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Functional Characterization of the Copper-Transporting P-Type ATPase Gene of *Penicillium janthinellum* Strain GXCR

Hongmin Lai[1,2,3†], Changbin Sun[1,2,3†], Huaying Tang[1,2,3†], Xianwei Fan[1,2,3], Yili Ma[1,2,3], and Youzhi Li[1,2,3*]

[1]*Guangxi Key Laboratory of Subtropical Bioresource Conservation and Utilization,*
[2]*College of Life Science and Technology,* [3]*Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering, Guangxi University, Nanning, Guangxi 530005, P. R. China*

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Copper (Cu)-transporting P-type ATPase (CTPA) genes have been documented to play an important role in resistance to heavy metals. However, our understanding of roles of CTPA genes of the filamentous fungi was based only on sequence similarity prediction before. In a previous study, we isolated a *Penicillum janthinellum* strain GXCR of higher tolerance to Cu (200 mM). In this study, we cloned the partial cDNA of CTPA gene, named *PcpA*, from the strain GXCR. Sequence alignment indicated that the cloned cDNA sequence has the highest identity (94.4%) with a predictive CTPA gene of *Aspergillus clavatus*. The *PcpA*-encoded protein, termed PcpA, has classical functional domains of CTPAs, and shows differences from reported CTPAs in some specific sequence motifs and transmembrane regions. Expression of the *PcpA* was induced by extracellular Cu, cadmium (Cd), and silver (Ag). *PcpA* RNA interference (RNAi) mutants with a reduced level of *PcpA* mRNA were more sensitive to Cu, iron, Cd, and Ag than the wild-type (WT) strain GXCR. When grown in the presence of Cu, iron, and Cd, intracellular Cu and iron contents in the *PcpA* RNAi mutant were significantly ($P<0.05$) lower than those in the WT; However, intracellular Cd content in the mutant was significantly ($P<0.05$) higher than that in the WT. Taken together, it can be concluded that the *PcpA* functions in Cu uptake and homeostasis, iron uptake, and Cd export from the cytosol to the extracytosol.

***Keywords*:** *Penicillium*, P-type ATPase, *PcpA* gene, resistance, heavy metal

Copper (Cu) has dual effects on the living organisms: beneficial to normal cellular processes at trace levels and toxic to cells at high concentrations (Valentine and Gralla, 1997; Kershaw *et al*., 2005). In order to prevent the damage to cells form intracellular excessive Cu, the living organisms have to employ resistance mechanisms, such as reduced influx, facilitated efflux, intracellular translocation, sequestration, and modification of Cu (Harrison *et al*., 2000; Clemens, 2001; Hall, 2002; Ahearn *et al*., 2004; Balamurugan and Schaffner, 2006). These mechanisms are controlled by a number of genes (Harrison *et al*., 2000; Clemens, 2001; Hussain *et al*., 2004; Silver and Phung, 2005). Of genes identified, Cu-transporting $P1_B$ ATPase (CTPA) genes are more important owing to their involvement of various aspects on Cu resistance mechanisms (Solioz *et al*., 1994; Solioz and Odermatt, 1995; Axelsen and Palmgren, 2001; Puig and Thiele, 2002; Voskoboinik *et al*., 2002). CTPAs are conserved in the organisms studied and characterized not by multimembrane-spanning regions but by some unique sequence motifs (Puig and Thiele, 2002; Argüello *et al*., 2007). Fungi are eukaryotic microbes. The number of CTPA genes reported in fungi is growing (http://ww w.ncbi.nlm.nih.gov/). Except for some yeast CTPA genes (Rensing *et al*., 1999), our understanding of CTPA genes of the other fungi was only based on sequence similarity prediction. The fungi *Penicillium* spp. are of great importance to environment, industry, and agriculture. The draft genome of *P. chrysogenum* Wisconsin strain 54-1255 indicates three possible genes encoding CTPAs, which are under accession numbers of AM 920421, AM920430, and AM920433 (http://www.ncbi.nlm.nih.gov/).

In our previous studies on fungal resistance to heavy metals, we isolated and identified a *P. janthinellum* strain GXCR from the sludge generated by Cu mining in Guangxi. This fungal strain is characteristic of high tolerance to various metals, including Cu (200 mM), Zn (>1200 mM), Mn (>200 mM), Cd (50 mM), Cr^{3+} (>60 mM), Cr^{6+} (>3 mM), Ni (20 mM), and Pb (50 mM) (Wei *et al*., 2006). This study focus on functional characterization of the CTPA gene of this fungus to lay a foundation for further insight into higher tolerance to heavy metals of this fungus.

Materials and Methods

Fungal strains and culture

The fungus studied in this study was a *P. janthinellum* strain GXCR (Wei *et al*., 2006). The fungi were cultured for indicated time at 32℃ on potato dextrose agar (PDA) medium (Shepherd and Sullivanm, 1976), in PDL medium (i.e. PDA without agar) or in Czapek's solution ($NaNO_3$ 2 g; K_2HPO_3 1 g; $MgSO{\cdot}7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4{\cdot}7H_2O$

† These authors contributed equally to this work.
∗ To whom correspondence should be addressed.
(Tel) 86-771-327-0103; (Fax) 86-771-327-0103
(E-mail) dyzl@gxu.edu.cn

0.001 g; sucrose 30 g; deionized water 1,000 ml).

Preparation of nucleic acids

Total RNA was extracted as the method described by Kramer (2007) and treated for elimination of contaminant DNAs with RQ1 RNase-free DNase (Promega, USA) according to the manufacturer's protocol. The RNA purity was examined at 260/280 nm. The RNA integrity was assayed by running formaldehyde agarose gels before further use. Genomic DNA was prepared as the report (Melo *et al*., 2006). Plasmid DNA was extracted with the TIANpure Midi kit (TIANGEN, China). Recovery of DNA from agarose gel was performed by using the Agarose Gel DNA Fragment Recovery kit Ver.2.0 (TaKaRa, China).

Cloning of the cDNA of the CTPA gene

Cloning of the cDNA of the CTPA gene was based on reverse transcriptase-polymerase chain reaction (RT-PCR). The first strand cDNA was used as and synthesized by reverse transcription using the RevertAid^{TM} First Strand cDNA Synthesis kit (Fermentas, USA).

First, the degenerate primer pair: forward; 5'-GAYAAGA CYGGYACCGMXCXXC-3' and reverse; 5'-TCGTTGATG CCRTCRCC-3' was deduced from a conserved part of the CTPA genes from *Aspergillus clavatus*, *A. fumigatus*, *A. niger*, *Candida albicans*, *Homo sapien*, and *Saccharomyces cerevisiae*, which were used to PCR-amplify the first fragment of cDNA from the wild-type (WT) strain of *P. janthinellum* strain GXCR.

Next, three pairs of sequence-specific primers were designed based on the sequences of the resulting cDNA and used to PCR-amplify other cDNA fragments in the coding region of the gene, which were F1; 5'-GCTGTCTTGACGA TTCCGGTCCTAGTC-3' and R1; 5'-GACGACTGAAAGC TTTCCCTGAGTCA-3', LF0.6; 5'-ATCCTGAGCATZTCZG GCATGACZTG-3' and LR0.6; 5'-CGCACTAGTACTCAA GACGATCAAGAGG-3', LF0.7; 5'-ACCACAAGGCATGC AGCCAACATAG-3' and LR0.7; 5'-GGACTATAAGATCT AAGCTAGATCCCTGG-3'. PCR was conducted in the 25 μl reaction mix by using the PCR Master Mix (2×) kit (Fermentas, USA) in the Primus 96^{Plus} thermal cycler (MWG AG Biotech, Germany).

