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Tmac1, a transcription factor which regulated high affinity copper transport in *Trichoderma reesei*

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ABSTRACT

The Mac1 protein was a transcriptional activator that sensed very low concentration of copper and regulated the copper transport in *Saccharomyces cerevisiae*. Here, we cloned a gene from *Trichoderma reesei* named *Tmac1*, whose deduced amino acid sequence showed 29% identical to Mac1p. Furthermore, two Cys-His repeats metal binding motifs of Tmac1p, one in the 354–369 C terminus and one in the 475–490 C terminus were also present in Mac1p. A deletion mutant of *Tmac1* was hypersensitive to the copper starvation and showed poor growth. Subsequently, the function was recovered by the gene complementation experiment. Furthermore, the *Tmac1* gene fully complemented growth defects of yeast Δ*Mac1* mutant. The expression of Tmac1p was activated at low concentration of copper and depressed when the concentration of copper excess 1 mM. Furthermore, the fluorescence intensity enhanced at copper starvation and decreased under copper excess by fusion the eGFP to the Tmac1p. It proved that the expression of Mac1p was exactly regulated by copper concentration, because eGFP and Mac1p were expressed under the control of the same one promoter. We also cloned a gene named *Tctr3* with bioinformatics. With a series of experiments, we proved it was the target gene of *Tmac1*. To sum up, *Tmac1* may encode a transcriptional activator regulated high-affinity copper transport in *T. reesei*.

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1. Introduction

Copper is an essential micronutrient for most eukaryotes. However, it would cause catastrophic damage to organisms when accumulated in excess (Robinson and Winge 2010). Therefore, cells have developed delicate mechanisms to acquire and maintain adequate intracellular copper concentrations (Pena et al. 1998). In *Saccharomyces cerevisiae*, copper is reduced from Cu (II) to Cu (I) by cell surface metalloreductases Fre1p and Fre2p, and transported across the plasma membrane by two high affinity membrane-associated transporters Ctr1p and Ctr3p (Hassett and Kosman 1995).

The *Ctr1* gene encodes a multispanning plasma membrane protein of 460 amino acids and is a necessary factor in high affinity copper uptake and supplies copper for all known copper-dependent physiological processes (Georgatsou et al. 1997; Sinani et al. 2007; Nakagawa et al. 2010). The *Ctr3* gene encodes another membrane-associated transporter which show little homology to Ctr1p. Under copper-limiting conditions, mRNA levels of *Ctr1* and *Ctr3* gene increase, while their expression is repressed under copper-replete conditions (Beaudoin and Labbe 2006). The delicate regulation was attributed to the copper sensing transcription factor Mac1p. Copper binds to the C terminus Cys-X_2-His metal binding motif of Mac1p and activate it DNA-binding activity. Subsequently, it binds to copper response element (5′-TTTGC(T/G)C(A/G)-3′) located in tandem or inverted repeats at the promoters of Ctr1p and Ctr3p, and regulate the copper adsorption of cell. Under copper-excess condition, another transcription factor Ace1p was activated to regulate the transcription of cellular copper-detoxifying gene, such as Cup 1, Crs5 and SOD1, which protected cells by chelating copper.

Trichoderma spp., one of the most widely distributed soil inhabitants, have been used in various fields of agriculture (Harman et al. 2004). In recent years, it also used for remediation of diverse pollutants and genetically enhanced transformants with bioremediation functions have been created (Akhtar et al. 2007). However, most studies focused on how to use *Trichoderma* to bioremediate metal pollutions. Molecular mechanisms of copper biosorption by *Trichoderma* remained less investigated.

We carried out a primary research on the copper biosorption mechanism in *Trichoderma reesei*. In the present study, we have identified a transcriptional factor *Tmac1*. With a series of experiments, we investigated the function of *Tmac1* in copper transport in *T. reesei*.

