9-fold (developing and developed appressoria).



Total RNAs of substrate mycelia (SM, cultured in liquid CM medium), aerial conidial mycelia (AM, cultured on solid CM medium), and conidia incubated for 2 h (germinating conidia), 6 h (developing appressoria), 20 h (maturing appressoria), 24 h and 25 h (matured appressoria) were extracted and synthesized to cDNA. The *β-tubulin* gene was selected as a control in RT-qPCR.

Fig.1 The expression pattern of *MoTCTP* in *M. oryzae*

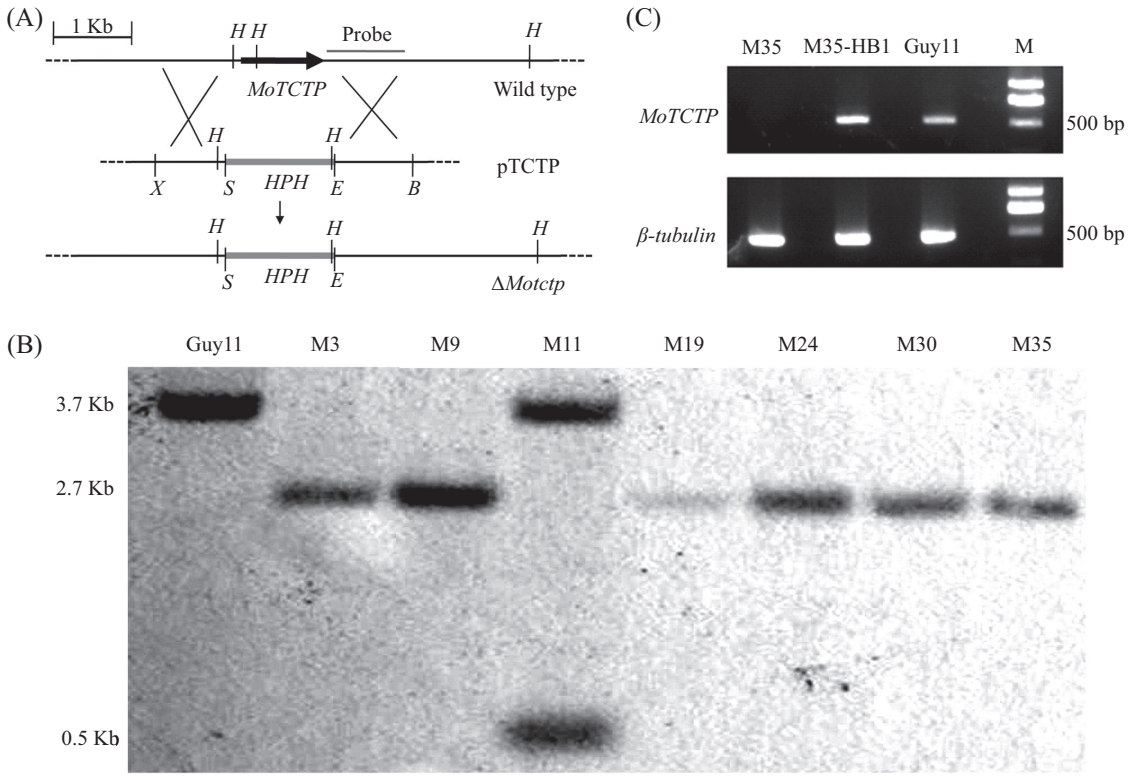
2.2 *MoTCTP* knockout and recovery in *M. oryzae*

To evaluate the function of *MoTCTP* at molecular level, we constructed *MoTCTP*-deleted mutants by targeted gene replacement as described in materials and methods. 1 277 bp DNA sequence covering whole coding sequence of *MoTCTP* gene was deleted in *M. oryzae* strain Guy11 (Fig.2A). The transformants were screened by PCR using internal primers (CKF/CKR) of *MoTCTP* coding sequence and 6 transformants were found losing *MoTCTP* genes. Then genomic DNA of *MoTCTP* mutants, wild type strain Guy11, and one ectopic transformant were digested with *Hin*d III for Southern blot analysis. After hybridized with a probe, a 998 bp downstream flanking sequence fragment of *MoTCTP*, a 2.7 Kb band that was 1 Kb smaller than that of the wild type appeared in the Δ*Motctp* mutants (Fig.2B). The ectopic transformant showed two bands, one is 3.7 Kb same with that of the wild type strain, the other is 0.5 Kb representing an ectopic insertion. Thus, six Δ*Motctp* mutants and one ectopic transformant were identified in this experiment.