Finally, 3'-end cDNA was synthesized as 3'-RACE method using the RNA PCR kit (AMV) Ver 3.0 (TaKaRa) following the manufacturer's instructions. All amplified cDNA fragments were recovered from the agarose gel, cloned into pUCm-T Vector (Sangon, China) and then subjected to double pass (both 5'→3' and 3'→5') sequencing. The resulting cDNA sequences were assembled according to alignment by using the vector NTI suite 9.0 programme (www.Invitrogen.com).

Construction of RNA interference (RNAi) mutants

A 413 bp cDNA fragment was amplified by PCR from the coding region of the CTPA gene with the primer pair: Forward; 5'-CTTGACTAGTGGAAAGCTTTCAGTCGTCGC-3', where the underlined indicates a *Spe*I site and Reverse; 5'-GACAAGATCTTCATCGTCGCCAGAAACGA-3', where the underlined represents a *Bgl*II site. This cDNA fragment was cloned into the *Spe*I-*Bgl*II and *BamH*I-*Xba*I sites of the plasmid pUCCRNAi (kindly provided by Professor Chengcai Chu. CAS, China), respectively, to form an inverted repeat spaced by an intron. The resulting recombinant plasmid was digested by *Hin*dIII to obtain the DNA fragment with the inverted repeat cDNA. This *Hin*dIII DNA fragment was then cloned into the *Hin*dIII site of the plasmid pCPXBn-1 (kindly provided by Professor Donald L. Nuss, Center for Biosystems Research, University of Maryland Biotechnology Institute, USA) to generate the RNAi construct that was predicted to form short double-stranded interfering RNA with a stem-loop structure. The plasmids carrying the RNAi construct were introduced into the fungal protoplasts by PEG/$CaCl_2$-mediated transformation (Churchill *et al*., 1990). The resulting fungal transformants were screened on PDA plate containing Benomyl (Bn; 0.75 μg/ml) to obtain Bn-resistant transformants. The Bn-resistant transformants were identified by Southern blot hybridization followed by PCR using primers of RNAi F; 5'-CATAGCTTCACACCAGCAG ACAACAC-3' and RNAi R; 5'-GAAACGAATAGAGTAGT ACGGTCCGTAC-3'.

Southern and Northern hybridization

Southern hybridization was performed as the standard method (Southern, 1992). Hybridized membranes were exposed for 24 h at -80°C to X-ray film (Fuji, USA) equipped with intensifying screen and then imaged in a Typhoon 9410 phosphorimager (Amersham Biosciences, USA).

For Northern hybridization, the total RNA was separated on agarose gels (1.5%) containing 0.67 M formaldehyde and 1×3-[N-morpholino] propanesulfonic acid, and subsequently transferred to Hybond-N^+ membranes. The resulting membranes were treated for 1 h at 42°C in hybridization solution containing 5× Denhardt's solution, 50% formamide, 5× SSC and 1% SDS, and hybridized for 18 h at 42°C in the hybridization solution with added the probe DNA (100 ng/ml). The hybridized membranes were washed

Table 1. Homology comparison of cDNA sequences of CTPA genes

	AM920433	AM920430	AM920421	XM_001276247	*PcpA*
AM920433	100	45.4	53.0	46.0	46.7
AM920430	45.4	100	44.2	45.7	44.5
AM920421	53.0	44.2	100	45.7	44.9
XM_001276247	46.0	45.7	45.7	100	94.4
PcpA	46.7	44.5	44.9	94.4	100

Shaded and non-shaded indicate percentages of identity and the similarity, respectively. The percentages were calculated using the EMBOSS programme 'Matcher' with default settings (Rice *et al*., 2000). Accession numbers for *A. clavatus*: XM_001276247; *P. chrysogenum*: AM920421, AM920430, AM920433; for *P. janthinellum*: *PcpA*=EU054430.

Table 2. Homology between CTPAs

	NW_ 001517097	XP_ 001276248	XM_ 747887	AM 920421	AM 920430	AM 920433	XM_ 001931863	PAA1	CCC2	CRP1	ClCCC1	ATP7A	ATP7B	PcpA
NW_001517097	100	28.4	28.6	47.8	46.3	63.6	27.1	30.5	27.6	39.4	29.2	29.6	29.8	29.0
XP_001276248.1	49.1	100	75.0	28.9	29.4	29.0	40.4	31.0	32.0	27.5	32.2	30.7	26.9	92.0
XM_747887	49.0	80.0	100	31.3	30.7	30.6	45.6	31.9	31.3	27.3	32.7	36.8	28.1	83.7
AM920421	68.5	49.4	50.5	100	52.6	47.6	28.9	32.7	26.5	39.3	28.9	32.2	34.2	29.5
AM920430	65.6	49.8	51.6	68.0	100	47.5	29.6	34.0	26.3	40.9	30.0	34.2	33.2	30.0
AM920433	77.0	51.0	50.3	67.6	65.5	100	26.7	31.1	27.4	39.5	29.2	31.1	32.2	30.0
XM_001931863	44.7	57.1	62.9	48.5	50.1	47.2	100	30.0	29.7	26.0	30.4	28.7	26.4	40.1
PAA1	49.4	49.5	50.3	51.8	53.7	51.2	47.5	100	36.2	29.9	34.0	34.0	34.9	30.2
CCC2	47.6	52.5	51.3	47.5	46.6	48.6	50.4	53.5	100	34.7	38.5	31.2	37.3	31.7
CRP1	58.7	50.6	50.6	59.3	61.1	59.6	48.1	51.7	53.2	100	34.1	33.1	33.1	27.8
ClCCC1	46.3	49.1	50.2	44.1	48.5	46.8	47.1	52.8	56.5	53.4	100	35.0	31.2	32.4
ATP7A	50.4	51.7	58.7	53.1	54.3	52.2	48.4	52.3	49.5	55.8	53.4	100	47.4	37.3
ATP7B	48.9	47.1	48.3	50.2	50.3	52.0	43.5	53.5	53.6	51.3	46.7	59.7	100	27.0
PcpA	49.4	95.1	89.4	49.7	50.2	51.5	56.8	49.4	51.9	50.3	49.5	58.6	46.3	100

Shaded and non-shaded indicate the percentages of identity and similarity at the amino acid level, respectively. The percentages were calculated using the EMBOSS programme 'Matcher' with default settings (Rice *et al*., 2000). Accession numbers for *A*. *clavatus*: NW_001517097, XP_001276248.1; for *A*. *fumigatus*: XM_747887; for *P*. *chrysogenum*: AM920421, AM920430, AM920433; for *Pyrenophora* tritici-repentis: XM_001931863; for *Arabidopsis thaliana*: PAA1= NM_202947; for *C*. *albicans*: CCC2=AY033085, CRP1=AF193508; for *Colletotrichum lagenarium*: ClCCC1=AB266101; for *H*. *sapiens* ATP7A=NP_ 000043.3, ATP7B=NM_001005918; for *P*. *janthinellum*: PcpA=EU054430.

once for 10 min at room temperature in the solution containing 6× SSC and 0.5% SDS, twice in the solution containing 2× SSC and 0.1% SDS, and finally once in the solution containing 0.1× SSC and 0.1% SDS. The washed membranes were exposed and then imaged.

The probe DNA used in the hybridization was amplified by PCR from the RNAi construct with primers of BnF; 5'-CATAGCTTCACACCAGCAGACAACAC-3' and IntrR; 5'-GAAACGAATAGAGTAGTACGGTCCGTAC-3', and labeled with [α-^{32}P] dCTP by using Random Primer DNA Labeling kit Ver 2.0 (TaKaRa).