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2. Materials and methods

2.1. Strains and plasmids

A recipient strain *T. reesei* QM6a, plasmid pCAMBIA1300 and *Agrobacterium tumefaciens* strain AGL1 (a T-DNA donor) were kindly provided by associate Professor Chu Long Zhang from institute of biotechnology at Zhejiang University of Hangzhou, China. Δ*mac1* mutant of *S. cerevisiae* was obtained from ATCC (NO. 4000596) and maintained on YEPD medium (ATCC medium 2241). YPMDA medium, containing 0.5% yeast exact, 0.5% peptone, 0.05% $MgSO_4{\cdot}7H_2O$, 2% dextrose, 1.4% agar (w/v) was used to maintain *T. reesei* strains. For analysis of the gene functions, stains were grown on YPMGA medium (dextrose of YPMDA was replaced by glycerol). $CuSO_4{\cdot}5H_2O$ was a donor of Cu^{2+}. Czapek agar (CA) medium containing 0.1% K_2HPO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% KCl, 0.001% $FeSO_4{\cdot}7H_2O$, 3% Sucrose, 1.4% agar (w/v) was used to screening the mutants.

2.2. Nucleic acid manipulations

DNA exaction was carried out by standard CTAB protocol. PCR primers (Table 1) were designed by primer premier 5.0 and synthesized from Sangon Inc. (Shanghai, China).

Total RNA was isolated by the Trizol method (Invitrogen), and semi-quantitative reverse transcriptase PCR (sqRT-PCR) were carried out according to the TaKaRa PrimeScript RT-PCR kit. Primers RTmU and RTmL were designed to amplify the *Tmac1* transcript (153 bp), and primers RgaU and RgaL were designed to amplify the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene (119 bp) as an inner reference.

2.3. Search for Tmac1 and its target genes

We queried the *T. reesei* genome and used BLAST to search gene based on the homolog domain of Mac1p of yeast. Subsequently, a homologous gene named *Tmac1* in *T. reesei* was identified and analyzed by multiple alignments using the program ClustalX (Thompson et al. 2002). Furthermore, we searched the target genes of *Tmac1* based on the homolog domain of Ctr1p and Ctr3p of yeast. With a series of experiments, a target gene of *Tmac1*, named *Tctr3*, was isolated.

2.4. Construction of gene replacement plasmids

Plasmid pCAMBIA1300 was digest by *Xho*I and self-ligation by T4 ligase for the purpose to remove *hph* cassette. The resulting plasmid was pC1300-h. Subsequently, A 1608 bp fragment containing the *hph* ORF (1026 bp), promoter regions (319 bp) and terminator of CAMV35S (263 bp) was amplified from plasmid 1003 by the HiFi polymerase using primers TkhU (upper) and TkhL (lower). *Xba*I site and *Bam*HI site were added to upper primer and lower primer, respectively. After digestion with the appropriate restriction enzymes, the fragment was gel purified and inserted into *Xba*I/*Bam*HI-digested pC1300-h to produce plasmid pC1300kh. Then, the resulting plasmid was digested with *Bam*HI/*Sac*I and ligated with an 1176 bp fragment (contain 3′ flanking sequences of *Tmac1*, digested by the same enzyme as above) to produce p1300KL. The 1205 bp fragment containing 5′ flanking sequences of *Tmac1* was amplified using primers Kma5U and Kma5L. *Pst*I site and *Xba*I site were added to upper primer and lower primer, respectively. Subsequently, the fragment was digested with appropriate restriction enzymes and inserted into *Hin*dIII/*Xba*I-digested p1300KL. The resulting plasmid pCkmac1 containing the *Tmac1* gene replacement cassette was used to gene *Tmac1* knockout (Fig. 1a). The *Tctr3* gene replacement cassette was constructed according to the *Tmac1* gene replacement cassette. The 834 bp 3′ flanking fragment of *Tctr3* was amplified by primers Kctr3U and Kctr3L, and then inserted into the plasmid pC1300kh (digested by *Bam*HI/*Sac*I). The 1070 bp 5′ flanking fragment of *Tctr3* was amplified by primers Kctr5U and Kctr5L, and then inserted into the plasmid (digested by *Hin*dIII/*Xba*I) to produce the gene replacement plasmid of *Tctr3*.

