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研究报告

Altered N-glycan processing in *Trichoderma reesei* affects the morphogenesis and improves the degradation of lignocellulose

Sharma Ghimire Prakriti[Δ1,2,4] OUYANG Haomiao[Δ1] ZHAO Guangya[1,2]
XIE Mingming[1,2] ZHOU Hui[1] YANG Jinghua[1] JIN Cheng[*1,2,3]

1 State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
2 University of Chinese Academy of Sciences, Beijing 100049, China
3 National Engineering Research Center for Non-Food Bio-Refinery, Guangxi Academy of Sciences, Nanning, Guangxi 530007, China
4 Himalayan Environment Research Institute (HERI), Bouddha-6, Kathmandu, Nepal

Abstract: [Background] *Aspergillus fumigatus* α-1,2-mannosidase MsdS is an enzyme that cleaves N-linked $Man_8GlcNAc_2$ in Golgi apparatus to produce $Man_6GlcNAc_2$ on mature secreted glycoproteins. MsdS has been shown to play a significant role in morphogenesis, cell wall synthesis and protein secretion in *A. fumi gatus*. Unlike *A. fumigatus*, *Trichoderma reesei* produces $Man_8GlcNAc_2$ on its mature secreted glycoproteins and grows normally. These observations suggest a species-specific N-glycan processing in filamentous fungi, however, its biological significance keeps unclear. **[Objective]** To evaluate the effects of the N-glycan processing on cell growth and protein secretion in *T. reesei*, *A. fumigatus* MsdS was introduced into *T. reesei* to change the glycoform on mature secreted proteins. **[Methods]** The recombinant plasmid haboring the *msdS* gene was constructed and transformed into *T. reesei* to obtain the *msdS*-expressing strain Tr-MsdS. The phenotypes, N-glycome, protein secretory pathway and cellulase activity were analysed. **[Results]** The *msdS*-expressing strain Tr-MsdS produced a major glycoform of $Man_6GlcNAc_2$ on its secreted glycoproteins, instead of $Man_8GlcNAc_2$ in the parent strain. Although the cell wall content of *msdS*-expressing strain Tr-MsdS was changed, it appeared that the cell wall integrity was not affected. However, phenotypes such as increased conidiation, multiple budding and random branching were observed in strain Tr-MsdS. In addition, expression of MsdS in *T. ressei* also affected protein secretion and increased the acivities of cellulose and β-mannan degradation by 9.9% and 32.2% at 50 °C, respectively. **[Conclusion]** Our results indicate that the N-glycan processing plays an important role in protein secretion in *T. reesei*, especially cellulases. Also, our results provide a new strategy to improve cellulases production by interfering the N-glycan processing in *T. reesei*.

Keywords: α-mannosidase, N-glycans processing, *Trichoderma reesei*, cellulase, protein secretion

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ΔThese anthors equally contributed to this work
***Corresponding author:** Tel: 86-10-64807425; E-mail: jinc@im.ac.cn

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*通信作者：Tel：010-64807425；E-mail：jinc@im.ac.cn

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N-糖链加工影响里氏木霉形态发生并提高木质纤维素降解能力

Sharma Ghimire Prakriti[Δ1,2,4] 欧阳浩淼[Δ1] 赵光亚[1,2] 解明明[1,2] 周慧[1] 杨静华[1] 金城[*1,2,3]

1 中国科学院微生物研究所真菌学国家重点实验室 北京 100101

2 中国科学院大学 北京 100049

3 广西科学院国家非粮生物质能源工程研究中心 广西 南宁 530007

4 Himalayan Environment Research Institute (HERI), Bouddha-6, Kathmandu, Nepal

摘 要:【背景】烟曲霉 α-1,2-甘露糖苷酶 MsdS 在高尔基体中将 N-糖链 $Man_8GlcNAc_2$ 加工为成熟分泌糖蛋白的糖型 $Man_6GlcNAc_2$，有研究表明 MsdS 与烟曲霉的形态发生、细胞壁合成及蛋白质分泌密切相关；与烟曲霉不同的是，里氏木霉的成熟分泌糖蛋白上的 N-糖链结构为 $Man_8GlcNAc_2$，细胞却能正常生长，说明丝状真菌 N-糖链的加工具有物种特异性，但其生物学意义不明。【目的】为研究 N-糖链加工对里氏木霉细胞生长及蛋白质分泌的影响，本研究将烟曲霉 MsdS 转入里氏木霉中以改变其成熟分泌糖蛋白的糖型。【方法】构建带有烟曲霉 *msdS* 基因的重组质粒并转入里氏木霉中，获得 *msdS* 表达菌株 Tr-MsdS，分析 Tr-MsdS 菌株的生长表型、N-糖组、蛋白质分泌途径和纤维素酶活性的变化。【结果】在里氏木霉 *msdS* 表达菌株 Tr-MsdS 中，分泌糖蛋白的主要糖型由出发株的 $Man_8GlcNAc_2$ 转变为 $Man_6GlcNAc_2$，细胞壁组分发生变化，但细胞壁完整性未受影响；与出发株相比，Tr-MsdS 菌株产孢、出芽及分枝增多；另外，MsdS 的表达还影响蛋白质分泌，在 50 °C 时降解纤维素和 β-葡聚糖的能力分别提高 9.9%和 32.2%。【结论】研究结果表明，N-糖链的加工可影响里氏木霉蛋白质，尤其是纤维素酶的分泌，干扰 N-糖链加工可能是提高里氏木酶纤维素酶产量的新策略。

关键词: α-甘露糖苷酶，N-糖链加工，里氏木霉，纤维素酶，蛋白质分泌

Trichoderma reesei (syn. *Hypocrea jecorina*), a mesophilic filamentous fungus identified as GRAS (generally recognized as safe) status by the FDA (U.S. Food and Drug Administration)[1-2], can produce a wide range of extracellular enzymes in large quantities. *T. reesei* can post-translationally modify proteins, while growing faster than plant, insect or mammalian cells; therefore, it has been thought as an attractive expression host. However, the expression of heterologous protein is less efficient than endogenous proteins in *T. reesei* and, therefore, efforts have been made to improve the production and secretion of heterologous proteins[3-5].

Enzymes secreted by *T. reesei* are N- and O-glycosylated[6]. It has been shown that O-glycosylation affects not only the function of these enzymes, such as proteolytic resistance, thermostability and cellulose-binding[7], but also their expression and secretion[8-9]. Interestingly, either increase or decrease of O-glycosylation level leads to an increase of secreted proteins[10-11]. However, it keeps unknown how N-glycan processing affects protein secretion.

In eukaryotic cells, secreted proteins are synthesized in the endoplasmic reticulum (ER) and transported to the Golgi apparatus. Within the secretory pathway, N-glycosylation is initiated in the lumen of the ER by transferring of $Glc_3Man_9GlcNAc_2$ from dolichol pyrophosphate to the nascent polypeptides. Once the $Glc_3Man_9GlcNAc_2$ is transferred to proteins, N-glycan processing is initiated sequentially in the ER and Golgi by two ER α-glucosidases and various 1,2-α-mannosidases[12-13]. In mammalian cells, $Man_9GlcNAc_2$ is converted to $Man_5GlcNAc_2$ by the action of ER and Golgi α-mannosidases, and $Man_5GlcNAc_2$ is the precursor for complex, hybrid and high-mannose type of N-glycans[12]. Golgi α-mannosidase is a Class-I α-mannosidase responsible for cleavage of

α-1,2-linked D-mannoses[14-16]. *In vitro* analysis reveals that this enzyme converts $Man_8GlcNAc_2$ or a mixture of $Man_{6-9}GlcNAc_2$ oligosaccharides to the respective $Man_5GlcNAc_2$ structures[15-16].

Our previous study has shown that in filamentous fungus *Aspergillus fumigatus* α-mannosidase MsdS is responsible for the processing of N-linked $Man_{8-9}GlcNAc_2$ to $Man_6GlcNAc_2$, which is the glycoform on mature secreted glycoproteins[16]. Deletion of the α-mannosidase gene *msdS* results in a conversion of the N-glycans on secreted glycoproteins from $Man_6GlcNAc_2$ to $Man_8GlcNAc_2$ and causes defective cell wall and abnormal polarity[16]. On the other hand, it is interesting to note that the N-glycan on secreted glycoproteins of *T. reesei* is $Man_8GlcNAc_2$[17], which is the same as that of the *A. fumigatus msdS*-knockout mutant, suggesting that N-glycan processing is different in these two species.

In an attempt to evaluate the effect of altered N-glycan processing on *T. reesei*, in this study a *msdS*-expressing strain Tr-MsdS was constructed by introducing the *A. fumigatus msdS* gene into *T. reesei*. As expected, the main N-glycan glycoform was converted from $Man_8GlcNAc_2$ to $Man_6GlcNAc_2$ in strain Tr-MsdS. Analysis of the Tr-MsdS strain revealed that the expression of MsdS led to abnormal polarity and altered protein secretion.

