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Adenine deaminase is encoded by *Tad*1 and participates in copper accumulation in *Trichoderma reesei*

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ABSTRACT

We cloned a novel *Tad*1 gene and demonstrated that this gene is closely involved in copper bioaccumulation in *Trichoderma reesei*. *Tad*1 gene encodes a 510 amino acids protein of the amidohydrolase superfamily which belongs to COG0402. We found that adenine was the most efficient substrate of Tad1 protein among the substrates used in this study. Gene function was also investigated by overexpression and RNA interference. Results showed that copper accumulation increased in mutant cells when *Tad*1 was overexpressed; by contrast, copper accumulation significantly decreased when *Tad*1 was inhibited. To investigate the function of *Tad*1 in copper bioaccumulation, we determined adenine, hypoxanthine, and xanthine concentrations by reversed phase HPLC. *Tad*1 overexpression induced a substantial production of xanthine, which functions in binding numerous copper ions and reducing copper concentration. We further compared the gene expression profile of AT01 with that of a wild-type *T. reesei* strain grown in a medium containing 1.0 mM Cu^{2+} by performing DNA microarray. Several upregulated genes in the mutant were associated with adenine or copper metabolism.

1. Introduction

Copper is a ubiquitous metal present in the environment and derived from mining, metal processing, and electroplating. This element is an essential micronutrient in many eukaryotes because it is a catalytic cofactor in various enzymes that catalyze redox reactions or oxygen chemistry (Labbé et al., 1997). At very high levels, copper in the environment is harmful to living cells because this element interacts with proteins, DNA, and lipids, causing adverse health conditions. Hence, studies have focused on the biological accumulation of pollutants in various microorganisms.

For several decades, *Trichoderma* spp., one of the most widely distributed soil microbes, has been used as either a biocontrol or bioremediation agent in agriculture. Copper accumulation in *Trichoderma* has also been intensively investigated (Anand et al., 2006). Furthermore, studies have focused on the practical applications of *Trichoderma* in the removal of copper from polluted environments. However, the mechanism of copper transport or copper resistance in microorganisms has rarely been investigated. For instance, (Martinez et al., 2008) found that *Trichoderma reesei* is an ascomycete widely used as a source of cellulases and hemicellulases that catalyze the hydrolysis of plant cell wall polysaccharides. *T. reesei* is also an ideal candidate used in the bioremediation of heavy metals because this ascomycete exhibits different traits, such as high adaptability to stressful environments and active absorption potential of different soil pollutants.

Saccharomyces cerevisiae has been used as an ideal model to study copper metabolism in eukaryotes because many molecular components involved in homeostatic copper metabolism in yeast are conserved in other eukaryotes (Huffman and O'Halloran, 2001). Studies have also developed the mechanism of high-affinity copper (activated under copper starvation) transport, manipulated by Mac1p (a transcriptional factor) as well as Ctr1p and Ctr3p (transmembrane proteins), in yeast. However, low-affinity copper (activated under excess extracellular copper) transporters, particularly in transmembrane copper ion channels, have been rarely studied (Peña et al., 2000). With excess extracellular copper, copper ions can be transported through a putative low-affinity copper transporter (Portnoy et al., 2001). Once transported in the yeast cytoplasm, copper is delivered to target organelles by copper chaperones, such as Atx1, Ccs, and Cox17, which are regulated by intracellular free copper ions. Ace1p, a transcription factor, is also activated by excess copper ions and regulates the expression of metallothionein, which may eliminate copper toxicity (Harrison et al., 2000).

Adenine deaminase (ADE) catalyzes the conversion of adenine to hypoxanthine; hypoxanthine is further oxidized to uric acid by xanthine oxidase via a xanthine intermediate (Kamat et al., 2011). ADE also participates in purine salvage pathway. Many studies on ADE have focused on bacteria, archea, and some fungi because this enzyme is present only in some organisms (Oestreicher et al., 2008). Some of the intermediate metabolites generated from purine

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metabolism can bind to copper in yeast cells and are possibly linked to the absorption activity of copper from exterior environments (Lippert, 2000). However, the functions of ADE isolated from *T. reesei* in copper absorption have not been reported yet. The relationship of ADE with copper metabolism in *Trichoderma* remains unclear.

Our previous study revealed that AT01 mutant can highly accumulate copper (Fu et al., 2010). In the present study, T-DNA insertion resulted in the upregulated expression of the *Tad*1 gene. We investigated the function of the Tad1 protein in copper accumulation in *T. reesei* by conducting RNA interference (RNAi) and DNA microarray.

2. Materials and methods

2.1. Strains and plasmids

Recipient strain *T. reesei* QM6a, plasmid pCAMBIA1300, and *Agrobacterium tumefaciens* strain AGL1 (a T-DNA donor) were provided by Chulong Zhang (Institute of Biotechnology, Zhejiang University, Hangzhou, China). Plasmid pSilent-1 was kindly provided by Dr. Nakayashiki (Nakayashiki et al., 2005). YPDA medium, containing 0.5% yeast exact, 0.5% peptone, 0.05% $MgSO_4{\cdot}7H_2O$, 2% glucose, and 1.4% agar, was used for the copper uptake. $CuSO_4{\cdot}5H_2O$ was used as the Cu^{2+} donor. AT01, a mutant of *T. reesei*, was constructed by *A. tumefaciens*-mediated transformation (ATMT). AT01 exhibits high copper accumulation capability (Fu et al., 2010).

