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Cadmium-inducible *BgMT2*, a type 2 metallothionein gene from mangrove species (*Bruguiera gymnorrhiza*), its encoding protein shows metal-binding ability

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ABSTRACT

Metallothioneins (MTs) are a family of low-molecular-weight Cys-rich proteins and are thought to play possible role in metal metabolism or detoxification. To shed light on the role of type 2 MT in *Bruguiera gymnorrhiza* (*BgMT2*) under heavy metal stress, *B. gymnorrhiza* seedlings were exposed to different concentrations of $CdCl_2$ (2 μM, 10 μM, 20 μM or 40 μM) for 3, 7 and 11 d. Real-time quantitative PCR analysis demonstrated that *BgMT2* gene transcripts in leaves of *B. gymnorrhiza* increased in all Cd concentrations and exposure durations. *BgMT2* was overexpressed in *Escherichia coli* BL21 (DE3) as a fusion protein (GST-BgMT2), and bacteria expressing the fusion protein had higher tolerance to Zn, Cu, Pb and Cd than control cells. Moreover, by analysis of metal-binding properties of GST-BgMT2 fusion protein, the expression of *BgMT2* endowed resistance in *E. coli* to Zn, Cu, Pb and Cd by *BgMT2* protein sequestering the four metals. Taken together, these data suggest that *BgMT2* gene plays an important role in resistance to heavy metals.

1. Introduction

Mangrove ecosystems are diverse communities growing in intertidal zones of tropical to subtropical coastal rivers, estuaries and bays. Like other wetlands, the mangrove ecosystems have been widely used as sites where effluents are discharged and solid wastes are dumped, including metallic anthropogenic wastes, and have a large capacity in retaining heavy metals (Zhang et al., 2007). Heavy metals are not biodegradable and are thus persistent in the marine environment. Among these heavy metals, cadmium (Cd) is one of the most abundant, ubiquitously distributed toxic elements in aquatic systems. Cd has a long biological half-life with a tendency to form strong complexes with S-containing peptides/proteins, which renders this metal a notable hazard to a wide range of organisms (Ranieri et al., 2005). Mangroves are woody communities, which form a part of mangrove ecosystems and possess great tolerance to high levels of heavy metal pollution (Huang et al., 2010; Usha et al., 2007). The mangrove plants, like other living organisms, have evolved various mechanisms for modulating heavy metal levels to adapt to changes in concentrations of heavy metals in the environment (Huang et al., 2010; Huang and Wang, 2009, 2010a, 2010b; Zhang et al., 2007). Among all the detoxification mechanisms, chelation by various ligands like metallothioneins (MTs) provides greater resistance to the toxic effects of heavy metals (Gonzalez-Mendoza et al., 2007; Huang and Wang, 2009, 2010a; Usha et al., 2007).

MTs are a family of low-molecular-weight, Cys-rich, metal-binding proteins that are widespread through the animal and plant kingdoms, as well as in some prokaryotes (Huang and Wang, 2009, 2010a). Plant MTs have been classified based on the arrangement of Cys residues into classes I, II and III. Most of the plant MTs are Class I proteins containing two smaller Cys-rich domains and a large spacer region devoid of this amino acid, and can be further divided into four types based on the position of Cys residues in the predicted proteins (Usha et al., 2009). In Class II MTs, Cys residues are distributed in a scattered manner in the entire protein sequence. Class III MTs differ markedly from class I and II MTs. They are enzymically derived and are most commonly composed of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2–11$), also called phytochelatins (PCs) (Robinson et al., 1993). Plant MT transcripts are detected in roots, stems, leaves, flowers, fruits, and seeds of different plant species, but they are expressed in a tissue-specific manner. Type 1 MTs are expressed more abundantly in roots (Guo et al., 2003; Kohler et al., 2004; Yang et al., 2009), whereas type 2 MTs are found mainly in leaves (Huang and Wang, 2009, 2010a). Type 3 MT RNAs are highly expressed in ripening fleshy fruits or in leaves (Guo et al., 2003; Kohler et al., 2004), while the expression of type 4 MTs, such as the wheat Ec MT, is restricted to developing seeds (Cobbett and Goldsbrough, 2002). In animals, MTs have been suggested to play a role in a number of processes, including the detoxication of nonessential metals such as Cd, Pb and Hg, the homeostasis of essential metals like Cu and Zn and the scavenging of free radicals (Foley et al., 1997). Despite the confirmation of the presence of MT genes in various plants, relatively little is known about the role and function of MTs in plants (Mir et al., 2004; Zhang et al., 2004). However, some plant MTs were found to be involved in cellular metal homeostasis or tolerance mechanisms (Cobbett and Goldsbrough, 2002; Mir et al.,