Quantitative real-time PCR analysis (qRT-PCR)

The total RNA (5 μg) was firstly reverse transcribed to the first strand cDNA. The first strand cDNA product was diluted tenfold with DNase/RNase-free water. The diluted cDNA product was used as template for qRT-PCR. qRT-PCR was performed using the iQTM SYBR Green Supermix kit (Bio-Rad Laboratories, Inc., USA) in an Option^{Tm} 2 Cotinuous Fluorescenc Detector (MJ Research, USA). The qRT-PCR thermal cycling conditions were at 95°C (3 min) followed by 45 cycles at 95°C (30 sec) and 55°C (30 sec), and then at 72°C (20 sec). The relative target mRNA level was calculated as $2^{-(\Delta CT_{RNAi} - \Delta CT_{Control})}$. Actin mRNA was used as an internal control to normalize the data of each sample. The qRT-PCR primers used were qPCRF; 5'-GTCGATGAAGCCAAACTGTCG-3' and qPCRR; 5'-ATCGTTAATGCCACCACAACC-3' for the *PcpA* gene, and ActinF; 5'-CTATTGGCAACGAGCGCTTC-3' and ActinR; 5'-TGGAGTTGAACGTGGTGACG-3' for the actin gene. The actin gene primers were designed according to actin gene sequence (GenBank accession no. AF056975) of *P*. *chrysogenum*.

Quantitative analysis of growth and intracellular ion content of fungi

The conidia (5×10^3) were inoculated in 100 ml PDL medium or in 100 ml Czapek's solution with or without supple-

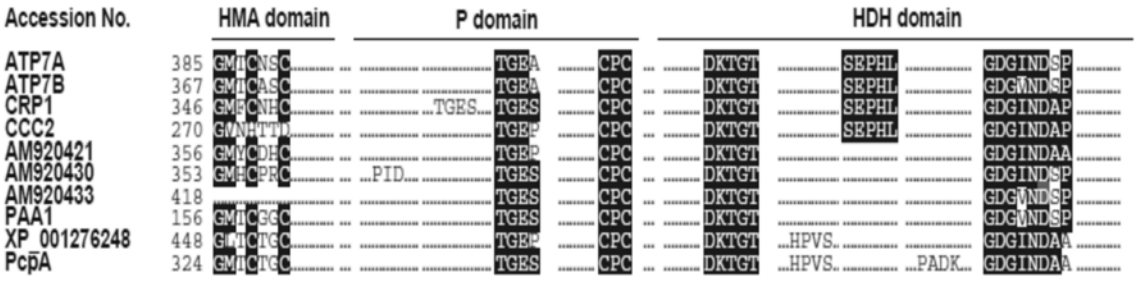

Fig. 1. Comparison of specific sequence motifs in conserved domains of CTPAs. The GenBank accession no. for the proteins are: NP_000043.3 (of APT7A for *H*. *sapiens*), NM_001005918 (of APT7B for *H*. *sapiens*), AF193508 (of CRP1 for *C*. *albicans*), AY033085 (of CCC2 for *C*. *albicans*), NM_202947 (of PAA1 for *A*. *thaliana*), AM920421, AM920430 and AM920433 (of PAA1 for *P*. *chrysogenum* Wisconsin strain 54-1255), and EU054430 (of PAA1 for *P*. *janthinellum* strain GXCR). The conserved domains were predicated by using the smart programme (http://smart.embl-heidelberg.de/). All the parameters used in prediction were default.

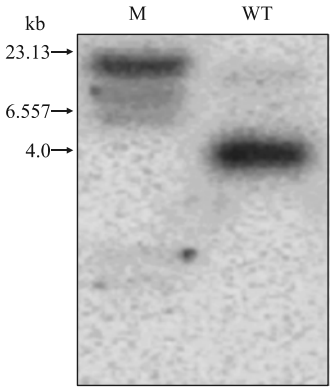

Fig. 2. Southern hybridization analysis of the *PcpA*. Genomic DNA was isolated from the WT grown for 3 days in PDL medium and digested with *Kpn*I. Twenty micrograms of digested genomic DNA were used for capillary transfer. M, marker (λ/*Hin*dIII DNA). WT, wild type.

mentation with metal salts, after that, cultured for adequate time at indicated temperature. The resulting mycelia were collected by centrifugation for 10 min at 12,000 rpm, dried for 5 h at 65°C and finally weighed.

As for measurement of intracellular ion content, the mycelia were washed twice in deionized water and washed once in 300 ml buffer (50 mM Tris-HCl, 20 mM EDTA, final pH 7.0) by 30 min centrifugation at 32°C at 200 rpm to remove the metal ions attached on the mycelial surfaces. The washed mycelia were dried at 65°C and finally ground to fine powder in liquid N_2. The 20 mg of powdered mycelia were digested for 1 h at 80°C in 0.5 ml solution composed of nitric acid and perchloric acid as a ratio of 5:2. After adding 2 ml of MilliQ H_2O, the metal content of each sample was determined as the atomic absorption spectroscopy (AAS) method by using the Hitachi Z-8000 atomic absorption spectrophotometer (Hitachi, Japan) equipped with a graphite tube atomizer. All samples were measured in duplicate, and the experiment was performed three times.

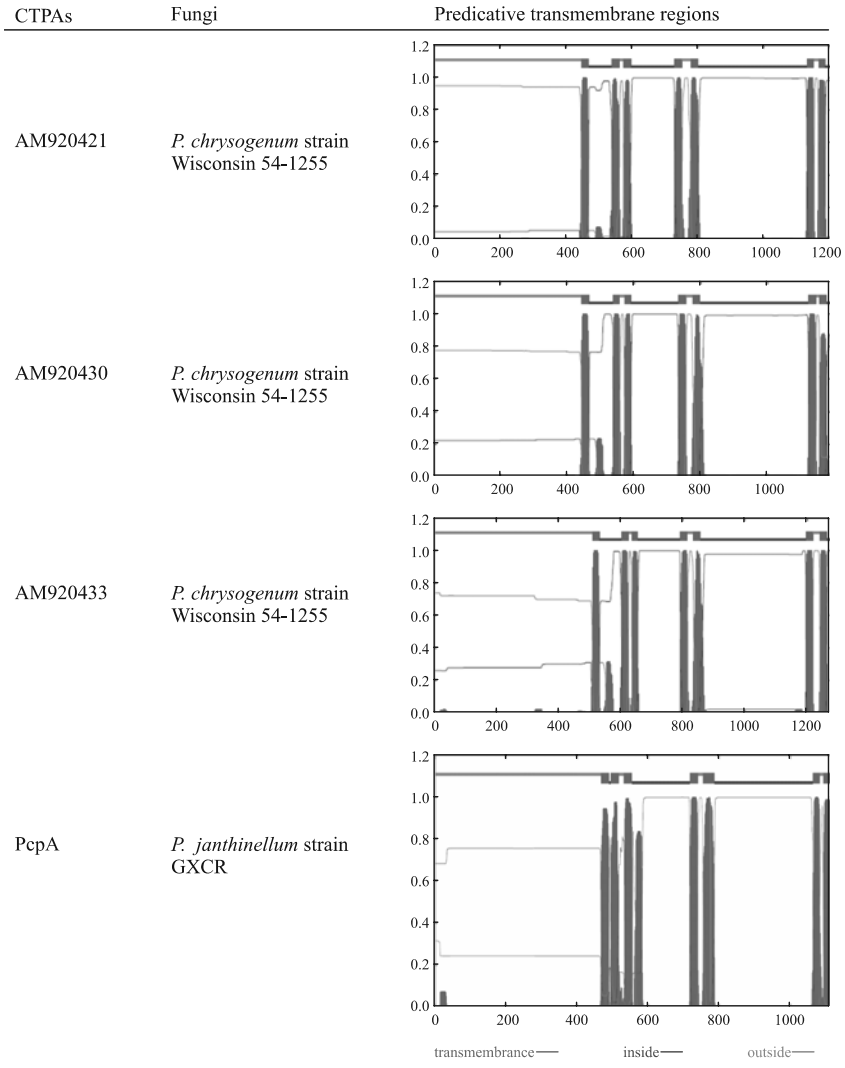

Fig. 3. Prediction of transmembrane regions of several CTPAs. Transmembrane regions were predicated by using the TMHMM-2.0 programme (http://www.cbs.dtu.dk/services/TMHMM-2.0/). All the parameters used in prediction were default.