1 Materials and Methods

1.1 Strains and growth conditions

T. reesei TU-6 (uridine auxotrophs, American Type Culture Collection, ATCCMYA-256, obtained from Prof. Zhiyang Dong, Institute of Microbiology, Chinese Academy of Sciences), was used as the recipient of the α-mannosidase gene from *A. fumigatus*. TU-6 was cultivated in minimal medium (MM medium) (g/L): Glucose 20.0, $(NH_4)_2SO_4$ 5.0 and KH_2PO_4 15.0. Microelements (0.005% $FeSO_4·7H_2O$, 0.001 6% $MnSO_4·7H_2O$, 0.001 4% $ZnSO_4·7H_2O$, 0.003 7% $CoCl_2$, 0.6% $CaCl_2$ and 0. 6% $MgSO_4·7H_2O$) and 0.2% uridine were also added in the medium to culture TU-6 at 28 °C on a rotary shaker (200 r/min). Conidia were prepared by cultivating *T. reesei* TU-6 strains on solid PDA medium with uridine for 5–7 days at 28°C. The spores were collected, washed thrice and resuspended in sterilized ddH_2O (added with 40% glycerol solution (1:1)) for long term storage at –70°C. The spore concentration was confirmed using haemocytometer counting and viable counting.

1.2 Reagents and equipment

pGEM-T Easy and trypsin were purchased from Promega Corporation; Peptide N-glycosidase F (PNGase F) was from New England BioLabs Inc.; DTT, fluorescent brightener 28, carboxymethyl cellulose, locust bean gum and 4,6-diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich Co.; RevertAid™ First Strand cDNA Synthesis Kit was from MBI Fermentas; Bradford protein assay kit was from Bio-Rad; TRIzol reagent was from Invitrogen/ Life Technologies; SYBR®Premix *Ex Taq*™ was from Takara. Scanning electron microscope was from Hitachi; MALDI-TOF/TOF was from AB Sciex Pte. Ltd.; Fluorescence microscope was from Carl Zeiss AG; PCR machine CFX manager 3.1 was from Bio-Rad.

1.3 Expression of *the A. fumigatus msdS* in *T. reesei*

Escherichia coli DH5α was used for plasmid propagation in order to insert the α-mannosidase *msdS* gene from *A. fumigatus* in TU-6. pCG (5.5 kb vector plasmid containing *chiB* promoter and GFP) vector was reconstructed into pGM vector by using GPDA as a promoter with oligonucleotides; *gpda*-F (5′-CAATT CCCTTGTATCTCTACACACAG-3′) with *Eco*R I restriction site and *gpda*-R (5′-GGTGATGTCTGCTC AAGCG-3′) with *Kpn* I restriction site and *msdS* as insert gene with oligonucleotides; *MsdS*-F (5′-ATGCA TTTACCCTCTTTGTCCGTG-3′) and *MsdS*-R (5′-TC ACGTATGATGAATTCGGACAGGGTG-3′) as forward and reverse primers at *Kpn* I and *Bam*H I restriction site respectively. Both *gpda* and *msdS* were cloned into pGEM-T Easy, confirmed by sequencing, inserted into the pCG vector at specific site, and confirmed by PCR[16] and sequencing. Transformation of the *msdS*-containing plasmid into *T. reesei* TU-6 protoplast was performed by a co-transformation with TAkulox *pyr4*-containing plasmid as a selectable marker. The transformants were regenerated in MM media containing sorbitol (1 mol/L D-sorbitol). The transformants were screened in MM media containing 1.5% agar and confirmed by PCR and sequencing.

1.4 Protein extraction

The extraction of proteins from different fractions of cells was done as described by Wang et al.[18] with some modifications. The extracellular proteins were precipitated with freshly prepared 2%

(*w*/*v*) sodium deoxycholate to the culture supernatant (1:100) for 30 min on ice followed by addition of 100% trichloroacetic acid (1:10) for 30 min on ice, and collected by centrifugation (12 000×*g* at 4 °C for 10 min). The precipitate was washed 3 times with ice-cold acetone (twice with 90% [*V*/*V*] and once with 100% [*V*/*V*]) and dried by exposure to air. For cytosolic and membrane proteins extraction, the mycelia obtained from culture was ground with buffer I (200 mmol/L Tris-HCl, 50 mmol/L EDTA, protease inhibitor cocktail) at 4 °C for 2 h, centrifuged at 4 000×*g* for 10 min and the supernatant obtained (containing cytosolic and membrane proteins) was ultracentrifuged 50 000×*g* at 4 °C for 1 h. After centrifugation, the supernatant containing cytosolic proteins fraction and the precipitate containing membrane protein fraction were collected separately. The mycelia obtained from the culture was dried, weighted and resuspended in 25 μL of Tris buffer (0.05 mol/L Tris-HCl, pH 7.8) per milligram of dried mycelia to extract cell wall protein. After centrifugation the pellets obtained were boiled three times in 25 μl of sodium dodecyl sulfate extraction buffer (50 mmol/L Tris-HCl, 2% SDS, 20 mmol/L Na-EDTA, and 40 mmol/L β-mercaptoethanol) per milligram of dried mycelium followed by centrifugation (12 000 r/min) at 4 °C for 10 min. The pellet was washed with distilled water three times, dried and treated with 10 μl of hydrofluoride (HF)-pyridine per microgram mycelium and put on ice for 3 h. The supernatant was separately collected and added with 9 volumes of 100% methanol buffer (100% methanol, 50 mmol/L Tris-HCl, pH 7.8) at 0 °C for 2 h. Finally, the cell wall proteins were collected by centrifugation (12 000 r/min) at 4 °C for 10 min after washing with 90% methanol buffer (90% methanol, 50 mmol/L Tris-HCl, pH 7.8) 3 times. The concentration of each fraction of proteins was determined by SDS-PAGE and quantitatively by Bradford protein assay.

1.5 SDS-PAGE and in-gel digestion

The proteins were separated by 10%−12% SDS-PAGE, stained with Coomassie Brilliant Blue R-250, and then cut out from gel. In-gel digestion of secreted proteins in SDS-PAGE was performed according to Liu et al. with a slight modification[19]. Briefly, each sliced band was de-stained by using 50% (*V*/*V*) acetonitrile in 40 mmol/L NH_4HCO_3, dehydrated using 100% acetonitrile and dried using SpeedVac. Proteins were then reduced with 10 mmol/L DTT/40 mmol/L NH_4HCO_3 at 56 °C for 20 min, alkylated with 55 mmol/L iodoacetamide/40 mmol/L NH_4HCO_3 in the dark for 25 min at room temperature followed by subsequent wash and dry. Peptides were then produced by trypsin digestion (50 ng trypsin)[19]. For MALDI-MS analysis, 0.4 μL aliquot of the reconstituted tryptic peptide mixture in 0.1% TFA was mixed with 0.4 μL of CHCA matrix solution (5 mg/mL CHCA in 50% ACN/0.1% TFA) and spotted onto a freshly cleaned target plate. After air drying, the crystallized spots were analyzed on the MALDI-TOF/ TOF 5800.

1.6 Analysis of N-glycan

N-glycans were released from membrane and secreted proteins of *T. reesei* strain by peptide N-glycosidase F (PNGase F)[18]. The enzyme reaction includes the process of denaturation of proteins at 95 °C for 5 min followed by addition 10% Nonidet P40 (NP40) treating with PNGase F. After digestion, the sample was centrifuged and the supernatant was subjected to C8 column, washed with 100% acetonitrile (ACN) and equilibrated with 0.1% trifluoroacetic acid (TFA) to separate N-glycans from proteins. The released N-glycans were collected and applied to a graphite column, washed with 0.1% TFA to remove salts and then eluted with elution buffer (60% ACN, 0.1% TFA) to collect N-glycans. The structure of released and purified N-glycans was analyzed by MALDI-TOF-MS.

1.7 Phenotypic analysis

The growth kinetics was determined by measuring colony diameter. Same amount of spore was inoculated on media plate and the colony growth was monitored by measuring the diameter of each colony at different time intervals[18]. Similarly, the sensitivity of the mutant to antifungal reagents was also analysed[16,18]. The conidiophores were spotted on solid MM medium with uridine (MMU) plates with same concentration in the presence of 100 μg/mL calcofluor white, 150 μg Congo red or 40 μg SDS and incubated at 28, 32 and 37 °C. After incubation the plates were analysed for colony growth, measured for colony diameter and photographed if required.

1.8 Electron microscopy

Scanning and transmission electron microscopy analyses were performed as described by Li et al.[16].