To confirm whether the phenotypical changes of Δ*Motctp* mutants were caused really by the loss of *MoTCTP*, we reintroduced *MoTCTP* into the Δ*Motctp* mutant strain M35. An original copy of *MoTCTP* which was inserted into binary vector pKD5 containing sulfonylurea resistance gene[28] was transformed into Δ*Motctp* germinating conidia through ATMT method. The expression of *MoTCTP* in the complementation transformant (M35-HB1) was confirmed by RT-PCR which assured the gene recovery event (Fig.2C).

2.3 Δ*Motctp* mutants display defects in mycelial growth and resistance to hydrogen peroxide stress

Δ*Motctp* mutants showed defects in mycelial growth on CM medium. Mutant M3/M35 colonies grew as much as 59.6%/53.1% of the wild type Guy11, while ectopic transformant M11 showed no significant differences to Guy11 on CM medium at 10 d after inoculation (Fig.3A). And the mycelial



A: *MoTCTP* locus and gene targeted deletion vector (pTCTP). The probe for Southern blot is shown in A. *H: Hin*d III; *E: Eco*R I; *S: Sal* I; *B: Bam*H I; *X: Xba* I. *HPH*: hygromycin resistance gene. B: Southern blot analysis of Δ*Motctp* mutants (M3, M9, M19, M24, M30, M35) and an ectopic transformant (M11). All genomic DNA samples were digested with *Hin*d III, and probed with a 998 bp downstream flanking sequence fragment of *MoTCTP*. As expected, the 2.7 Kb bands were detected in Δ *Motctp* mutant (M3, M9, M19, M24, M30, M35) and the 3.7 Kb band was detected in wild-type strain Guy-11 and an ectopic transformant (M11), whereas a 0.5 Kb band was also detected in ectopic transformant M11. C: confirmation of *MoTCTP* expression in the complementation transformant (M35-HB1) by RT-PCR. Total RNAs were isolated from mycelia of the wild-type Guy11, Δ*Motctp* mutant M35, and *MoTCTP*-rescued strain M35-HB1 grown on CM medium plates. The *β-tubulin* gene was selected as a control. RT-PCR results confirmed that *MoTCTP* transcripts were absent in the Δ*Motctp* mutant and present in *MoTCTP*-rescued strain M35-HB1. M: DNA marker (Trans2k Plus II, TransGen Biotech, China).

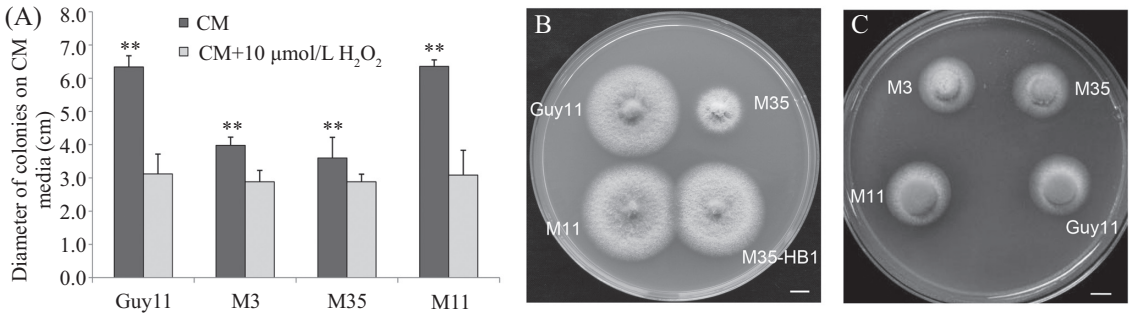
Fig.2 Knockout and recovery of *MoTCTP*

growth in *MoTCTP*-rescued strain M35-HB1 was recovered when compared to mutant M35 and wild type (Fig.3B).

As yeast mutant lacking MMI1, a homolog of TCTP, which displayed resistance to H_2O_2 stress[14], we assayed the resistance of Δ*Motctp* to hydrogen peroxide stress in CM medium. After trying 0.1 μmol/L~100 μmol/L H_2O_2, we found 10 μmol/L H_2O_2 is a suitable concentration. In this concentration wild type strain could grow in a speed nearly half of that in a controlled CM medium. The growth inhibit rate of 10 μmol/L H_2O_2 on CM medium at 10 d after inoculation is 55.1% for wild type Guy11, 31.5% for mutant M3, 23.1% for mutant M35 and 56.1% for ectopic transformant M11, respectively when compared to the growth under control CM medium. However, there were no significant differences among these four tested strains in term of the mycelial growth of colonies on CM medium containing 10 μmol/L H_2O_2 (Fig.3A and 3C). These results suggested that Δ*Motctp* increase resistance to hydrogen peroxide stress.