Results

Characterization of the CTPA gene of *P. janthinillum* strain GXCR

Five cDNA fragments were amplified by PCR from *P. janthinillum* strain GXCR and sequenced. These cDNA sequences were assembled, generating a 3,753 bp cDNA sequence with a polythymidylic acid tail at the 3' end, which has been published in NCBI database under accession number EU054430. This cDNA sequence shows the highest identity (94.4%) with a putative CTPA gene of *A. clavatus* (accession number NRRL1 XM_001276247) (Table 1). It contains an incomplete open reading frame and putatively encodes a protein of 1,114 amino acid residues. This putative protein has the C-terminal end but loses the N-terminal end due to failure to PCR-amplify 5' end cDNA. The amino acid sequence of the protein shows the highest identity (95%) with CTPA of *A. clavatus* NRRL1 (accession number XP_001276248.1) (Table 2). In this study, the cloned gene and encoded protein were named *PcpA* and PcpA, respectively. The PcpA was predicted to contain several functional domains: heavy-metal-associated (HMA), P-type ATPase (P), and haloacid dehalogenase-like hydrolase (HDH) domains. Each domain has one or more specific sequence motifs (Fig. 1), including a GMXCXXC for transition-metal binding in HMA domain (Bayle *et al.*, 1998; Camakaris *et al.*, 1999; Riggle and Kumamoto, 2000), a TGES for ATP binding (Rensing *et al.*, 1999) and a CPC for ion translocation in P domain (Lutsenko and Petris, 2003), and DKTGT, HPLG-like, PADK and GDGINDAP-like sequences (Riggle and Kumamoto, 2000) in HDH domain. The Southern hybridization signal was detected at the about 4-kb size and

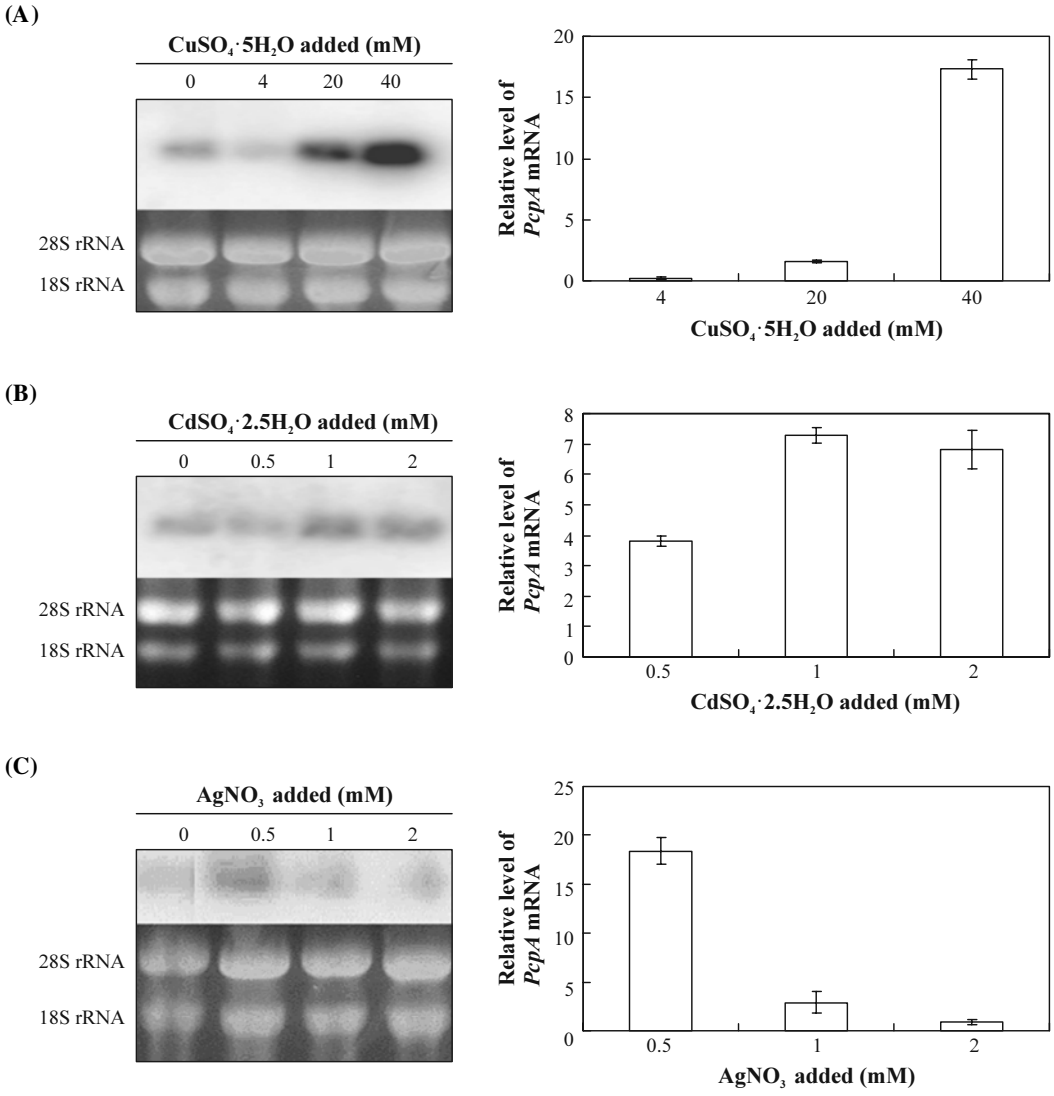

Fig. 4. Expression analysis of the *PcpA* of the WT strain GXCR by Northern hybridization (left) and qRT-PCR (right). The fungal conidia (5×10^3) were inoculated in 100 ml PDL with or without supplementation with heavy metal salts and grown for 3 days to prepare total RNA. Twenty to thirty micrograms of the total RNA were used for capillary transfer of each lane in Northern hybridization. M, marker (λ/*Hin*dIII DNA). Error bars in the figures (right) indicate standard error of the mean from three independent tests. $P=0.05$.

indicated the single copy of the orthologous gene (Fig. 2). The PcpA was predicted to contain seven transmembrane (TM) regions (Fig. 3).

PcpA expression of the WT strain GXCR in the presence of $CuSO_4 \cdot 5H_2O$

When the WT strain GXCR was grown for 30 min at different concentrations of $CuSO_4 \cdot 5H_2O$, the mRNA level between control and treatment with 4 mM did not significantly differ ($P>0.05$), and was increased to 1.5- and 17-fold, respectively, at 20 and 40 mM (Fig. 4A). Such expression pattern of *PcpA* was similar to that of CaCRP1 gene of *C. albicans* grown in the Cu-added medium (Weissman *et al*., 2000).

PcpA expression of the WT strain GXCR in the presence of $CdSO_4$ and $AgNO_3$

It has been reported that the yeast CTPA gene expression can be also affected by Cd and silver (Ag) (Riggle and Kumamoto, 2000). To gain further insight into *PcpA* roles, its expression was detected in the presence of $CdSO_4 \cdot 2.5H_2O$ (Fig. 4B) and $AgNO_3$ (Fig. 4C). When compared to control, expression level of the *PcpA* was significantly enhanced with increasing $CdSO_4 \cdot 2.5H_2O$ concentration (Fig. 4B), showing an approximately 3.8-, 7.3-, and 6.8-flod increase, respectively, at 0.5, 1 and 2 mM.

PcpA expression under $AgNO_3$-added conditions was distinct from that in the presence of $CuSO_4 \cdot 5H_2O$ and $CdSO_4 \cdot 2.5H_2O$, with a 18.4- and 2.89-flod increase, respectively, at 0.5 and 1 mM. At a higher concentration (2 mM), *PcpA* expression was reduced to 89% of that in control mycelia (Fig. 4C).