Culture samples were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) and then examined with scanning electron microscope. While for transmission electron microscopy, culture sample were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate at room temperature or 4 °C for 2 h or overnight, washed 3 times with 0.1 mol/L phosphate buffer, post-fixed in 1% osmium tetroxide, and incubated at room temperature for 2−4 h in 0.1 mol/L phosphate. The cells were washed with 0.1 mol/L phosphate buffer 2−3 times and then dehydration was done by treating with each of 30%, 50%, 70%, 85%, 95%, and 100% methanol for 15−20 min each. Infiltration was done by using LR white resin: ethanol (1:1) for 4 h followed by rinsing with LR white resin (100%) overnight once and rinsed for 2 h again. Cells were embedded in mold at 55 °C for 24 h followed by sectioning and staining (uranyl acetate for 25 min and lead citrate for 5 min) and the sections were examined with JEM-1400.

1.9 Germination

10^6 freshly harvested conidia were inoculated in 10 mL of minimal liquid medium in a Petri dish containing 5−6 glass coverslips at 32°C. The coverslips with adhering germinated conidia were taken out and counted for the number of germ tubes germinated at the specified times under differential interference contrast microscopy[16]. For the observation of nuclei and septum the conidia adhered on the glass coverslip were fixed in fixative solution (4% formaldehyde, 50 mmol/L phosphate buffer, pH 7.0, and 0.3% Triton X-100). After 30 min of fixation, coverslips were washed with phosphate-buffered saline (PBS), incubated with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) for 20 min and washed with PBS. Then the germinated conidia on glass coverslips was dyed with a 5 μg/mL solution of fluorescent brightener 28 for 5 min and washed with PBS for 5 times[18,20]. The conidia were then observed and photographed using a fluorescence microscope.

1.10 Quantitative real time PCR

One hundred milliliters of complete liquid medium were inoculated with 10^6−10^7 conidia and the harvested mycelia were disrupted by grinding. Total RNA was extracted using TRIzol reagent[18, 21]. The cDNA synthesis was carried out with 5 μg RNA using the RevertAid™ First Strand cDNA Synthesis Kit. The quantitative PCR reaction was done with SYBR®Premix *Ex Taq*™ with the thermal cycling condition of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Quantification of mRNA levels of different genes was performed using the $2^{-\Delta\Delta C_t}$ method. The 18S rRNA gene was used to standardize the mRNA levels of the target genes. Each assay and each experiment were repeated 3 times. To avoid or detect any possible contamination or carryover, appropriate negative controls containing no template were also subjected to the same procedure. Primers used in this study are listed in Table 1.

1.11 Enzyme activity assays and hydrolysis yield

The secreted proteins from different strains were analyzed for cellulase and β-mannanase activity as well as reducing sugar yield at 30, 40 and 50 °C using carboxymethyl cellulose or locust bean gum as a substrate. The activity assay was determined by using 3,5-dinitrosalicylic acid (DNS) method[22-23]. The reaction was started by mixing 5 mg/mL carboxymethyl cellulose or 5 mg/mL locust bean gum in 0.1 mol/L citrate-phosphate buffer with 0.1 mL secretory proteins and incubated at different temperatures. One unit of cellulase or endo-1,4-β-mannosidase activity is defined as amount of enzyme releasing 1 μmol of glucose or 1 μmol mannose per minute and the hydrolysis yield (μmol) of reducing sugar was determined by measuring the amount of reducing sugar produced from the reaction. Triplicates of each sample were analyzed and mean values were calculated.

Table 1 Primer pairs used for RT-PCR
表 1 用于 RT-PCR 的引物

Gene name	Primers sequence (5′→3′)
hac1	CAGATAAGAAGCCTGCCAAGAA
	GACGCTGTTCCTTTTCATCTTC
msdS	GCTTGCTTTGATGGAGGAAG
	TGACGCGGTACGCATAGTAG
rho3	ATACGGCGGGACAGGAGGAATT
	GTCGTAGGAAATCGTGGGCGGT
sec61	ATCCACACCGCCGTCTACATCA
	TTGGCAGCAATTTCAAAGTAGC
rab5	ATTGGAGCGGCTTTCCTCACCC
	GCTTATCGGGCTGTTCGTTGAC
ftt1	CAAACTCCTACCGTCAAAATGG
	GCCTCGATCTTCTGGCGGTACT
ypt1	TTCCGAACCATCACCTCGTCTT
	GGCGAGCCATGGTCAGGAAAGC
snc1	CGCCAGCAGGCACGCTCATTAG
	GGTGAGGGTTTGGGCGGTATGT

2 Results and Analysis

2.1 Introduction of the *A. fumigatus msdS* gene into *T. reesei*

We have previously cloned the *A. fumigatus* α-1,2-mannosidase *msdS* gene[16], whose reading frame encodes a polypeptide of 503 amino acids (55 kD) with homology to other members of the glycosyl hydrolase family 47. These glycosidases catalyze the hydrolysis of the terminal 1,2-linked-α-D-mannose residues from oligomannosidic N-glycans such as $Man_9GlcNAc_2$. The blast score of MsdS exhibits 82.0% amino acid identity with *Aspergillus clavatus* α-mannosidase, 77.8% with *Aspergillus oryzae* α-mannosidase, 73.7% with *Aspergillus niger* mannosyl-oligosaccharide α-mannosidase, and 41.7% with *T. reesei* (*Hypocrea jecorina*) α-mannosidase (TRIREDRAFT_45717).

As described under methods, an expression vector (pGM) containing the *gpda* promoter and *msdS* gene was constructed (Figure 1A and 1B). By transforming the expression vector pGM into *T. reesei* TU-6 strain, the *msdS*-expressing strain Tr-MsdS was obtained. RT-PCR analysis revealed that the *msdS* gene was expressed in Tr-MsdS strain but not its parental strain TU-6 (P<0.001) (Figure 1C). To detect if the heterologous MsdS protein was expressed, we prepared an anti-MsdS antibody using recombinant MsdS expressed in *E. coli*[16], however, the antibody we obtained was not specific (data not shown). Therefore, we further analyzed proteins extracted from the Tr-MsdS strain with LC-MS/MS. To this end, the secreted proteins of strain Tr-MsdS were separated by polyacrylamide gel, and protein bands corresponding to 40–60 kD were subjected to LC-MS/MS analysis. As a result, 16 peptides were identified as fragments of MsdS (gi159131578) (Figure 2A and Figure 1). Although, the

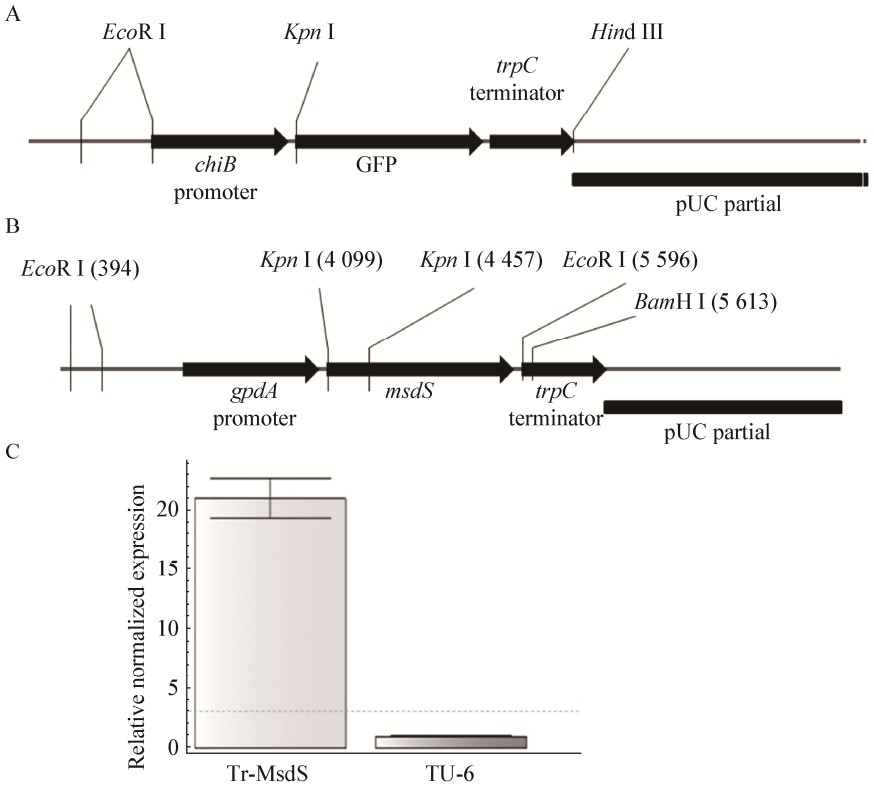

Figure 1 Expression of the *msdS* gene

图 1 *msdS* 基因的表达分析

Note: A and B: Schematic of the *msdS*-expressing vector construction. The parent vector containing the *chiB* promoter, GFP fragment and terminator *trpC* was used to generate a new recombinant vector by replacing the *chiB* promoter with the *gpdA* promoter and the GFP fragment with the *msdS* gene. Insertion of the *gpdA* promoter was done at restriction site *Eco*R I and *Kpn* I, while insertion of the *msdS* gene was done at *Kpn* I and *Bam*H I restriction site. C: Expression of the *msdS* gene in *T. reesei*. The expression of the *msdS* gene was measured with total RNA from the parental strain or Tr-MsdS cultured at 32 °C. RNA extraction and cDNA synthesis were carried out as described in method section. Results are repeated three times and presented as mean ± SD