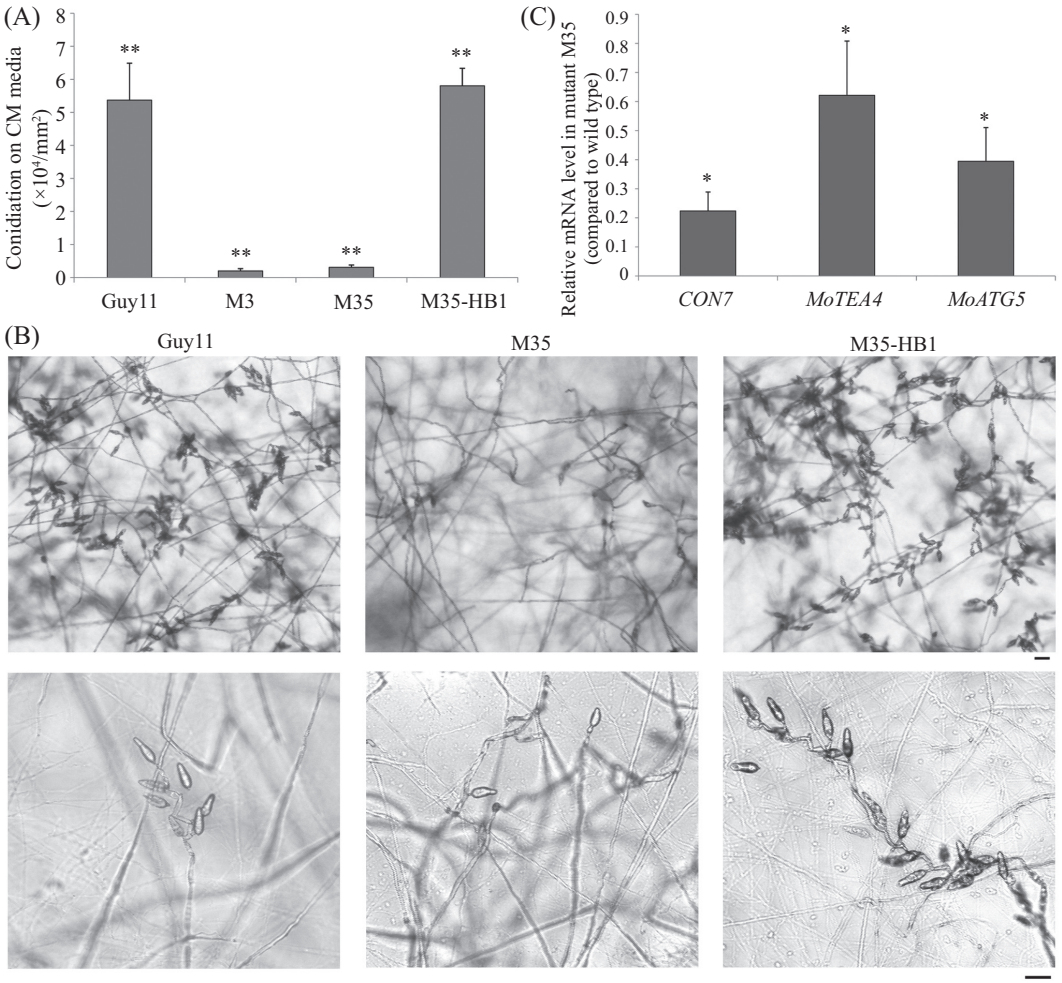
2.4 Δ*Motctp* mutants display defects in conidiation

MoTCTP is necessary for the conidiation in rice blast fungus. Conidial production of Δ*Motctp* mutants M3 and M35, wild type Guy11 and *MoTCTP*-rescued transformant of Δ*Motctp* (M35-HB1) was measured on CM medium. Wild type Guy11 produced $(5.37\pm1.12)\times10^4$ conidia/mm^2, while Δ*Motctp* mutants M3 and M35 produced less conidia than wild type at $(0.20\pm0.07)\times10^4$ conidia/mm^2 and $(0.31\pm0.07)\times10^4$