PcpA RNAi mutants

If the *PcpA* is associated with Cu resistance, mutants with reduced levels of *PcpA* mRNA should be sensitive to Cu. To test this hypothesis, *PcpA* RNAi mutants were constructed.

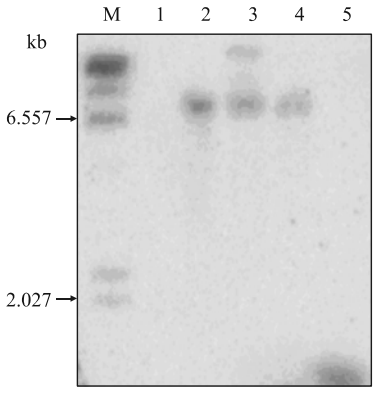

Fig. 5. Southern hybridization analysis of *PcpA* RNAi mutants. Genomic DNA was isolated from the WT mycelia grown for 3 days in PDL without supplementation with heavy metal salts and digested with *Kpn*I. Twenty micrograms of digested genomic DNA were used for capillary transfer. M, marker (λ/*Hin*dIII DNA). 1, WT; 2, The mutant MB; 3, The mutant MT; 4, The mutant MP; 5, Positive control (The blotted DNA was amplified by PCR from the RNAi construct with primers of BnF and IntrR).

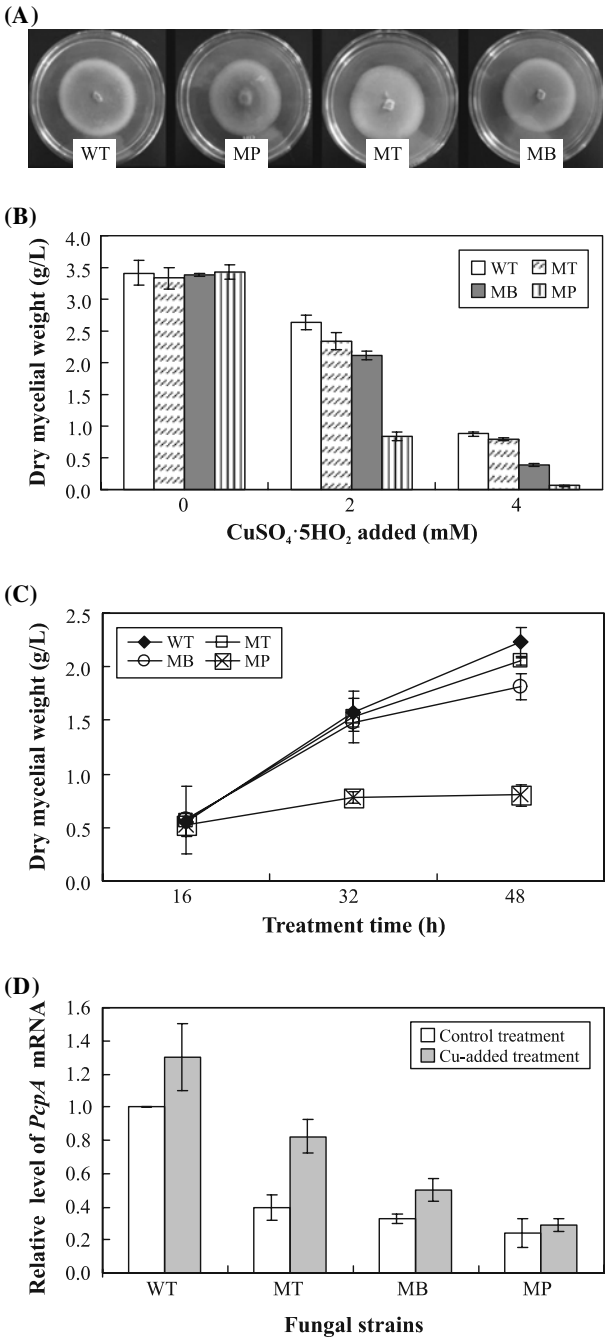

Fig. 6. Comparision between the WT and the *PcpA* RNAi mutants in growth phenotypes and *PcpA* mRNA levels. (A) Colonies grown for 3 days on PDA plate without additional $CuSO_4 \cdot 5H_2O$. (B) Dry weight of mycelia grown for 3 days in PDL containing different concentrations of $CuSO_4 \cdot 5H_2O$. (C) Dry weight of mycelia grown for indicated time in PDL with additional 2 mM $CuSO_4 \cdot 5H_2O$. (D) Relative *PcpA* mRNA levels. For analysis of dry mycelial weights, conidia (5×10^3) were cultured in 100 ml PDL. The mRNA levels were determined by qRT-PCR. For qRT-PCR, the conidia were grown for 3 days in 100 ml PDL, the resulting mycelia were cultured for 30 min in PDL with supplementation with 4 mM $CuSO_4 \cdot 5H_2O$ and then analyzed for the *PcpA* mRNA level by qRT-PCR The basal level in WT was set at 1. Error bars indicate standard errors of the mean from three independent tests. $P=0.05$.

According to Ben resistance together with Southern hybridization analysis (Fig. 5), three *PcpA* RNAi mutants were obtained and named MB, MP, and MT, respectively. Southern hybridization analysis indicated that one RNAi construct integrated into the genome of MB and MP while two RNAi constructs integrated into the MT genome.

PcpA RNAi mutants showed no differences from the WT in colony (Fig. 6A) and growth ability (Fig. 6B) under the control conditions without additional $CuSO_4 \cdot 5H_2O$. In presence of $CuSO_4 \cdot 5H_2O$, there was no significant ($P>0.05$) difference between WT and the mutant MT in dry mycelial weight; However, the dry mycelial weights of the mutants MB and MP were, respectively, 80.7% and 32.3% of that of WT at 2 mM, and 43.1% and 6.8% of that of WT at 4 mM.

Change in growth of the strains with the treatment time course was monitored (Fig. 6C). For 16 h of treatment at 2 mM $CuSO_4 \cdot 5H_2O$, mutants and the WT did not exhibit a significant difference in growth ability. Compared to WT, MB, and MT, the mutant MTP presented significant growth inhibition at 32 h. Growth inhibition of the mutant MB occurred at 48 h compared to WT. Within 48 h of treatment, the mutant MT did not significantly differ from WT in growth.

All results indicated that the growth ability in the presence of $CuSO_4 \cdot 5H_2O$ was arranged in order of WT=MT> MB>MP.

Relative levels of *PcpA* mRNA of the mutants

To demonstrate whether growth ability of the mutants correlates with the level of the *PcpA* mRNA, transcription of the *PcpA* was assayed by qRT-PCR. When grown under the control condition without additional $CuSO_4 \cdot 5H_2O$, the mRNA amounts of the *PcpA* in mutants MT, MB, and MP were, respectively, 39.8%, 32.5%, and 24.3% of that in WT (Fig. 6D). When grown in the presence of $CuSO_4 \cdot 5H_2O$, *PcpA* mRNA levels of WT and mutants MT and MB were significantly ($P<0.05$) higher than those of their controls; However, the mutant MP treated by $CuSO_4 \cdot 5H_2O$ showed no significant difference from its control in the *PcpA* mRNA level. The mRNA levels of all mutants were much lower than those of WT. The relative mRNA level followed the order of WT>MT>MB>MP ($P<0.05$), showing a strongly positive correlation with growth ability of the strains (Fig. 6B and C).