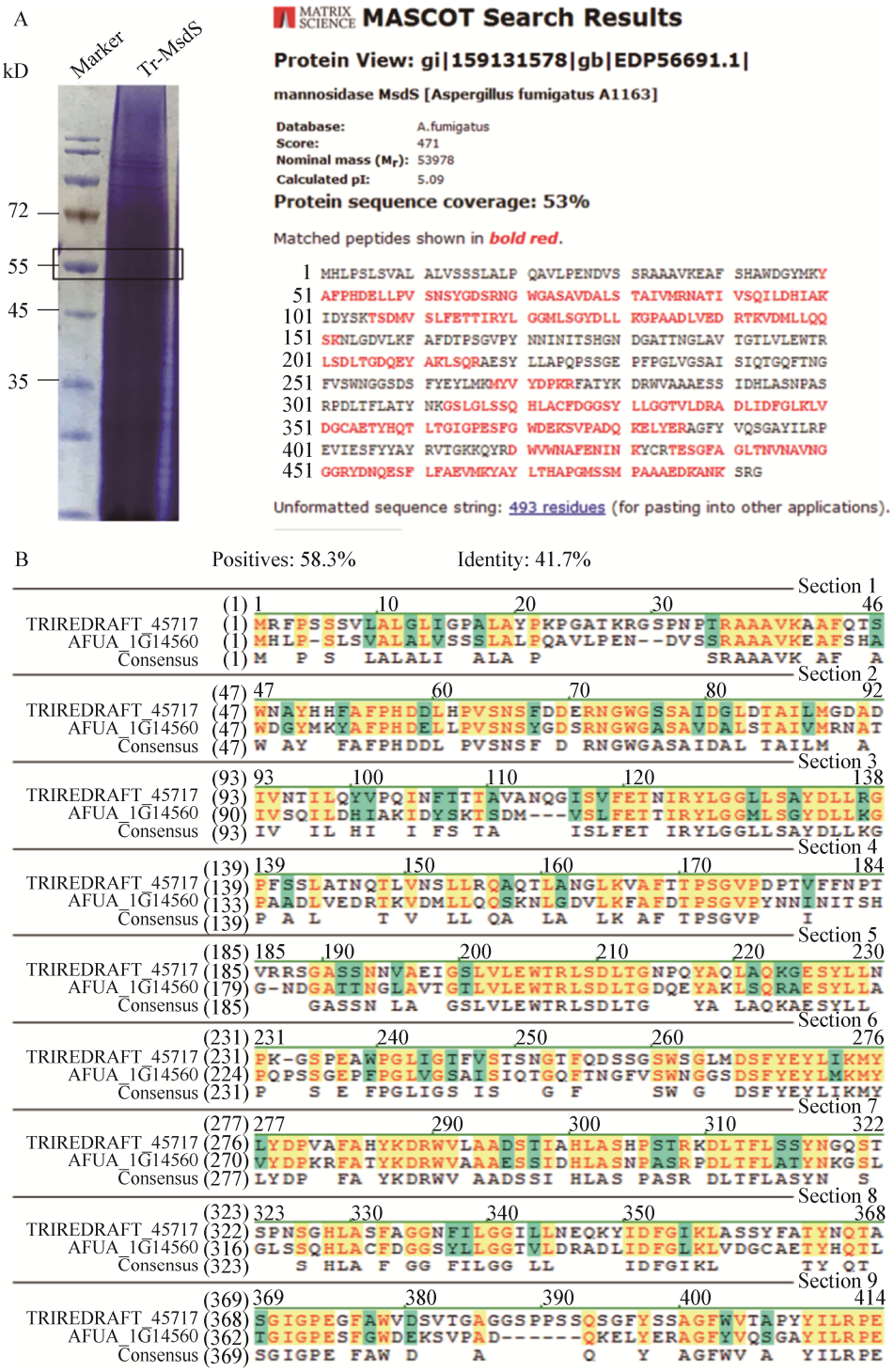

Figure 2 Confirmation of MsdS expression in *T. reesei* with LC-MS/MS

图 2 LC-MS/MS 分析确认 MsdS 在 *T. reesei* 中的表达

Note: In A, proteins extracted from the *msdS*-expressing strain Tr-MsdS were separated by 10% SDS-PAGE and stained with Coomassie R-250. Proteins corresponding to 40−60 kD were cut from polyacrylamide gels and analyzed using LC-MS/MS. The sequences were identified by SEQUEST with FASTA database of *A. fumigatus* from NCBI. In B, alignment of *A. fumigtus* MsdS and *T. reesei* TRIREDRAFT_45717

MsdS homolog (TRIREDRAFT_45717) was found in *T. reesei* (Figure 2B), no corresponding peptide was identified. These results demonstrated that *A. fumigatus* MsdS was successfully produced in strain Tr-MsdS, while the endogenous orthologue was not detected.

2.2 Phenotype of Tr-MsdS strain

The growth kinetics of *T. reesei* strains were studied on solid media by measuring colony diameter. The result showed that 28−32 °C were the favorable temperatures for both parent and Tr-MsdS strains, however Tr-MsdS strain was slightly temperature-sensitive at 42 °C as compared with its parent strain (Figure 3). Additionally, at 32 °C, the conidia produced by parent strain was more than that produced by Tr-MsdS strain (47%, 61% and 38% at 12 h, 24 h and 48 h, respectively), whereas after 72 h the conidia produced by Tr-MsdS strain appeared higher. This suggests that expression of MsdS leads to a slightly slower conidiation at early phase (Table 2).

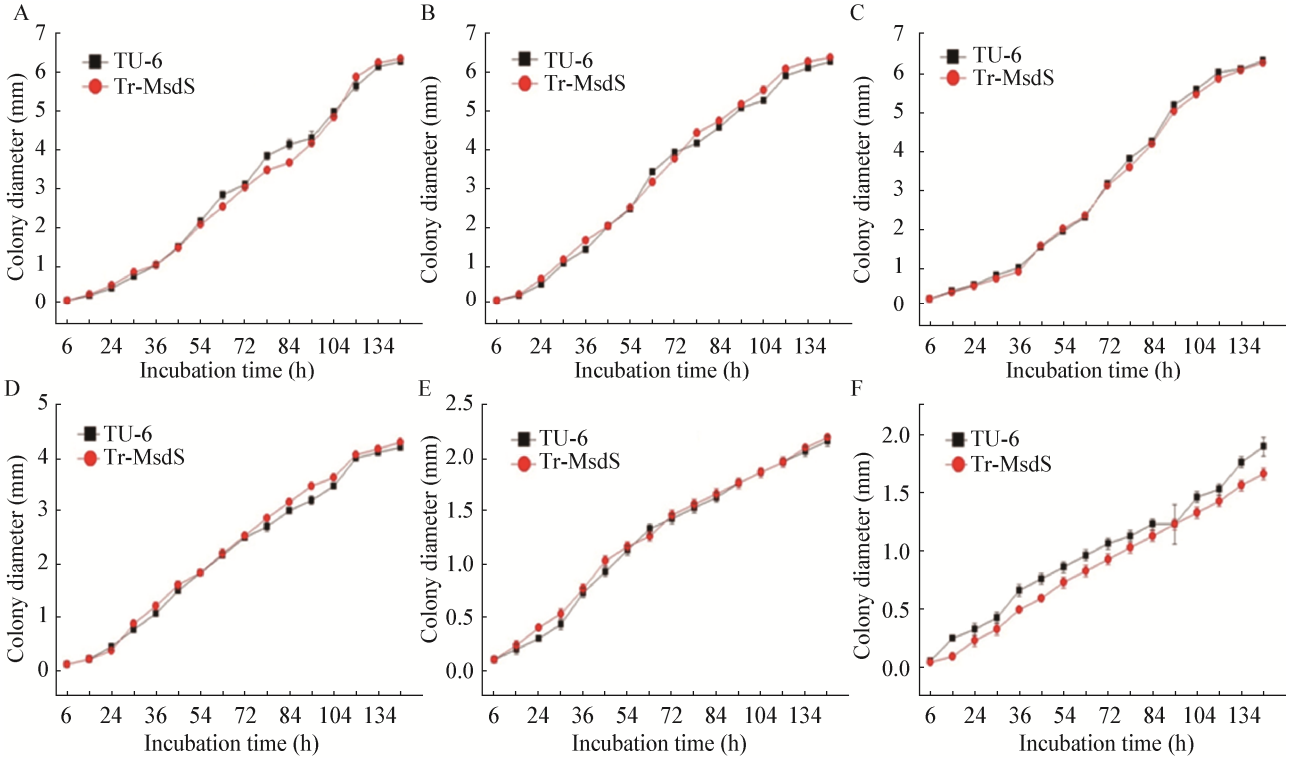

Figure 3 Growth kinetics of the TU-6 and Tr-MsdS strain

图 3 TU-6 和 Tr-MsdS 菌株的生长曲线

Note: Change in colony diameter with different time when same amount of conidia were inoculated and incubated at 28 (A), 30 (B), 32 (C), 38 (D), 40 (E) and 42 °C (F)