A: the mycelial growth of wild type Guy11, Δ*Motctp* mutants M3 and M35, and an ectopic transformant strain M11 on CM media or CM media containing 10 μmol/L H_2O_2 at 10 DPI (days post inoculation). $^{**}P \leqslant 0.01$ indicates significant differences between wild type strain/ectopic transformant strain and mutants estimated by Duncan's test. B: the colonies of Guy11, M35 and *MoTCTP*-rescued strain M35-HB1 on CM media. The photo was taken at 6 DPI with point mycelial block. C: the colonies of Guy11, M3 and M35, and M11 on CM media containing 10 μmol/L H_2O_2. The photo was taken at 4 DPI with 0.5 cm mycelial block. Bar=1 cm.

Fig.3 Growth defects of Δ*Motctp* mutants



A: conidiation of wild type Guy11 and Δ*Motctp* M3 and M35, and *MoTCTP*-rescued transformants M35-HB1 on CM medium. $^{**}P \leqslant 0.01$ indicates significant differences between wild type strain/*MoTCTP*-rescued transformant and mutants estimated by Duncan's test. B: development of conidia on the conidiophores of wild type Guy11, Δ*Motctp* M35 and *MoTCTP*-rescued transformants M35-HB1. The photos were taken on strains grown on CM medium over microscope slides for 4 days. Bar=40 μm. C: expression levels of *CON7*, *MoATG5* and *MoTEA5* genes in mutant M35. Total RNAs were isolated from mycelia grown in liquid CM medium. The data from RT-qPCR were firstly normalized using *β-tubulin* and then the expression levels in the wild type used as a control. $^{*}P \leqslant 0.05$ indicates significant difference between wild type strain and mutant estimated by Duncan's test.

Fig.4 Conidiation defects of Δ*Motctp* mutants

conidia/mm^2 respectively, just 3.7% or 5.8% of that in wild type (Fig.4A). The *MoTCTP*-rescued transformant of Δ*Motctp* showed no significant difference to Guy11 in conidiation.

Conidial development was observed by careful inspection for mycelial mat grown on CM medium over microscope slides. There were fewer conidiophores developed in Δ*Motctp* mutant M35, and most conidia were singly developed in mutant M35 compared to the wild type, while *MoTCTP*-rescued transformant of Δ*Motctp* (M35-HB1) produced normal conidiophores and conidia as the wild type Guy11 (Fig.4B).

Conidiation are often weakened in many reported gene deletion mutants in rice blast fungus. However, the causes leading to weakened conidiation are still unrevealed. We selected three genes: *CON7*, *MoATG5* and *MoTEA4*, which resulted in defective conidiation when they were deleted[29-31], and assayed their expression in mutant M35 and wild type Guy11. We found that these three genes were downregulated compared to wild type (Fig.4C), and the mRNA level of *CON7*, *MoATG5* and *MoTEA4* in mutant is only 22.4%, 39.4% and 62.1% of the wild type. These results implied that the weakened conidiation in Δ*Motctp* mutants was possibly caused by the expression changes of some conidiation-relative genes expression.

2.5 Delayed conidial germination and normal appressorium formation in Δ*Motctp*

The germination of conidia in Δ*Motctp* mutants M3 and M35 was delayed. At 2 hpi (hours post incubation), conidial germination rates were (74.47±5.89)% in M3 and (76.77±5.85)% in M35, compared to (92.30±0.50)% in Guy11 and (93.53±1.70)% in ectopic transformant M11. At 4 hpi, conidial germination rate of Δ*Motctp* mutants was comparable to wild type and ectopic transformant (Fig.5). However, the ability to form appressorium in Δ*Motctp* mutants didn't show any abnormity (Fig.5).

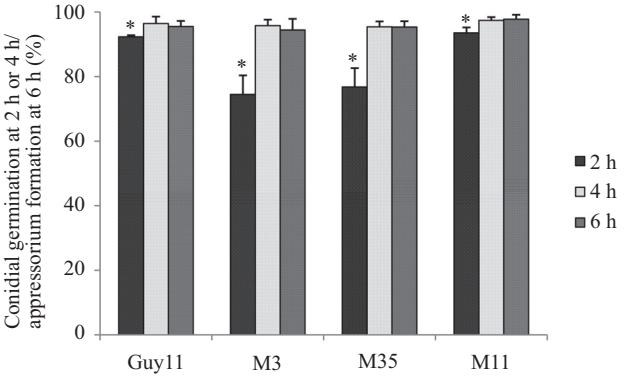
2.6 Expression level of genes related to cell cycle is affected by *MoTCTP*

The reduced mycelial growth and weakened conidiation in Δ*Motctp* implied *MoTCTP* functions in cell growth, cell proliferation and differentiation. It is known that cyclin proteins control cell progression by activating cyclin-dependent kinases (CDK). To see whether the expression of cyclin genes was affected by MoTCTP, the mRNA level of 10 cyclin or cyclin-dependant proteins (Table 2) were detected using RT-qPCR in both the wide type and mutants.