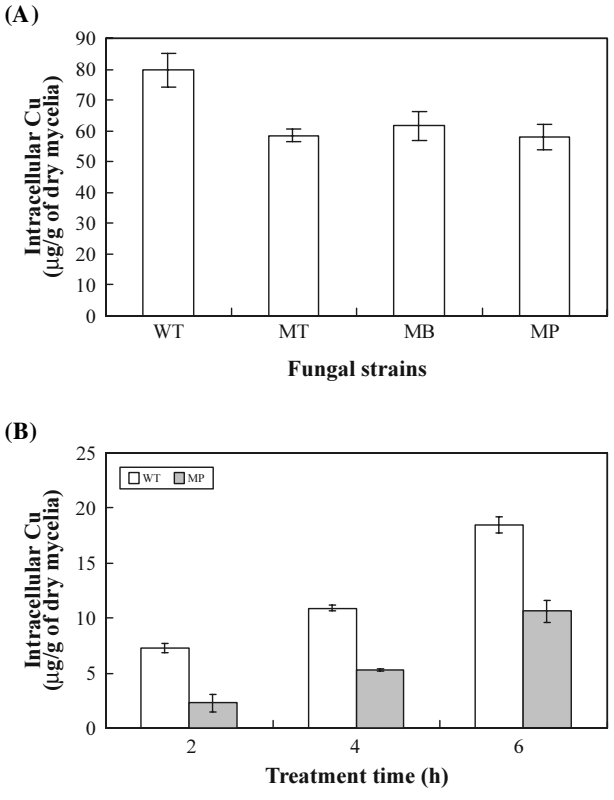

Fig. 7. Intracellular Cu content of the WT and mutants. (A) Intracellular Cu content of WT and mutants grown for 2 days at 2 mM $CuSO_4 \cdot 5H_2O$. (B) Change in intracellular Cu content with the time course of treatment at 2 mM $CuSO_4 \cdot 5H_2O$. Conidia (5×10^3) were cultured for indicated time in 100 ml PDL with or without supplementation with $CuSO_4 \cdot 5H_2O$, the resulting mycelia were analyzed for Cu content by AAS. Error bars indicate standard errors of the mean from three independent tests. $P=0.05$.

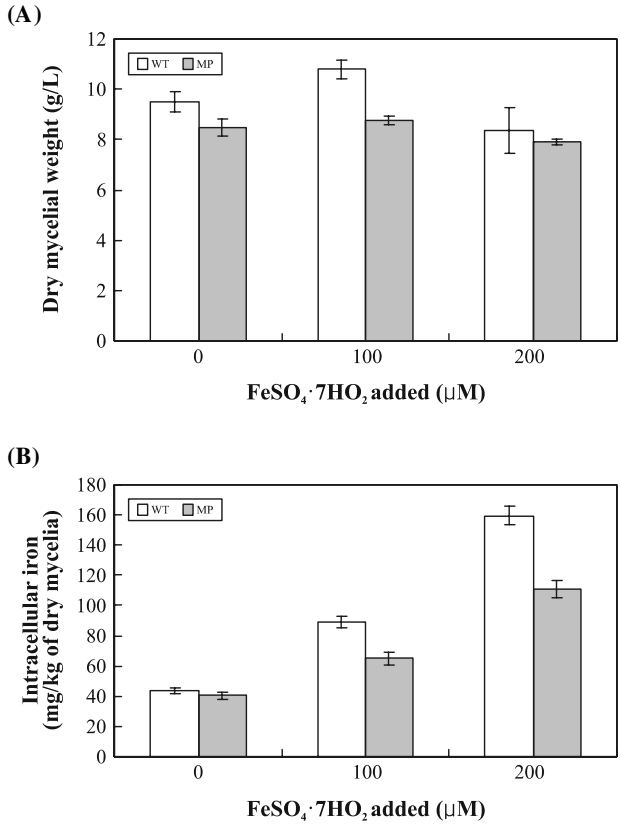

Fig. 8. Growth and intracellular iron levels of the WT and the mutant MP. The fungal conidia (5×10^3) were inoculated for 6 d in the Czapek's solution with or without supplementation with $FeSO_4 \cdot 7H_2O$. The resulting mycelia were then analyzed for dry mycelial weights and iron content. Iron content was analyzed by AAS. Error bars indicate standard errors of the mean from three independent tests. $P=0.05$.

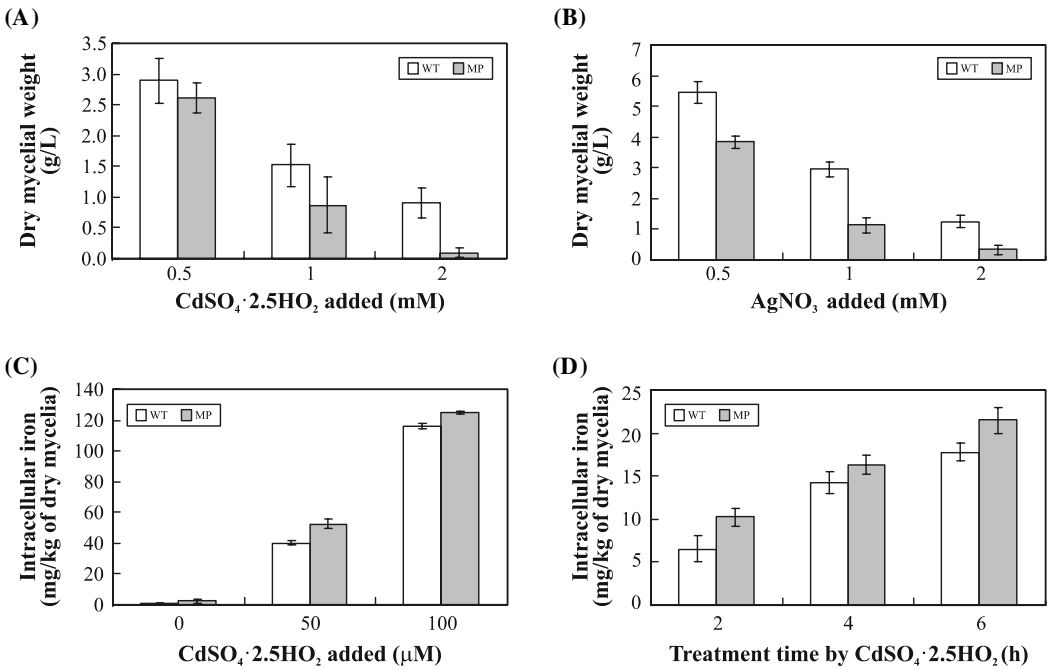

Fig. 9. Comparsion between the WT and the mutant MP in growth, intracellular Cd and Ag content in the presence of $CdSO_4 \cdot 2.5HO_2$ and $AgNO_3$. (A) Growth in PDL supplemented with $CdSO_4 \cdot 2.5HO_2$. (B) Growth in PDL supplemented with $AgNO_3$. (C) Intracellular Cd content of the strains grown at different concentrations of $CdSO_4 \cdot 2.5H_2O$. (D) Intracellular Cd content of the strains with the time course of treatment at 100 μm $CdSO_4 \cdot 2.5H_2O$. For analysis of dry mycelial weights, the fungal conidia (5×10^3) were inoculated for 6 days in PDL with or without supplementation with heavy metals. Cd content was analyzed by AAS. *Error bars* indicate standard errors of the mean from three independent tests. $P=0.05$.

Cu accumulation in mycelial cells

To characterize the roles of the *PcpA*, intracellular Cu contents of RNAi mutants and WT grown in the presence of $CuSO_4 \cdot 5H_2O$ were measured by AAS. When grown for 2 days at 2 mM $CuSO_4 \cdot 5H_2O$, intercellular Cu contents in *PcpA* RNAi mutant accounted for 72.7 to 77.38% of that in WT (Fig. 7A); However, difference in intercellular Cu contents was not significant ($P>0.05$) among the mutants (Fig. 7A).

Subsequent experiments were performed on the mutant MP because it had a lowest mRNA level. Change in Cu accumulation in this mutant was monitored over the time course of treatment at 2 mM $CuSO_4 \cdot 5H_2O$. As a result, Cu accumulation was significantly increased in both WT and MP with increasing treatment time but significantly lower ($P<0.05$) in MP than in WT at each treatment time point (Fig. 7B).