Table 2 Conidia produced by Tr-MsdS strain at 32 °C

表 2 Tr-MsdS 菌株在 32 °C 时的产孢统计

Time (h)	Strain	Spore count ($\times10^4$)	Time (h)	Strain	Spore count ($\times10^4$)
12	TU-6	3.5±1.3	96	TU-6	402.3±16.6
	Tr-MsdS	1.3±0.5		Tr-MsdS	453.8±11.7
24	TU-6	10.3±1.7	120	TU-6	531.8±28.7
	Tr-MsdS	2.5±1.0		Tr-MsdS	552.8±10.4
48	TU-6	16.0±2.5	144	TU-6	658.0±28.4
	Tr-MsdS	7.3±2.1		Tr-MsdS	897.5±35.5
72	TU-6	267.3±24.8	168	TU-6	644.5±41.3
	Tr-MsdS	376.0±12.0		Tr-MsdS	891.8±29.4

Note: Same amount of conidia was spotted on solid complete medium and incubated at 32 °C. Conidia were harvested at the specified times, resuspended in ddH_2O, and counted under microscopy using haemocytometer. A triplet of each strain was counted. The same experiment was repeated twice

As shown in Figure 4A, in the presence of antifungal reagent, the hyphal growth of strain Tr-MsdS was not affected at 28, 32 or 37 °C. Under a transmission electron microscope (TEM) (Figure 4B), the hyphal cell wall of strain Tr-MsdS grown at 32 °C was 30% more thickened as compared with that of the parental strain. When the temperature was raised to 37 °C, the thickness of the Tr-MsdS mycelia cell wall was only 13% more as compared with its parental strain. Additionally, the conidial cell wall of the Tr-MsdS strain formed at 32 °C was 9% thicker than at 37 °C, whereas the cell wall of the parental strain was 27% less thick at 37 °C than that at 32 °C (Figure 4C and Table 3). Interestingly, the Tr-MsdS strain showed less dense and irregularly scattered filamentous material around spore and hypha, whereas its parent strain showed denser, regular and prominent filamentous material surrounding spore and hyphae. These results indicate that hyphal cell wall thickness is changed in strain Tr-MsdS.

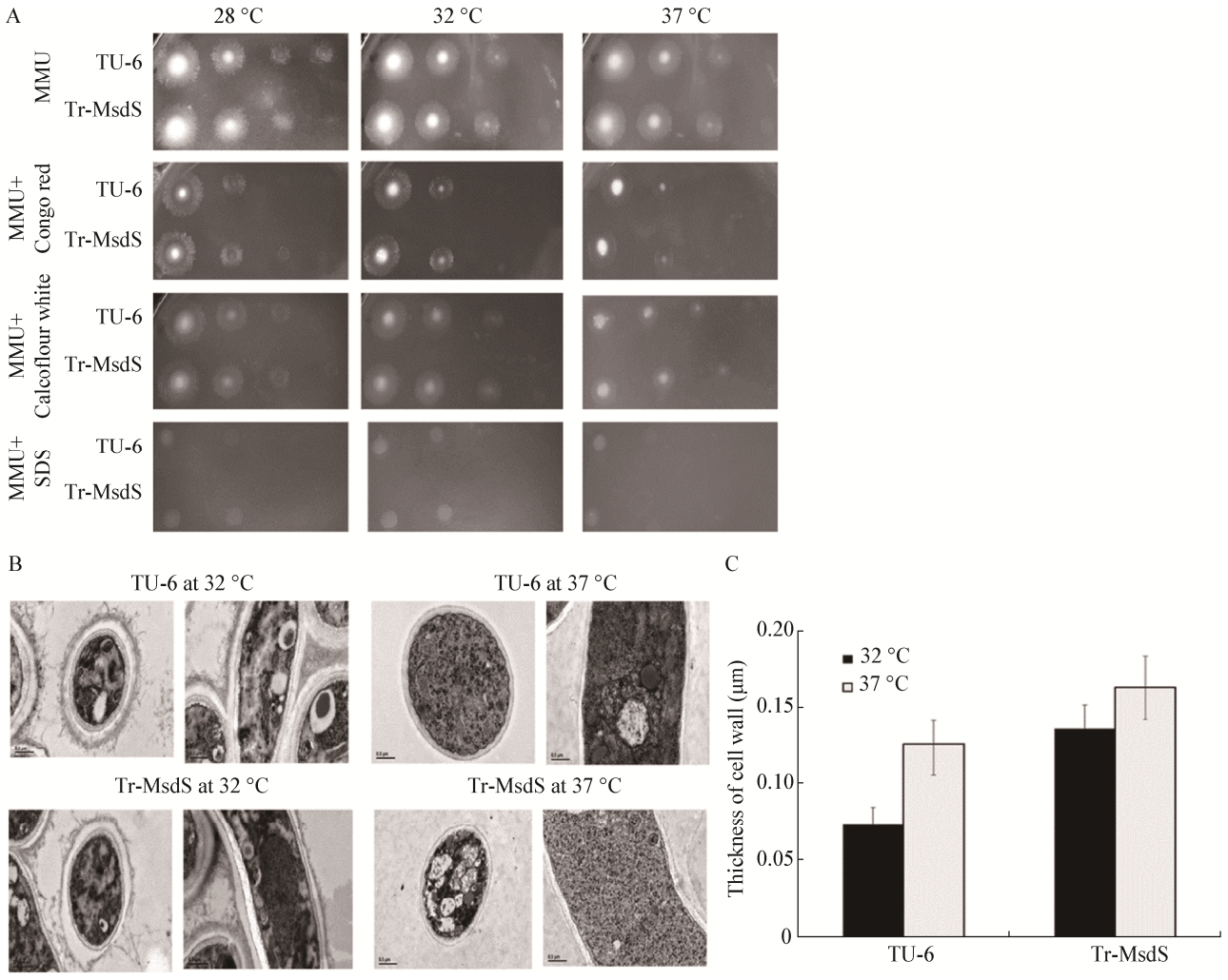

Figure 4 Phenotype and morphology of Tr-MsdS strain

图 4 Tr-MsdS 菌株的表型与形态

Note: In A, sensitivity to cell wall perturbing compounds was carried out by spotting conidiophores on solid MMU plates supplemented with 100 μg/mL calcofluor white, 150 μg Congo red or 40 μg SDS and incubating at 28, 32 and 37 °C. In B, the mycelia and conidial cells were fixed as described in method section and examined with transmission electron microscopy (TEM) (JEM-1400). In C, thickness (μm) of cell wall was measured under TEM

Table 3 Thickness of the cell wall of parent and Tr-MsdS cells
表 3 Tr-MsdS 与亲本的细胞壁厚度

Temperature	TU-6		Tr-MsdS	
	Hyphal (μm)	Conidia (μm)	Hyphal (μm)	Conidia (μm)
32 °C	0.06	0.06	0.14	0.07
	0.08	0.04	0.12	0.07
	0.08	0.07	0.11	0.09
37 °C	0.11	0.11	0.12	0.1
	0.12	0.12	0.19	0.12
	0.15	0.13	0.14	0.12

Note: Mycelia and conidial cells were fixed as described in method section. The thickness of cell wall was measured under transmission electron microscopy (TEM) (JEM-1400)

The cell wall components, including glycoprotein, glucan and chitin, were further analysed (Table 4). The glycoprotein content was reduced by 30%−40% in the Tr-MsdS strain at both 32 °C and 37 °C, while cell wall chitin was reduced by 15% at 32 °C and by 10% at 37 °C. Interestingly, α/β-glucan was found to be increased by 25% in strain Tr-MsdS. On the other hand, the cell wall glycoprotein was reduced in strain Tr-MsdS. Previously, 10%−27% reduction of α-glucan, mannoprotein, β-glucan and chitin were observed in the *A. fumigatus msdS*-knockout mutant[16]. The unfolded protein response (UPR) genes in strain Tr-MsdS was also analysed in this study. Real-time PCR analysis revealed a reduced transcript level of the UPR transcription activator factor *hac1* (Figure 5A). This could indicate that UPR is not induced in the Tr-MsdS strain, and there is no protein misfolding, which could have improved the growth and proteins secretion in strain Tr-MsdS. Besides, slightly enhanced expression of *sec61* and *rho3,* which mediates protein translocation across ER and controls the cell shape respectively, were observed in the Tr-MsdS strain (Figure 5A). Apparently, these results indicate a change in expression of the genes involved in the secretory pathway of *T. reesei* (Figure 5B).