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2004). For instance, the induction of MT transcripts has been observed in plants exposed to heavy metals (Gonzalez-Mendoza et al., 2007; Schor-Fumbarov et al., 2005; Usha et al., 2007). When expressing a *Typha latifolia* MT gene (*tyMT*) in copper sensitive MT-deficient strain of yeast, it restored the yeast's metal tolerance (Zhang et al., 2004). Recently, Yang et al. (2009) reported that the rice MT gene (*OsMT1a*) expression was induced specifically by Zn treatment. In addition, both transgenic plants and yeasts harboring *OsMT1a* accumulated more Zn than wild type control.

Bruguiera gymnorrhiza is a dominant mangrove species along south china coast. Like other mangrove species, i.e. *Kandelia candel* and *Avicennia marina*, *B. gymnorrhiza* also plays a critical role in maintaining mangrove ecosystems. However, basic understanding of *B. gymnorrhiza* molecular physiology is still limited. In previous work, a cDNA clone, designated *BgMT2*, was isolated from *B. gymnorrhiza* by RT-PCR (GenBank accession no. DQ494173). It had an open-reading frame encoding a protein comprising 79 amino acids. The deduced amino acid sequence of *BgMT2* contained the Cys-rich regions typical of plant type 2 MT proteins. A Real-time quantitative PCR protocol was developed to directly evaluate the expression of *BgMT2* mRNA in previous studies, when *B. gymnorrhiza* seedlings were exposed to different concentrations of zinc (Zn), copper (Cu) or lead (Pb). Real-time quantitative PCR results demonstrated that the regulation of *BgMT2* mRNA expression by Zn, Cu and Pb was strongly dependent on concentration and time of exposure. In addition, significant increase in the transcripts of *BgMT2* gene was also found in response to Zn, Cu and Pb, at least under some experimental conditions (Huang and Wang, 2009). To date, the information about its putative function is derived from mRNA expression studies, but existing data on the effect of metal ions is inconsistent. In this paper, in order to provide further information on *BgMT2* gene expression and its function in response to heavy metals, the expression pattern of *BgMT2* mRNA was analyzed in leaves of *B. gymnorrhiza* in response to Cd using Real-time quantitative PCR. Moreover, the metal-binding characteristics of *BgMT2* protein and its possible role in homeostasis or detoxification of heavy metals were investigated by overexpressing it in *Escherichia coli* BL21 (DE3) as a carboxy-terminal extension of glutathione-S-transferase (GST).

2. Materials and methods

2.1. Plant material and treatment

B. gymnorrhiza was planted in pots filled with water-washed sand and watered with 1/2 Hoagland's solution (containing 10‰ NaCl) under greenhouse conditions. For the experiment of heavy metal exposure, eight-month-old *B. gymnorrhiza* was submerged in liquid solution (as described above) containing different concentrations of $CdCl_2$ (2 μM, 10 μM, 20 μM or 40 μM) for 3, 7 and 11 d. These doses were established based on previous studies (Huang and Wang, 2010b). Control plants were submerged in liquid solution without heavy metals. Following heavy metal treatment, plants were prepared for RNA extraction.

2.2. RNA isolation and first strand cDNA synthesis

RNA isolation and first strand cDNA synthesis were carried out according to the method devised by Huang and Wang (2009). Leaf portions were homogenized in liquid nitrogen in a mortar and total RNA was extracted using concert™ plant RNA reagent (Invitrogen). After treatment with RNase-free DNase, reverse transcription was performed using 0.8 μg of total RNA, random primers, and reverse transcription system (Promega) in a total volume of 20 μL. The reaction was incubated at 25 °C for 10 min followed by incubation at 42 °C for 60 min. The first strand cDNA was checked using the 18S rRNA gene to assess the quality of the reverse transcription (conventional PCR). PCR products were run on gelred (Biotium) stained 2% agar gels. The first strand cDNA was diluted to 1:5 and stored at −40 °C until used in Real-time quantitative PCR assays.