The results showed that the mRNA level of six cell cycle genes (*MoCCNK*, *CYC1*, *MoCLB3*, *MoCTK2*, *MoCDC14* and *MoPCL1*) was downregulated, while three CDK genes (*MoCDC15*, *MoCDC28* and *MoCDC42*) were upregulated (Fig.6). However, three cyclin proteins (*CYC2*, *MoCCL1* and *MoCCNL2*) were little affected at mRNA expression level by MoTCTP. It is known that *CYC1*, *MoCDC15* and *MoCDC42* are required for fungal growth or conidiation[32-34]. These results suggested that the transcription of several cell cycle genes was regulated by MoTCTP, and the defects in the fungal growth and conidiation might be possibly caused by the altered expression of cell cycle relative genes in Δ*Motctp* mutants.

2.7 Cellular localization of MoTCTP

GFP-MoTCTP fusion expression transformants were built to investigate the localization of MoTCTP in *M. oryzae*. In yeast, the translocations of Mmi1 (TCTP) between cytoplasm and mitochondria were observed under oxidative stress[14]. So, we checked the MoTCTP distribution in the transformant hyphae after staining



Mutants M3 and M35, wild type Guy11, and ectopic transformant M11 were cultured in H_2O on plastic cover slides for 2 h and 4 h (conidial germination), and 6 h (appressorial formation). *$P \leqslant 0.05$ indicates significant difference between wild type strain/ectopic transformant and mutants estimated by Duncan's test.

Fig.5 Conidial germination rate (%) and appressorial formation rate (%) of Δ*Motctp* mutants

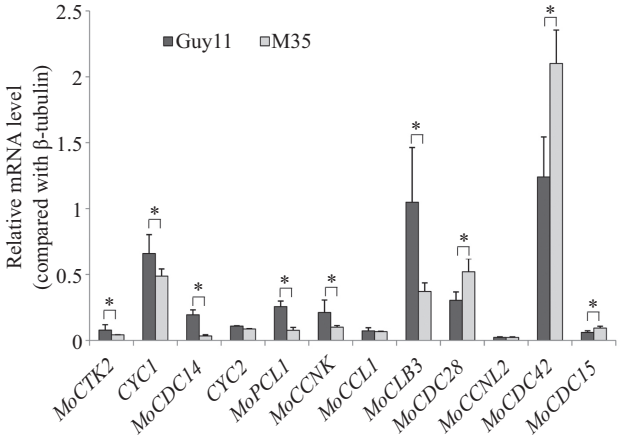
Table 2 Genes encoding cyclins and cell division proteins in *Magnaporthe oryzae*

Gene	Locus No.	Description	Reference
MoCTK2	MGG_11817.7	C-type cyclin, ortholog to ctk2 in yeast	[35]
CYC1	MGG_05646.7	B-type cyclin, ortholog to clb2 in yeast	[32,36]
MoCDC14	MGG_00757.7	CDC14	[37]
CYC2	MGG_07065.7	B-type cyclin, ortholog to clb3 in yeast	[32]
MoPCL1	MGG_01361.7	PHO85 cyclin, ortholog to pcl1 in yeast	[38]
MoCCNK	MGG_01258.7	Cyclin K, ortholog to human Ccnk	[39]
MoCCL1	MGG_15176.7	Cyclin H, ortholog to ccl1 in yeast	[40]
MoCLB3	MGG_03595.7	B-type cyclin, ortholog to clb3 in yeast	[41]
MoCDC28	MGG_01362.7	Cyclin-dependent kinase, ortholog to cdc28 in yeast	[42-43]
MoCCNL2	MGG_01437.7	Ortholog to cyclin L2 in human	[44]
MoCDC42	MGG_00466.7	CDC42	[33]
MoCDC15	MGG_04100.7	CDC15	[34]

with dyes for mitochondria, vacuole and DNA under fluorescence microscope.