Growth and iron uptake of the mutant MP in iron-added medium

It has been reported that deletion of yeast gene *ccc2* results in diminished iron uptake and growth retardation under iron-limiting conditions (Yuan *et al*., 1995). Therefore, growth and iron uptake of WT and mutant MP in the presence of $FeSO_4 \cdot 7H_2O$ were compared. At 100 μm $FeSO_4 \cdot 7H_2O$, WT grew much better ($P<0.05$) than the mutant MP, with higher dry weight. At 200 μm $FeSO_4 \cdot 7H_2O$, growth of both WT and MP was significantly ($P<0.05$) inhibited when compared to their growth in Czapek's solution with or without supplementation with 100 μm $FeSO_4 \cdot 7H_2O$; However, the difference between WT and MP in growth was not significant ($P>0.05$) at this iron concentration (Fig. 8A). Intracellular iron content of both WT and MP was significantly ($P<0.05$) enhanced with increasing $FeSO_4 \cdot 7H_2O$ concentration. The iron content of MP accounted for 72.8% and 69.6% of that in WT, respectively, at 100 and 200 μm (Fig. 8B). These results suggest (1) that the *PcpA* is associated with iron uptake of the fungal strain GXCR, (2) that higher concentration (200 μm) of $FeSO_4 \cdot 7H_2O$ is disadvantageous to normal growth of this fungus.

Growth inhibition of the mutant MP by $CdSO_4$ and $AgNO_3$

The sensitivity of the mutant MP to $CdSO_4$ and $AgNO_3$ was also assayed. This mutant was more sensitive to $CdSO_4 \cdot 2.5H_2O$ (Fig. 9A) and $AgNO_3$ (Fig. 9B) than WT, Intracellular Cd content of both WT and MP was significantly ($P<0.05$) elevated either with increasing $CdSO_4 \cdot 2.5H_2O$ concentration (Fig. 9C) or with increasing treatment time at 100 μm $CdSO_4 \cdot 2.5H_2O$ (Fig. 9D), However, accumulated Cd in MP was 1.1- to 1.56-fold that in WT.

Discussion

In this study, we successfully cloned partial cDNA fragment of CTPA gene *PcpA* from *P. janthinellum* strain GXCR.

The *PcpA* and *PcpA*-encoded protein PcpA shows lower sequence identities with the CTPAS reported in *P. chrysogenum* Wisconsin strain 54~1255 (Table 1 and 2). The PcpA differs from other reported CTPAs in some specific sequence motifs. The major difference from other CTPA lies in the sequence motifs in HMA and HDH domains (Fig. 1). In addition, the first three TM regions of PcpA differ apparently in those of AM920421, AM920430, and AM920433 (Fig. 3). Differences in topology of TMs can, to some extent, affect cation binding specificity of P-type ATPases (Fatemi and Sarkar, 2002). In yeast, three CTPA genes have been reported, which are *ScCcc2* from *S. cerevisiae*, and *CaCcc2* and *CaCrp1* from *C. albicans* (Weissman *et al*., 2000, 2002). In encoded proteins, CaCcc2 and ScCcc2 have two GMXCXXC sequence motifs, and the CaCrp1 possesses three GMXCXXC and two TGES sequence motifs (Weissman *et al*., 2002). It was found that differences in GMXCXXC motif in HMA domain is coupled with yeast Cu resistance (Weissman *et al*., 2002). The PcpA has only one GMXCXXC motif and one TGES sequence motif (Fig. 1). Obviously, the *PcPA* is a novel CTPA gene.

In eukaryotes, Cu uptake is mediated mainly by Cu transporters belonging to Ctr family, such as h/mCtr1 in mammalian cells, high-affinity Ctr1 and Ctr3, and a low-affinity Fet4 in yeast cells (Puig and Thiele, 2002), and Ctr1B in *Drosophila* cells (Balamurugan and Schaffner, 2006). Nevertheless, in prokaryotic bacteria, Cu uptake is governed by a CTPA enconded by *CopA* (Dameron and Harrison, 1998). Besides an uptake via specific Ctr importers, Cu can also be imported by less specific general metal transporters (Balamurugan and Schaffner, 2006). Disruption of these related genes can cause defect in Cu uptake, therefore resulting in a phenotype with a low level of intracellular Cu (Yuan *et al*., 1995). To date, three CTPAs have been reported to involve Cu extrusion, including bacterial CopB and the human CTPAs (Wilson and Menkes disease proteins) (Dameron and Harrison, 1998), and *C. albicans* CaCrp1 (Weissman *et al*., 2002). Deletion of these genes usually leads to extreme Cu accumulation in the cells and growth inhibition by Cu of the mutants (Weissman *et al*., 2000). Some CTPAs also function in Cu homeostasis by Cu translocation, such as CaCrp1 (Weissman *et al*., 2000). Phenotypes of the *PcpA* RNAi mutants, such as the decreased growth ability and lower *PcpA* mRNA levels in the presence of Cu, strongly indicate that *PcpA* is closely associated with Cu tolerance. The question arising is about *PcpA* roles in Cu utilization. When grown in Cu-added medium, intracellular Cu content of *PcpA* RNAi mutants was much lower than that in WT (Fig. 7), strongly suggesting that the *PcpA* plays a role in Cu uptake; For the mutants, decreased growth ability (Fig. 6B) was coupled with lower Cu content (Fig. 7), strongly indicating that the *PcpA* is also associated with Cu homeostasis.

As for the mutant MP, decreased growth ability under the conditions with or without supplementation with iron can be inferred to be caused by decreased of intracellular iron content (Fig. 8) because iron is required for normal growth of the living organisms. Such decrease in intracellular iron content should be imputable to decrease in intracellular Cu content (Fig. 7). This is because the low Cu level leads to decrease in activities of Cu-dependent enzymes responsible for iron uptake. For instance, in yeast, it has been evidenced that activity of ferroxidase Fet3 necessary for high-affinity iron uptake is Cu-dependent. When yeast cells lack a CTPA such as Ccc2p, Cu is not incorporated into Fet3, and subsequently the cells lose the ability of high-affinity iron uptake (Yuan *et al*., 1995; Stearman *et al*., 1996).

With regard of WT grown in the presence of Ag, its *PcpA* expression level seems to be counterintuitive (Fig. 4C). Such expression pattern is probably associated with high toxicity of Ag to all microorganisms, perhaps due to poisoning of the respiratory electron transport chains and components of DNA replication (Silver and Phung, 2005).

Heavy metal-transporting P-type ATPases are usually classified into several subfamilies by metal ion specificity (www.Patbase.kvl.dk). P-type ATPases responsible for transport of Cu, Ag, and Cd belong to the same subfamily P_{1B} ATPases (Argüello *et al*., 2007). However, whether they are absolutely specific for heavy metals was unclear (Argüello *et al*., 2007). Our data clearly indicate that the *PcpA* is of multiple-heavy-metal response. Elevated Cd accumulation in cells of the mutant MP suggests that the *PcpA* is also responsible for the export of Cd from the cytosol to the extracytosol. Similarly, *C. albicans* CRD1 can respond to Cu, Cd, and Ag (Riggle and Kumamoto, 2000) and the *Escherichia coli* CopA is of response to Cu, Ag, and gold (Stoyanov *et al*., 2001). All these results indicate that CTPAs are non-specific for heavy metals. The probable reasons are as follows: (1) the GMX CXXC sequence motif required for metal ion binding (Dameron and Harrison, 1998) is of lower metal binding specificity, (2) the CTPA promoter can be activated by multiple metals (Outten *et al*., 2000; Stoyanov *et al*., 2001; Stoyanov and Brown, 2003), (3) Ag ion is similar to Cu ion in structures (Solioz and Odermatt, 1995), and (4) more likely non-specificity for heavy metals is required for the microorganisms to survive environments containing various heavy metals, such as *P. janthinellum* strain GXCR (Wei *et al*., 2006).