When the dormant conidia start to germinate in *A. fumigatus*, a series of nuclear division occur, which is accompanied with an order of morphological events (switch from isotropic to polar growth) including appearance of first/second germ tubes and septation[18, 24]. In the *A. fumigatus msdS* mutant random budding and septation at an early stage of germination were observed[16]. In *A. nidulans*, the temperature-sensitive mutant which is unable to switch from isotropic to polar growth was also reported, although multiple points of polarity were established[18,24]. In some other studies, the abnormal morphology with balloon-like structures, swollen hyphae tips, and altered cell cycle were described with a chitin synthase deficient mutant of *F. oxysporum*[25].

Table 4 Cell wall components of Tr-MsdS strain at 32 °C and 37 °C
表 4 32 °C 与 37 °C 生长时 Tr-MsdS 菌株的细胞壁组分

Temperature	Strain	Alkali soluble		Alkali insoluble	
		Glycoprotein (μg)	α-glucan (μg)	Chitin (μg)	β-glucan (μg)
32 °C	TU-6	104.2±2.8 (100)	215.2±1.1 (95.9)	202.9±7.6 (100)	268.2±1.7 (81.4)
	Tr-MsdS	81.6±0.3 (71.3)	224.0±3.1 (100)	175.1±2.6 (86.6)	298.4±3.1 (100)
37 °C	TU-6	185.4±14.8 (100)	228.0±3.0 (94.4)	282.4±3.9 (100)	329.2±3.8 (78.2)
	Tr-MsdS	154.8±3.4 (61.3)	241.4±1.9 (100)	230.9±8.3 (89.7)	363.5±1.7 (100)

Note: Cell wall components were isolated and determined as described by Li et al.[16]. Ten milligrams of lyophilized mycelia were used as independent samples for cell wall analysis with three different biological repeats. The values shown are microgram of cell wall component per 10 mg dry mycelia (±SD)

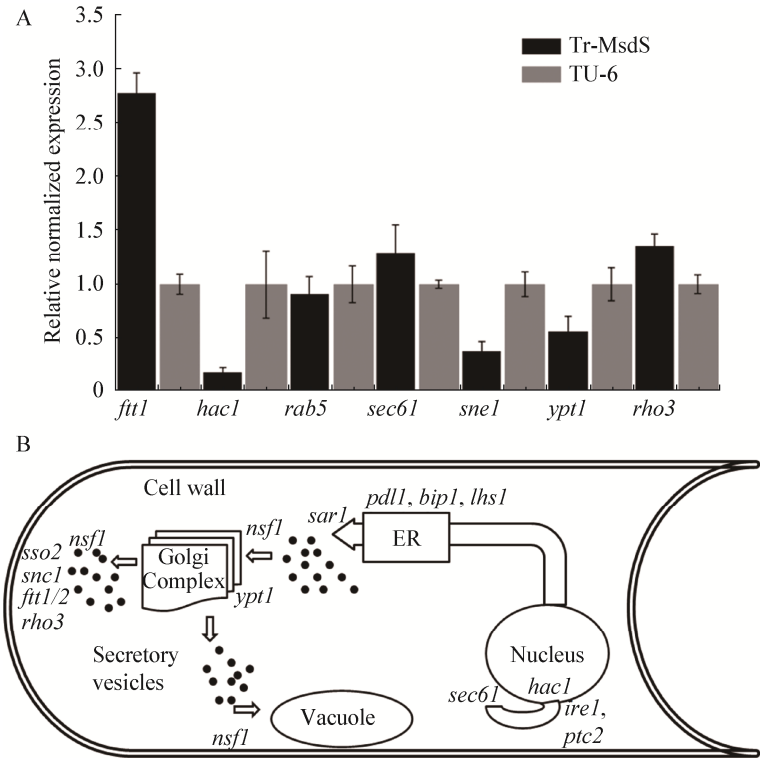

Figure 5 Activation of the genes involved in protein transport and the secretory pathway in *T. reesei*

图 5 里氏木霉蛋白质运输与分泌途径相关基因的激活

Note: In A, 10^6−10^7 conidia were inoculated 100 mL of complete liquid medium and cultured at 32 °C. Mycelia were harvested and disrupted by grinding. Total RNA was extracted and quantification of mRNA levels was performed as described under method section. The 18S rRNA gene was used to standardize the mRNA levels of the target genes. Primers used in this assay are listed in Table 1. Each assay and each experiment were repeated 3 times. Results were presented as mean±SD. In B**,** the pathway components constitute gene characterized in *T. reesei*. *hac1*, transcription factor of the UPR; *ire1*, sensor of UPR; *ptc2*, phosphatase acting as a negative regulator of UPR; *sec61*, major component of the protein translocation complex in ER membrane; *pdi1*, protein disulphide isomerase; *bip1* and *lhs1*, HSP70 family chaperones involved in protein folding in ER; *sar1*, small GTPase involved in vesicle budding from the ER; *ypt1*, Ras-type small GTPase involved in vesicle fusion into Golgi; *nsf1*, general fusion factor involved in multiple vesicle fusion steps; *snc1*, v-SNARE protein involved in vesicle fusion to the plasma membrane; *sso1/2*, t-SNARE proteins involved in vesicle fusion to the plasma membrane; *ftt1/2*, 14-3-3 type proteins involved in the last step of the secretory pathway; and *rho3*, Ras-type small GTPase involved in cell polarity and vesicle fusion with plasma membrane (adapted from Ref. 27)

In the present study, the Tr-MsdS and its parental strain were grown in media containing glucose as the carbon source and observed for the germination of conidium. As shown in Figure 6 and Table 5, both strains produced the first germ tube at 6 h and initiation of the second germ tube at 7 h. In the parental strain, the germination showed a typical and regularly branched apical growth usually at the early stage of germination, while less-branched hyphae at the later stage of germination. However, in strain Tr-MsdS, slightly swollen hyphal tips were observed together with multiple budding sites and random branching. In addition, during the early stage, the first germ tube grew longer, and then lateral germ tube appeared in strain Tr-MsdS. The spores of the parental strain germinated in a typical polarized pattern in which the first and second germ tubes occurred at 5 h and 6 h, respectively, and the first septation occurred after 9 h. Surprisingly, the occurrence of the first and second germ tubes was rapid until 8 h in parent strain and then slowed down, whereas strain Tr-MsdS started to generate the second germ tube more rapidly after 8 h. The chitin accumulation was observed at the basal and lateral portion of hyphae. Very few septation events occurred in both parental and Tr-MsdS strain. Previous studies have also reported that there is a proportional correlation of the increased level of chitin with the level of glucan depicting that the cells will

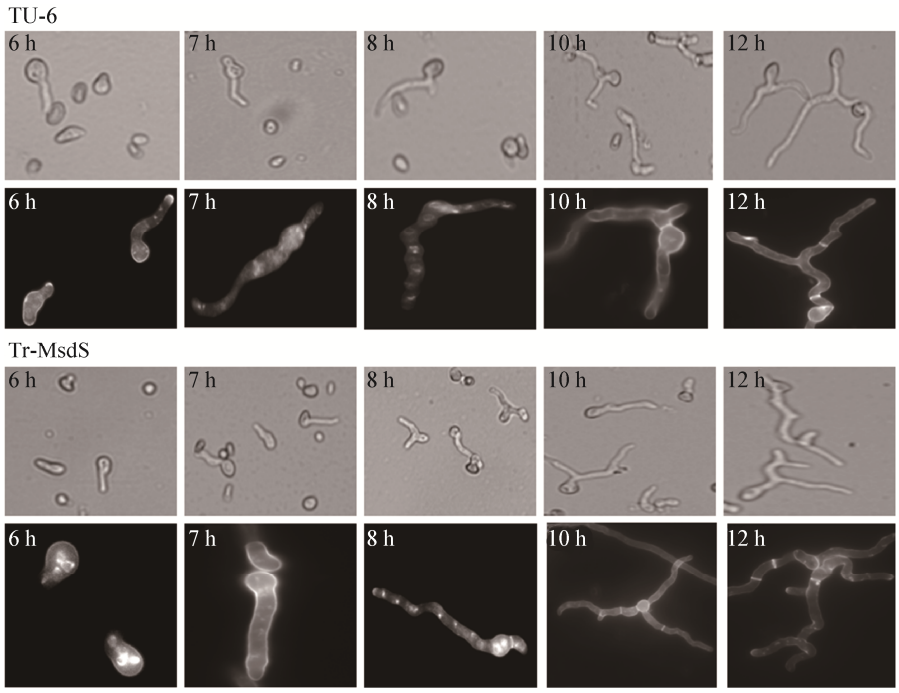

Figure 6 Germination of the TU-6 and Tr-MsdS strain

图 6 TU-6 和 Tr-MsdS 菌株的孢子萌发

Note: Freshly harvested conidia were inoculated on petri dish containing 10 mL minimal media and the germinated conidia were observed under microscope (40×) at the specified times. The germlings of the TU-6 and Tr-MsdS strain grown and fixed as described under method section. The nuclei and cell wall of the germlings were stained with DAPI and Calcofluor white respectively (100×)