Firstly, the hyphae were stained with 1 mmol/L Mitotracker red, a red-fluorescent mitochondrial dye, for 40 minutes to check if GFP-MoTCTP was co-localized with mitochondria. The results showed the mitochondria marked by red fluorescence were encircled by green GFP-MoTCTP fusion proteins in some hyphal cells grown in liquid CM media (Fig.7A), and cytoplasm nearby vacuoles and mitochondria were also localized by GFP-MoTCTP fusion proteins in some aerial hyphal cells (Fig.7B). To identify the co-localization of MoTCTP with mitochondrion furthermore, DAPI which binds strongly to A-T rich regions in DNA of mitochondrion or nucleus were used to stain hyphae cultured in liquid CM media. GFP-MoTCTP fusion protein showed co-localization with mitochondrion stained by DAPI (Fig.7C) under fluorescence microscope.

To check if MoTCTP could also be localized to vacuoles, aerial hyphae were further stained with 7.5 μmol/L vacuole membrane-specific dye FM4-64 for 2.5 h. The results showed GFP-MoTCTP fusion protein appeared in cytoplasm or in circular balls, but not in vacuoles marked in red by FM4-64 (Fig.7D-7F). These data implied that MoTCTP might locate in mitochondrial outer membrane, cytoplasm, cytoplasm nearby vacuoles and other unidentified cellular organelles, but not in vacuoles.



Total RNAs (n=6) were isolated from mycelia grown in liquid CM medium. The data from RT-qPCR were normalized using *β-tubulin* (=1). *$P \leqslant 0.05$ indicates significant difference between wild type strain Guy11 and mutant M35 estimated by Duncan's test.

Fig.6 Expression in mRNA level of cyclins and cell division proteins in Δ*Motctp* mutant

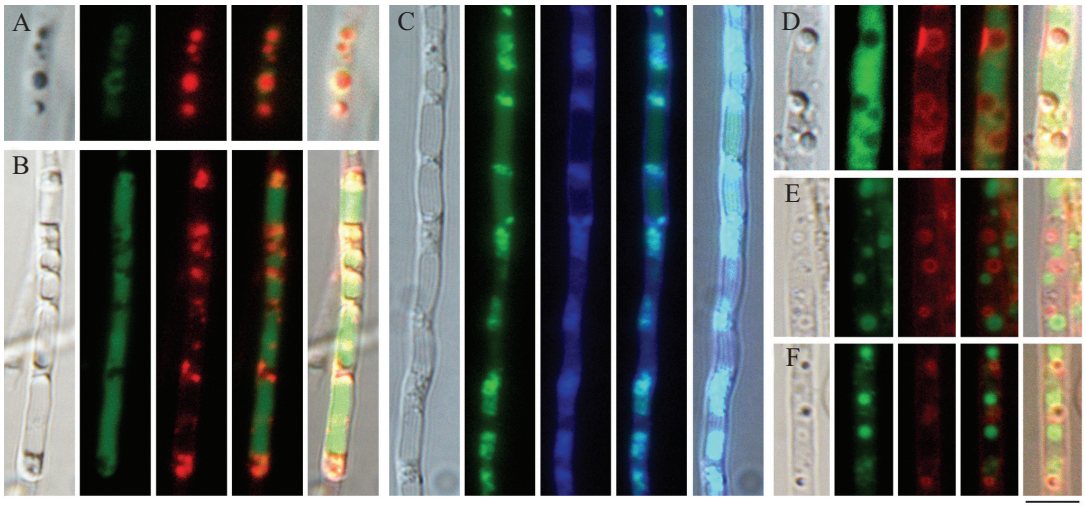
2.8 Pathogenicity in Δ*Motctp* mutants

Pathogenicity of Δ*Motctp* mutants was tested on barley. After being inoculated on leaf explants of barley with 5 mm mycelium blocks, Δ*Motctp* mutant M35 displayed weakened lesions compared with wild type Guy11 and *MoTCTP*-rescued strain M35-HB1 (Fig.8).