2.2. Nucleic acid manipulation

DNA was extracted using a standard cetyltrimethylammonium bromide protocol. Total RNA was isolated using the Trizol method (Invitrogen); cDNA was then synthesized using TaKaRa PrimeScript™ II First Strand cDNA synthesis kit (6210A). Quantitative real-time PCR (qRT-PCR) was performed using the TaKaRa PrimeScript RT-PCR kit (DRR081A) on Biotech Ftc-3000. A total of 25 μl was used in the PCR system containing 12.5 μl of SYBR Premix Ex Taq, 1 μl of forward primer, 1 μl of reverse primer, 1 μl of cDNA template, and 9.5 μl of deionized water. The PCR reaction conditions used were as follows: preheating at 95 °C for 30 s; 35 cycles of 95 °C for 20 s; 55 °C for 30 s; and 72 °C for 20 s. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene (119 bp) was set as the internal reference. The expression levels were relative to the wild-type (WT) strain and calculated using the following equation:

$$\text{Relative mRNA} = 2^{-[(\text{Ctt}-\text{Ctti})-(\text{Ctw}-\text{Ctwi})]}$$

where Ctt Ct value of the target gene template, Ctti Ct value of the internal reference of the target gene template, Ctw Ct value of the WT strain template, and Ctwi Ct value of the internal reference of the WT strain template.

Twofold differences in the expression level were set as significant differences. The PCR primers (Table S1) were designed using Primer Premier 5.0 and synthesized by Sangon Inc. (Shanghai, China).

2.3. Plasmid construction

pCAMBIA1300 plasmid was digested using *Eco*RI/*Xho*I to cleave the Camv35S promoter and *hph* open reading frame (ORF). The fragment was then gel purified, forming pC1300-h plasmid. A 1361 bp fragment containing the *hph* ORF (1026 bp) promoter region (318 bp) was amplified from the plasmid 1003 by HiFi polymerase using primers ThphU (upper) and ThphL (lower). *Eco*RI and *Xho*I sites were added to upper and lower primers, respectively. The fragments were digested by the appropriate restriction enzymes, gel purified, and inserted into pC1300-h plasmid to produce pC1300th plasmid. A 252 bp fragment containing CAMV35S terminator was amplified using T35SU and T35SL primers, digested with *Sac*I/*Eco*RI, and inserted into *Sac*I/*Eco*RI-digested pC1300th plasmid to produce pC1300thTE plasmid. To construct the overexpressed plasmid, we amplified a 3188 bp fragment of *Tad*1, in which an ORF of 1533 bp in length and a promoter of 1640 bp in length were found, by using OTad1U and OTad1L primers. The amplified products were then inserted into pC1300thTE plasmid (digested with *Xba*I/*Sac*I) to produce the overexpressed pC1300Tad1oe plasmid (Fig. S1A). To construct an RNAi plasmid, we cloned a 918 bp fragment containing the Trpc terminator and CUT intron from pSilent-1 plasmid. The fragment was then inserted into pC1300th plasmid and digested with *Hin*dIII. The Trpc promoter was also amplified from pSilent-1 plasmid and then inserted into the plasmid digested with *Sac*I to produce pC1300silent plasmid. We inversely inserted a 944 bp fragment of *Tad*1 cDNA (amplified by RiTad15U/RiTad15L and RiTad13U/RiTad13L) into the two sides of the CUT intron to express the hairpin RNA and produce pC1300silent-Tad1 plasmid (Fig. S1B). The methods used to transform and screen the target mutants were performed according to our previous studies (Fu et al., 2012).

2.4. Prokaryotic expression of Tad1

The ORF of *Tad*1 was amplified with pTad1U and pTad1L primers. *Nde*I and *Eco*RI sites were respectively added to the upper and lower primers. The fragments were then ligated using pMD18-T simple plasmid (TaKaRa Biotech Co., Ltd.). These fragments were digested using appropriate restriction enzymes, gel purified, and inserted into *Nde*I/*Eco*RI-digested pet28a plasmid to produce pet28a-Tad1, a prokaryotic expression plasmid. This plasmid was subsequently transformed into *Escherichia coli* BL21 (DE3) competent cells. A single colony was grown overnight at 37 °C in 20 ml of Luria–Bertani (LB) medium containing 50 μg/ml kanamycin. 5 ml of the bacterial suspension was inoculated in 1 L of LB medium. The cell cultures were grown at 37 °C until an optical density (OD_{600}) of 0.6 was reached; afterward, 0.5 mM isopropyl β-thiogalactoside (IPTG) was added to induce protein expression. After 4 h of cultivation, the cells were collected by centrifugation and ultrasonicated. These cells were further centrifuged and resuspended in 50 mM PBS containing 8 M urea for 2 h. The soluble proteins were separated by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant liquid was collected and filtered through a Millipore membrane filter with an average pore diameter of 0.45 μm and then purified with a Ni agarose gel chromatographic column eluted with 50 mM PBS containing 8 M urea. The proteins were refolded by dialysis in a gradient urea solution and separated by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant liquid was collected and filtered through a Millipore membrane filter with an average pore diameter of 0.45 μm. Protein concentration was determined using a BCS kit (Beyotime Institute of Biotech Ltd, China.).

2.5. ADE activity

The deamination ability of Tad1 protein was determined using a coupled assay with glutamate dehydrogenase (GDH). The protein powder of Tad1 was diluted with PBS to obtain a concentration of 0.25 mg/l. NADPH (0.2 mM), α-ketoglutarate (15 mM), GDH (50,000 U/L), and different substrates (adenine, cytosine, folic acid, and guanine) at various concentrations (10, 15, 20, 25, and 30 mM) were used (Kamat et al., 2011). The ADE activity was calculated using the following equation:

$$\text{ADA(U/L)} = \Delta A/\min \times V_t/V_{sw} \times 1000/\varepsilon$$

where V_t is the total volume of the reaction system, V_s is the volume of the sample, and ε is the molar extinction coefficient of NADPH.