Table 5 Statistics of germination of Tr-MsdS strain

表 5 Tr-MsdS 菌株的孢子萌发统计

Time (h)	Number of germ tube (Tr-MsdS strain)				Number of germ tube (TU-6 strain)			
	0	1	2	Hyper-branching	0	1	2	Hyper-branching
5	156.0±9.5	3.0±1.0	0.0	0.0	149.0±15.7	3.3±1.5	0.3±0.6	0.0
6	100.0±10	6.7±1.2	0.7±0.6	0.0	87.3±16.3	12.0±6.3	1.0±1.4	0.0
7	78.3±4.7	23.0±3.6	1.7±1.5	0.0	88.7±4.2	27.3±2.1	2.0±1.0	0.0
8	65.3±3.5	35.0±2.7	9.0±2.7	0.0	64.0±3.6	33.0±4.4	4.3±1.5	0.3±0.6
9	39.3±2.5	44.7±4.5	19.0±2.7	2.0±1.0	51.7±3.1	39.0±4.0	8.7±1.5	1.3±0.6
10	10.7±2.5	46.0±10.8	30.0±3.6	3.3±0.6	12.3±1.5	48.0±2.7	16.0±2.7	1.7±0.6
12	7.0±2.0	21.0±7.6	43.7±2.5	7.0±2.0	6.3±3.8	28.6±2.1	28.3±4.4	5.7±3.1

Note: Freshly harvested conidia were poured into a Petri dish containing glass coverslips and incubated in 10 mL of liquid medium. The adhered germlings on the coverslips were counted for number of germ tubes appear during different stages of germination under microscope. For each independent experiment approximately 100 conidia were counted and three independent experiments were carried out

turn on the cell wall compensatory mechanism as a reaction under stress[26]. These results demonstrate that the insertion of the *msdS* gene leads to more branched and random budding at the later stage of germination.

2.3 Glycosylation and protein expression in Tr-MsdS strain

To compare the N-glycosylation between parent and Tr-MsdS strain, the N-glycans were released from membrane and secreted proteins with PNGase F and analysed by MALDI-TOF. As shown in Figure 7, signals of $Man_{5-8}GlcNAc_2$ were detected in parent strain, among them $Man_8GlcNAc_2$ was the major glycoform. Although signals corresponding to $Man_{5-9}GlcNAc_2$ were detected in Tr-MsdS strain, the glycoform $Man_8GlcNAc_2$ was greatly reduced, suggesting an action of MsdS on N-glycans.

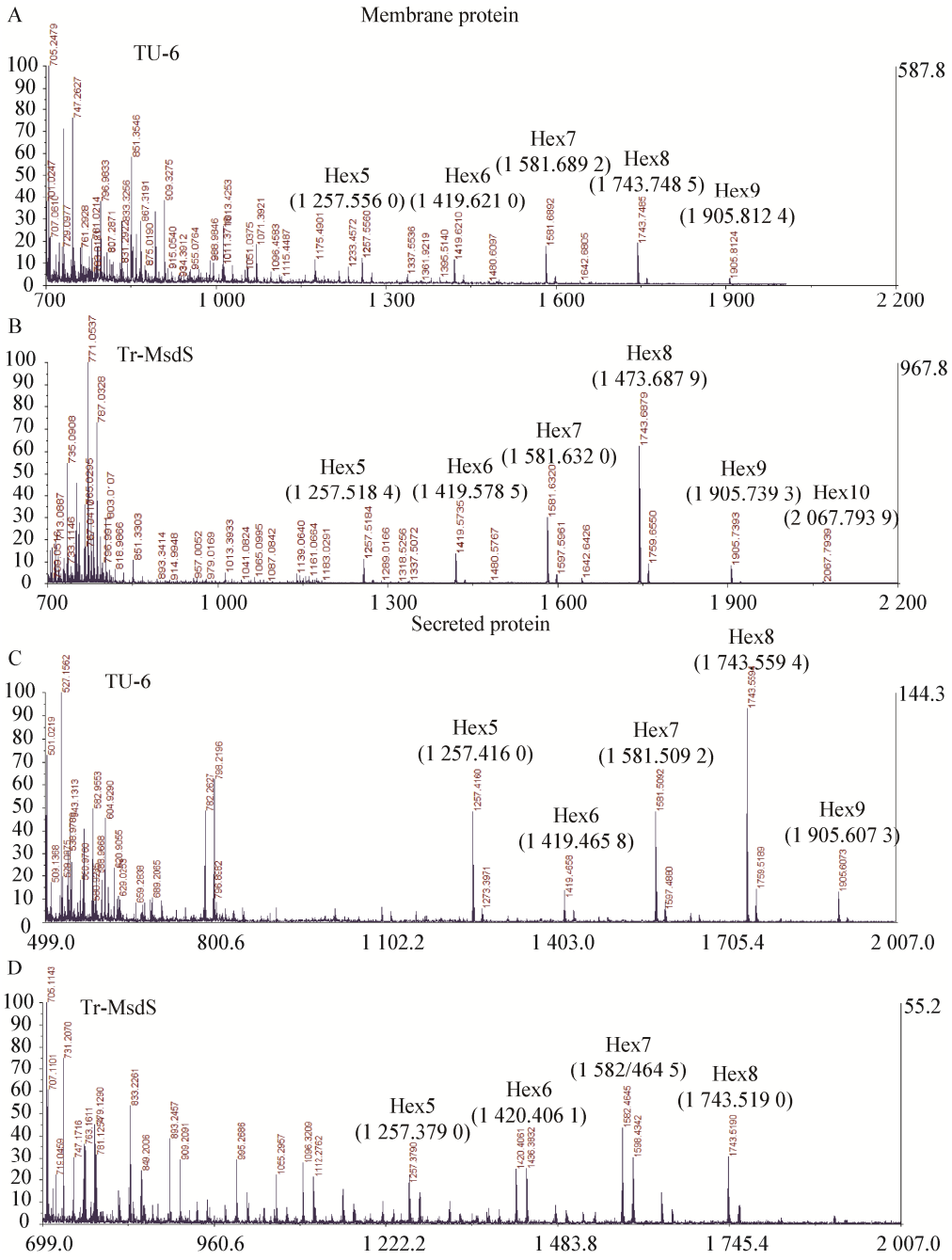

Figure 7 Detection of N-glycans on membrane and secreted proteins

图 7 细胞膜与分泌蛋白上 N-糖链测定

Note: N-glycans from membrane proteins (A) and secreted proteins (B) were released by PNGase F and subjected to mass spectrometry analysis

The MS/MS analysis of secreted proteins identified some proteins involved in the secretory and regulatory pathway of *T. reesei*, such as 14-3-3-like protein (gi|12054274), HSP70 (gi|30961863) and small GTPase of the Rab/Ypt family (gi|340519278). Saloheimo and Pakula reviewed the literature on the secretory pathway in filamentous fungi, including *T. reesei*[27]. Based on their overview[27], as summarized in Figure 5B, we performed real-time PCR of selected transcripts (Figure 5A). Interestingly, it was observed

that the genes encoding Sec61 (the major component of protein translocation complex in ER membrane), Ftt1 (a 14-3-3 type involved in the last step of the secretory pathway) and Rho3 (Ras-type GTPase involved in cell polarity and vesical fusion with plasma membrane) were found to be expressed higher in strain Tr-MsdS as compared with its parental strain, while expressions of the genes encoding Hac1, Rab5, Snc1 and Ypt1 were reduced in strain Tr-MsdS. These results suggest that the expression of MsdS affects protein transportation and secretion in *T. reesei*.