3 Discussion

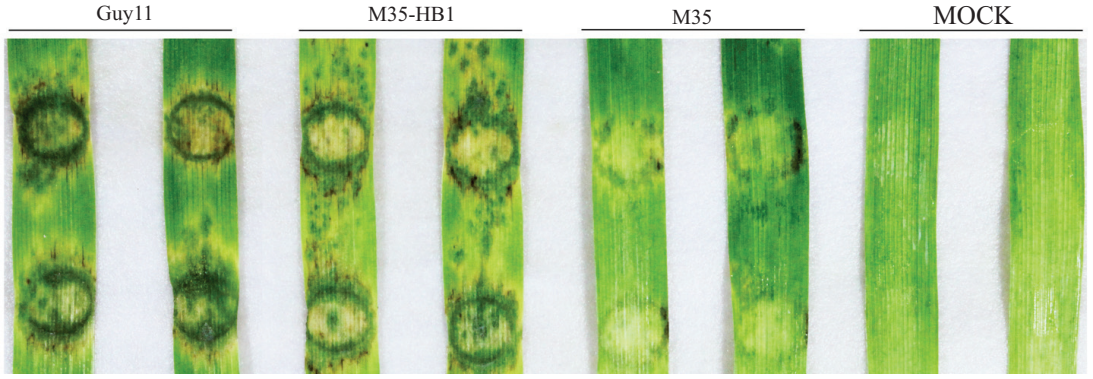
3.1 Structure, function, expression and localization of TCTP

TCTPs (translationally controlled tumor proteins)



A,B: GFP-MoTCTP protein co-localization with mitochondrion. Left 1, light microscopy; left 2, green fluorescence for GFP-MoTCTP; middle, red fluorescence for mitochondria stained by Mitotracker red; right 1 and 2, merged pictures. C: GFP-MoTCTP protein co-localization with mitochondria. Left 1, light microscopy; left 2, green fluorescence for GFP-MoTCTP; middle, blue fluorescence for mitochondrial DNA stained by DAPI; right 1 and 2, merged pictures. D~F: GFP-MoTCTP protein not co-localization with vacuoles. Left 1, light microscopy; left 2, green fluorescence for GFP-MoTCTP; middle, red fluorescence for vacuolar membranes stained by FM4-64; right 1 and 2, merged pictures. Bar=5 μm.

Fig.7 Co-localization of GFP-MoTCTP fusion protein in hyphal cells



5 mm mycelium blocks (Guy11, M35 and M35-HB1) were inoculated on barley leaves and cultured in a wet box at 25 ℃ for 4 days.

Fig.8 Pathogenicity assays on barley leaves

from metazoans, plants and fungi exhibit extensive conservation in protein sequence[2,45]. It is proved that TCTP sequences have several binding sites in different proteins. With these binding sites, TCTPs could interact with microtubules in mammal[18,46-47] and yeast[14], actin in mammal[46,48], Bcl-2 family proteins in mammal[49-50], P53 in mammal[51-52], Rab proteins act as a guanine nucleotide exchange factor (GEF) to activate TOR pathway in yeast, plant, insect and mammal[12,17,53-55], and Na-K-ATPase in mammal[56], and Ca^{2+}[15], etc. TCTPs could also bind to DNA and function as a transcription factor. For example, TCTP regulates the expression of *Oct4* gene in mammal cells via binding its promoter[57-58]. In accord with its binding to diversified proteins, TCTP plays different roles in various cellular processes, such as cell growth, cell differentiation, cell cycle, gene expression, cellular stress responses and apoptosis[2-8,12,59]. In the rice blast fungus, MoTCTP is also required for various fungal processes, such as mycelial growth, conidiation, conidial germination and cellular stress response. After deletion, *MoTCTP* null mutants display many defects, such as decreased growth, greatly weakened conidiation, and delayed conidial germination, but increased resistance to H_2O_2 stress.

TCTP is one of most abundantly expressed

proteins in many cells[2]. TCTP expression is highly regulated at transcription level or translation level in response to various cellular stimuli and stresses[2,7]. In rice blast fungus, *MoTCTP* is also a highly expressed gene in various developmental stages, including mycelium, germinating conidium, forming appressorium and mature appressorium. Coinciding with its diversified functions, TCTP is localized to cytoplasm and nucleus[4,6,14,60], and outer membrane of mitochondria[14]. The translocations of TCTP from cytoplasm to nucleus and mitochondria, from mitochondria to cytoplasm were regulated by cellular stress, such as oxidative stress and glucose level[6,14,60]. The localization of MoTCTP in cell is also variable in rice blast fungus. It was detected in the mitochondrial outer membrane, cytoplasm and cytoplasm nearby vacuoles in hyphae. Considering its diversity in function, MoTCTP might locate in other cellular organelles as well. The translocations of MoTCTP among cellular organelles are still needed to be further revealed in the future in response to cellular stress or during cell differentiation.