2.5. Construction of gene complementation plasmid

A 795 bp fragment containing neomycin resistance gene ORF was amplified by primers TnroU (upper) and TnroL (lower), and digest with *Xho*I/*Eco*RI. Then, the fragment was inserted into *Xho*I/*Eco*RI-digested plasmid pCAMBIA1300. Subsequently, the resulting plasmid was digest by *Eco*RI and connected with the 318 bp fragment (promoter of *trpC* of *Aspergillus nidulans*), which produced the plasmid pC1300NR. A 252 bp fragment containing Terminator of CAMV35S was digested by *Sac*I and inserted into *Sac*I digested pC1300NR. Then, the produced plasmid was digested by *Xba*I and *Kpn*I, and was connected with *Xba*I/*Kpn*I digested fragment (3138 bp, containing the promoter and ORF of gene *Tmac1*), which produced plasmid pCcmac1 (Fig. 1b). To locate the Mac1p in cells, Mac1p was expressed with the eGFP (Enhance Green Fluorescence Protein) fused to the C-terminus (Mac1-eGFP), and unfused eGFP set as control. Expression plasmid construction was according to methods of complementation plasmid construction (Fig. 1c). Fluorescence microscopy was carried out using a Leica DM2500 equipped with Leica DFC425C camera. Images were obtained using Leica application suite software.

Functional complementation of yeast Δ*mac1* mutants was carried out by the plasmid PRS416-Tmac1, which contained the Mac1 ORF of *T. reesei*. Plasmid construction was performing as follow: a 1526 bp fragment containing the *ADH1* promoter of *S. cerevisiae* was amplified from plasmid pGADT7-Rec using primers PadhU and PadhL, and inserted into the plasmid pRS416 (digested by *Hin*dIII/*Bam*HI) to produce the plasmid pRS416P. Furthermore, a 371 bp fragment containing the *ADH1* terminator of *S. cerevisiae* was amplified from plasmid pGADT7-Rec using primers TadhU and TadhL, and inserted into pRS416P (digested by *Xba*I/*Sac*I) to produce the plasmid416-ADH1. ORF of *Tmac1* was inserted into the plasmid pRS416-ADH1 digested by *Bam*HI/*Xba*I. The transformants method was according to Pena et al. (2000).

2.6. Methods of transformants

A. tumefaciens-mediated transformation (ATMT) Transformation was carried out according to (De Groot et al. 1998) with some modifications. AGL1 containing plasmid pctr2 was grown on LB supplemented with kanamycin (50 μg/ml) and rifampin (50 μg/ml). After incubation at 28 °C for 24 h on a rotary shaker (220 rpm), the bacterial cells were diluted to OD_{600} = 0.20, and inoculated in induction medium (IM) and cultured at 28 °C and 250 rpm for 6 h. Subsequently, conidia of *T. reesei* strain and AGL1 cells were mixed at a ratio of 1:1 and a total of 200 μl mixture was spread onto a cellophane sheet and placed on IM plate (90 mm in diameter) containing 0.2 mM acetosyringone. After 48 h of incubation at 23 °C, the cellophane sheet was transferred to CA plate containing 200 μg/ml cefotaxime and 150 μg/ml hygromycin B and incubated at 25 °C. Each putative mutant was subsequently transferred to PDA medium containing 200 μg/ml cefotaxime and 150 μg/ml hygromycin B. Mitotic stability was test by subculturing five generations on PDA medium without hygromycin B.

2.7. Screening of ideal transformants

PCR were used to screen the ideal gene knock-out transformants. First, primers FhphU and FhphL were used to amplify the

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