RNAi is an RNA-dependent gene silencing process (Lenz, 2005; Liu *et al*., 2007) and used as a good alternative to gene disruption in organisms where no adequate markers are available or when the likelihood of homologous recombination is low. The RNAi strategy used in this study is generally adopted by laboratories, but it sometimes leads to integration of one or more RNAi constructs into different sites in the genome (Fig. 5), likely leading to the potential positional effects. In addition, the integration of the RNAi construct into the genome also likely occurs at the sites that are related to the normal growth, therefore, ones usually question that alteration in growth ability of the RNAi mutants is not caused by decreased mRNA levels of the target genes. Our findings underlined that *PcpA* RNAi did not affect the growth ability of the fungi under the normal growth conditions (Fig. 6A and B).

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References

Ahearn, G.A., P.K. Mandal, and A. Mandal. 2004. Mechanisms of heavy-metal sequestration and detoxification in crustaceans: a review. *J. Comp. Physiol.* 174, 439-452.

Argüello, J.M., E. Eren, and M. González-Guerrero. 2007. The structure and function of heavy metal transport P_{1B}-ATPases. *Biometals* 20 (3-4), 233-248.

Axelsen, K.B. and M.G. Palmgren. 2001. Inventory of the superfamily of P-type ion pumps in Arabidopsis. *Plant Physiol.* 126, 696-706.

Balamurugan, K. and W. Schaffner. 2006. Copper homeostasis in eukaryotes: teetering on a tightrope. *Biochim. Biophys. Acta* 1763, 737-746.

Bayle, D., S. Wangler, T. Weitzenegger, W. Steinhilber, J. Volz, M. Przybylski, K.P. Schafer, G. Sachs, and K. Melchers. 1998. Properties of the P-type ATPases encoded by the *copAP* operons of *Helicobacter pylori* and *Helicobacter felis*. *J. Bacteriol.* 180, 317-329.

Camakaris, J., I. Voskoboinik, and J.F. Mercer. 1999. Molecular mechanisms of copper homeostasis. *Biochem. Biophys. Res. Commun.* 261, 225-232.

Churchill, A.C.L., L.M. Ciuffetti, D.R. Hansen, H.D. Van Etten, and N.K. Van Alfen. 1990. Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. *Curr. Genet.* 17, 25-31.

Clemens, S. 2001. Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* 212, 475-486.

Dameron, C.T. and M.D. Harrison. 1998. Mechanisms for protection against copper toxicity. *Am. J. Clin. Nutr.* 67(suppl), 1091S-1097S.

Fatemi, N. and B. Sarkar. 2002. Structural and functional insights of Wilson disease copper-transporting ATPase. *J. Bioenerg. Biomembr.* 34, 339-349.

Hall, J.L. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.* 53, 1-11.

Harrison, M.D., C.E. Jones, M. Solioz, and C.T. Dameron. 2000. Intracellular copper routing: the role of copper chaperones. *Trends Biochem. Sci.* 25, 29-32.

Hussain, D., M.J. Haydon, Y. Wang, E. Wong, S.M. Sherson, J. Young, J. Camakaris, J.F. Harper, and C.S. Cobbett. 2004. P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* 16, 1327-1339.

Kershaw, C.J., N.L. Brown, C. Constantinidou, M.D. Patel, and J.L. Hobman. 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. *Microbiology* 151(Pt4), 1187-1198.

Kramer, C. 2007. Isolation of total RNA from *Neurospora mycelium*. *Methods Mol. Biol.* 362, 291-303.

Lenz, G. 2005. The RNA interference revolution. *Braz. J. Med. Biol. Res.* 38, 1749-1757.

Liu, G., F. Wong-Staal, and Q.X. Li. 2007. Development of new RNAi therapeutics. *Histol. Histopathol.* 22, 211-217.

Lutsenko, S. and M.J. Petris. 2003. Function and regulation of the mammalian copper-transporting ATPases: insights from biochemical and cell biological approaches. *J. Membr. Biol.* 191, 1-12.

Melo, S.C., C. Pungartnik, J.C. Cascardo, and M. Brendel. 2006. Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa*. *Genet Mol. Res.* 5, 851-855.

Outten, F.W., C.E. Outten, J. Hale, and T.V. O'Halloran. 2000. Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal *MerR* homologue, *cueR*. *J. Biol. Chem.* 275, 31024-31029.

Puig, S. and D.J. Thiele. 2002. Molecular mechanisms of copper uptake and distribution. *Curr. Opin. Chem. Biol.* 6, 171-180.

Rensing, C., M. Ghosh, and B.P. Rosen. 1999. Families of soft-metal-ion-transporting ATPases. *J. Bacteriol.* 181, 5891-5897.

Rice, P., I. Longden, and A. Bleasby. 2000. EMBOSS: The european molecular biology open software suite. *Trends Genet.* 16, 276-277.

Riggle, P.J. and C.A. Kumamoto. 2000. Role of a *Candida albicans* P1-type ATPase in resistance to copper and silver ion toxicity. *J. Bacteriol.* 182, 4899-4905.

Shepherd, M.G. and P.A. Sullivanm. 1976. The production and growth characteristics of yeast and mycelial forms of *Candida albicans* in continuous culture. *J. Gen. Microbiol.* 93, 361-370.

Silver, S. and I.T. Phung. 2005. A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *J. Ind. Microbiol. Biotechnol.* 32(11-12), 587-605.

Solioz, M. and A. Odermatt. 1995. Copper and silver transport by CopBATPase in membrane vesicles of *Enterococcus hirae*. *J. Biol. Chem.* 270, 9217-9221.

Solioz, M., A. Odermatt, and R. Krapf. 1994. Copper pumping ATPases: common concepts in bacteria and man. *FEBS Lett.* 346, 44-47.

Southern, E.M. 1992. Detection of specific sequences among DNA fragments separated by gel electrophoresis. 1975. *Biotechnology* 24, 122-139.

Stearman, R., D.S. Yuan, Y. Yamaguchi-Iwai, R.D. Klausner, and A. Dancis. 1996. A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* 271, 1552-1557.

Stoyanov, J.V. and N.L. Brown. 2003. The *Escherichia coli* copper-responsive *copA* promoter is activated by gold. *J. Biol. Chem.* 278, 1407-1410.

Stoyanov, J.V., J.L. Hobman, and N.L. Brown. 2001. CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. *Mol. Microbiol.* 39, 502-512.

Valentine, J.S. and E.B. Gralla. 1997. Delivering copper inside yeast and human cells. *Science* 278, 817-818.

Voskoboinik, I., J. Camakaris, and J.F. Mercer. 2002. Understanding the mechanism and function of copper P-type ATPases. *Adv. Protein Chem.* 60, 123-150.

Wei, M.K., H.Y. Tang, L. Liang, S. Huang, and Y.Z. Li. 2006. Characteristics of high resistance to heavy metal salts and uptake of Cu^{2+} and Zn^{2+} ions of *Penicillium janthinellum*. *Mycosystem* 25, 616-623. (in Chinese)

Weissman, Z., I. Berdicevsky, B.Z. Cavari, and D. Kornitzer. 2000. The high copper tolerance of *Candida albicans* is mediated by a P-type ATPase. *Proc. Natl. Acad. Sci. USA* 97, 3520-3525.

Weissman, Z., R. Shemer, and D. Kornitzer. 2002. Deletion of the copper transporter Ca*CCC2* reveals two distinct pathways for iron acquisition in *Candida albicans*. *Mol. Microbiol.* 44, 1551-1560.

Yuan, D.S., R. Stearman, A. Dancis, T. Dunn, T. Beeler, and R.D. Klausner. 1995. The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc. Natl. Acad. Sci. USA* 92, 2632-2636.