3.2 MoTCTP and Cyclins in fungal growth

The mycelial growth depends on two developmental processes, cell growth (increase in cell size) and cell division (increase in cell number). Cyclins and cell division control proteins play key roles in the regulation of cell division and cell growth. Cyclin gene *CYC2* is necessary for normal hyphal growth in *M. oryzae*[32]. Deletion of *MoCDC15* leads to defects in hyphal growth and conidial production[34]. The *MoCDC42* null mutant doesn't exhibit obvious deficiency in hyphal growth on several tested media, but its aerial growth is reduced and conidiogenesis weakened[33]. In this study, six cyclin and cell division control protein genes (*MoCCNK*, *CYC1*, *MoCLB3*, *MoCTK2*, *MoCDC14* and *MoPCL1*) were downregulated and three cyclin-dependent kinase genes (*MoCDC15*, *MoCDC28* and *MoCDC42*) were upregulated in Δ*Motctp* mutants.

Human cyclin-K, homolog of MoCCNK, is a positive regulator of cyclin-dependent kinase (CDKs)[39]. B-type cyclins (CLB1, CLB2 and CLB3, homologs of CYC1, CYC2 and MoCLB3) are activators of cyclin-dependent kinases in yeast[61], and have been shown to be essential for vegetative growth in *Ustilago maydis*[62] and *Candida albicans*[63-64]. Cyclin-K and B-type cyclins may regulate transcription through activation of CDK kinases (cyclin-dependent kinase, such as CDC28) and then phosphorylation of the C-terminal domain (CTD) of the large subunit of RNA polymerase II[61,64]. CTK2 is a β-subunit of CTDK1 (C-terminal domain kinase I), which is required for transcription, mRNA maturing and translation[65]. CDC14 is a protein phosphatase essential for inactivation of mitotic cyclins and meiotic progression[66]. *PiCDC14* is expressed during sporulation but not hyphal growth in oomycete *Phytophthora infestans*[37]. PCL1 is a cyclin interacting with the CDK kinase Pho85[67] and required for polarized growth and morphogenesis in cell cycle[68]. The expression changes of cyclins and cell division control proteins will affect expression of lots of genes and subsequently changes cell division and cell growth. The reduction of mycelial growth and conidial production in *MoTCTP* null mutant might be caused by the defects in the expression of cyclin proteins and cell division control proteins.

3.3 MoTCTP and Asexual differentiation

Conidial production is a cell differentiation process, where there are lots of genes involved. 603 (4.42%) of 13 666 checked genes were upregulated and 557 genes (4.08%) were downregulated more than two-fold respectively during conidiation in the rice blast fungus[69]. Many reported works also showed that the defect in conidiation was a common phenotype when some genes were deleted in rice blast fungus, such as autophagy-related genes[70], transcription factors *MoHOX2*[71], *COM1*[72] and *MNH6*[24], phosphodiesterase genes *PDEL* and *PDEH*[73], chitin synthase genes[74], genes encoding Pmk1-interacting proteins[75] and cell division control proteins[33-34], and *MTP1*[76]. In our study, the deletion of *MoTCTP* dramatically reduced conidial production. However, the up-regulation of *MoTCTP* was not strong, and actually, no more than two folds during conidiation[69]. In order to make sure if conidial production reduced in Δ*Motctp* mutants is

caused by the expression change of known conidial production related genes, we assessed the expression level of three genes, *CON7*, *MoATG5* and *MoTEA4*, in the mycelia of the wild type and Δ*Motctp*. It was reported that deletion of these three genes separately resulted in defective conidiation[29-31]. As expected, the expression of *CON7*, *MoATG5* and *MoTEA4* were downregulated in Δ*Motctp* mutants. Considering the expression changes of cyclin and cell division control protein genes in mutants, these data implied that *MoTCTP* might affect fungal conidiation through regulating conidiogenesis related genes directly or indirectly.

Taken together, *MoTCTP* is necessary for fungal growth, conidiation, conidial germination and has a role in resistance to hydrogen peroxide stress. *MoTCTP* is a highly expressed gene in mycelia, conidia and appressoria, and its protein localizes to mitochondrial outer membrane, cytoplasm and cytoplasm nearby vacuoles. Deletion of *MoTCTP* changes the mRNA expression levels in cyclins, cell division control genes and conidiogenesis related genes greatly.

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