2.3. Real-time quantitative PCR

The protocol described by Huang and Wang (2009) was adapted for Real-time quantitative PCR measurement of *BgMT2* transcripts. Briefly, a constitutive expression gene, the 18S rRNA gene, was used as internal control to verify Real-time quantitative PCR. *BgMT2* (GenBank accession no. DQ494173) quantitative PCR primers were 5′-TCTTGCTGTGGTG GAAACTG-3′ (sense) and 5′-ATCTCGGCTCCCTCAAAGT-3′ (antisense). *B. gymnorrhiza* 18S rRNA (GenBank accession no. AB233615) quantitative PCR primers were 5′-CGGGGGCATTCGTATTTC-3′ (sense) and 5′-CCTGGTCGGCATCGTTTAT-3′ (antisense). Real-time quantitative PCR amplification was performed using the SYBR® Green Real-time PCR Master Mix (Toyobo) according to the instructions provided by the manufacturer. To ensure gene-specific amplification, melting curve analyses were conducted for all samples. Fold induction in *BgMT2* mRNA expression relative to the control was determined by the standard $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). This formula was determined as: $\Delta C_{T\ \text{treatment}}$ (the threshold cycle of MT of treatment sample) $= C_{T\ \text{MT treatment}} - C_{T\ 18S\ \text{rRNA treatment}}$; $\Delta C_{T\ \text{control}}$ (the threshold cycle of MT of control sample) $= C_{T\ \text{MT control}} - C_{T\ 18S\ \text{rRNA control}}$; $\Delta\Delta C_T = \Delta C_{T\ \text{treatment}} - \Delta C_{T\ \text{control}}$. Real-time RT-PCR was performed in duplicates for each sample. All data were given in terms of relative mRNA expressed as mean ± SD. Student-Newman-Keuls was performed to determine the significant difference between treated and control group using SPSS 13.0. Significant difference is indicated by an asterisk ($P \leq 0.05$).

2.4. Cloning of BgMT2 in pGEX-4T-1

The coding region of *BgMT2* was amplified by PCR with oligonucleotide primers, containing a *Bam*HI and an *Xho*I site, respectively: sense primer 5′-CGCGGATCCATGTCTTGCTGTGGTGGAAACT-3′; antisense primer 5′-CCGCTCGAGTCATTTACAAGTGCAGGGGTC-3′ (restriction sites underlined). The 40-cycle amplification reaction was performed with PCR Master Mix (Promega) under the following conditions: 45 s at 94 °C (denaturation), 45 s at 60 °C (hybridization) and 45 s at 72 °C (elongation). Then the PCR products were digested with *Bam*HI and *Xho*I and then inserted into pGEX-4T-1 vector, in-frame with a sequence encoding an N-terminal GST tail. Plasmid construction was made in *E. coli* DH5α for integrity and identity analysis, and gene expression was performed in *E. coli* BL21 (DE3). Both plasmid-containing strains of *E. coli* were grown in LB medium supplemented with 50 μg/mL of ampicillin.

2.5. Heavy metal tolerance analysis of transformed E. coli

To address tolerance of *E. coli* BL21 (DE3) transformed with pGEX-*BgMT2* or pGEX-4T-1 to metals in the growth medium, an overnight culture of *E. coli* BL21 (DE3) cells was diluted 100-fold in fresh LB medium containing 50 μg/mL of ampicillin for induction culture. When the OD_{600} reached 0.5, the expression of *BgMT2* gene was induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) followed by the addition of 400 μM $ZnSO_4$, $CuSO_4$, $Pb(NO_3)_2$ or $CdCl_2$, respectively. Bacterial growth was monitored by OD_{600} measurements at 1 h intervals for 8 h. Each sample was analyzed in duplicate.

2.6. Analysis of metal-binding properties of BgMT2 protein

An overnight culture was diluted 1:100 in fresh LB medium containing 50 μg/mL of ampicillin and grown at 37 °C until mid-exponential-growth phase ($OD_{600} = 0.5–0.8$). Induction with 100 μM IPTG was performed, and cultures were grown for a further 3 h in the presence of 400 μM