2.4 Cellulase and β-mannanase in Tr-MsdS strain

In the present study, cellulase and β-mannanase activity were analyzed using carboxymethyl cellulose and locust bean gum as substrates, respectively. Both activity and hydrolysis yield were found to be higher in strain Tr-MsdS as compare with its parental strain. As shown in Figure 8, glucose yield was 17.5%, 12.6% and 9.9% higher in strain Tr-MsdS at 30, 40 and 50 °C, respectively (Welch two sample *t*-test, $P<0.05$). Also, mannose yields in strain Tr-MsdS were 27.1%, 25.7% and 32.2% higher than that in the parental strain at 30, 40 and 50 °C, respectively (Welch two sample *t*-test, $P<0.05$). These observations suggest that the introduction of MsdS into *T. reesei* not only affects the glycosylation and cell wall synthesis, but also improves the lignocellulose degradation. Furthermore, the yield was found to be increased at temperature up to 50 °C (an industrially applicable temperature). It suggests that our knock-in strategy could be useful in optimizing the degradation of complex substrates in paper manufacturing or biofuel production.

3 Discussion and Conclusion

The primary steps of protein N-glycosylation are common among fungi and mammals including the site-specific transfer of $Glc_3Man_9GlcNAc_2$ from ER lumen to the de novo synthesized protein followed by subsequent trimming by glucosidase I, glucosidase II and a specific ER-residing α-1,2-mannosidase to form $Man_8GlcNAc_2$ structures (isomer Man8B), which later export the predominant Man8-isomer to the Golgi[28]. In human Golgi α-1,2-mannosidase (IA-IC) removes Man to yield the $Man_5GlcNAc_2$ structure (the precursor for complex N-glycans)[29-31], whereas in *Saccharomyces cerevisiae*[29], the N-glycan processing involves the addition of numerous mannose sugars

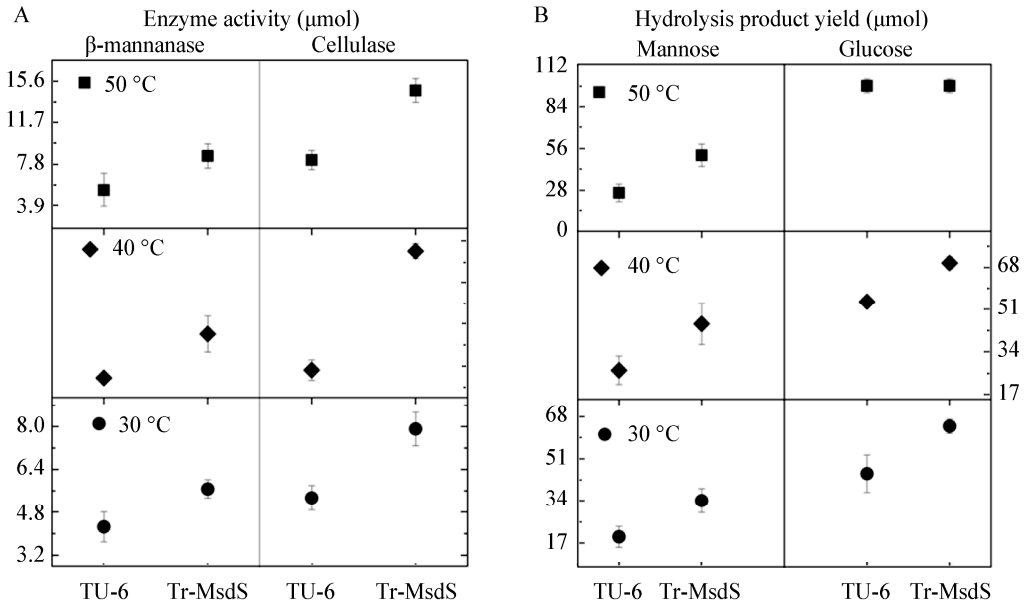

Figure 8 Hydrolysis yield and enzyme activity of secreted protein produced by Tr-MsdS strain

图 8 Tr-MsdS 菌株的分泌蛋白水解甘露聚糖与纤维素的活性分析

Note: The secreted proteins obtained from both TU-6 and Tr-MsdS strains were mixed with locust bean gum and CMC-cellulose. After reaction the enzyme activity of β-mannanase or cellulase (A) and release of mannose or glucose (B) were measured. Results were presented as mean ± SD

throughout the entire Golgi to form hyper-mannosylated N-glycan structures. Previous studies have explained the consequences of α-mannosidase deficiencies in different species[32-33]. The mutations in a human class I mannosidase result in a variety of MAN1B1-CDG phenotypes, such as a relevant congenital defect of glycosylation causing autosomal recessive intellectual disability. In contrast, the lack of endoplasmic reticulum mannosidase MNS1 has a little effect on outer chain synthesis in *S. cerevisiae*[34].

In *T. reesei*, the most common N-glycan structure is found to be mono-glucosylated high-mannose glycan $GlcMan_{7-8}GlcNAc_2$, which is similar to a transient structure of $GlcMan_9GlcNAc_2$ found in the ER[35]. However, many other N-glycan structures also have been reported in different strains. For example, the N-glycan of Cel7A in *T. reesei* strain QM9414 and ALKO2877 is single GlcNAc residues in three (Asn45, Asn270 and Asn384) out of four potential sites characterized in the catalytic domain[36], while Garcia et al. also identified complex N-glycans on endoglucanase I (Cel7B) from strain QM9414[17]. In hyper-producing glucosidase II-deficient strain Rut-C30, more complex N-glycan structures, including single GlcNAc and $Hex_{8-9}GlcNAc_2$ structures, are found on cellulases Cel7B, Cel6A and Cel5A[37-38]. These observations suggest that the N-glycan structures on cellulases produced by different mutant strains are different. *T. reesei* α-1,2-mannosidase can sequentially cleave all α-1,2-linked mannose sugars from a $Man_9GlcNAc_2$ oligosaccharide with broad substrate specificity that allows different oligosaccharide conformations[14]. However, it is also possible that some enzymes in glycosylation pathway or some glycosylation sites on cellulases were mutated as these mutants were screened for cellulases production or high-yield of protein expression. Somehow, it is no doubt that N-glycosylation is involved in protein secretion.

In contrast to the $Hex_{8-9}GlcNAc_2$ structures in *T. reesei*, the major glycoform N-glycan in *A. fumigatus* is $Man_6GlcNAc_2$[16]. In *A. fumigatus*, deletion of the Golgi α-mannosidase gene *msdS* causes defective N-glycan processing and gives rise to $Man_8GlcNAc_2$ glycoform, which is similar to that of the *T. reesei* TU-6 strain. Interestingly, the conversion of glycoform from $Man_6GlcNAc_2$ to $Man_8GlcNAc_2$ leads phenotypes such as defective cell wall, reduced conidiation and abnormal polarity in *A. fumigatus*[16], suggesting a species-specific N-glycan structure in different filamentous fungi.

As several studies have successfully reported the gene manipulation in *T. reesei* under the *A. nidulans gpdA* gene promoter and *trpC* (indole-3-glycerol phosphate synthase) terminator [39-44], in this study we constructed the *msdS*-expressing strain by generating the vector, in which the *msdS* gene is controlled under the *gpdA* promoter, and co-transforming the resulting vector into *T. reesei* with plasmid Takulox, which carries the *pyr4* gene as a selective marker. As a result, the *msdS*-expressing strain Tr-MsdS was successfully obtained. Analysis of strain Tr-MsdS revealed that, although its cell wall integrity was not affected, the expression of MsdS in *T. reesei* led to morphological changes, including increased cell wall thickness, abnormal polarity, and ballooned hyphal tips. Our results also demonstrated that the genes encoding proteins involved in protein translation, folding and vesicle transportation were induced, such as Sec61, HSP70, Rab/Ypt family GTPase, Rho3 and Ftt1. These results indicate that alteration of the N-glycan processing by MsdS affects the transport and secretion of proteins in *T. reesei*. Therefore it is not surprised to observe the changes in cell wall synthesis and polarized growth in strain Tr-MsdS as these two processes require proper transport of proteins in cell.

Filamentous fungi are thought as the most efficient lignocelluloses degraders, especially *T. reesei*[45-46]; however, there is still insufficient hydrolysis of lignocellulose due to the moderately low levels of mannan-degrading activity[47]. The hydrolysis has been improved by adding some exogenous mannosidases to the cellulase system. For example, in *Talaromyces cellulolyticus* the approximately 80% mannose yield was increased with high β-mannanase and β-mannosidase activities when supplemented with *A. niger* derived commercial enzyme[47]. It has been shown that the crystal of mannan can be degraded when endo-α-1,4-mannanase from *T. reesei*, endo-α-1,4-mannanase from *A. niger* and exo-α-1,4-mannosidase from *A. niger* are used[48]. To evaluate the effects of N-glycan processing on cellulases secretion, in this study, we further analyzed cellulase and β-mannanase activities in strain Tr-MsdS. Our

results showed that both cellulase-degradation and mannan-degradation activities were increased by 10%–32% (Figure 8) in strain Tr-MsdS, confirming that introduction of MsdS into *T. ressei* affects protein secretion and could be potentially used as a new strategy to improve the degradation of lignocellulose in an industrial context.

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