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Research paper

Cloning and characterization of metallothionein gene (*HcMT*) from *Halostachys caspica* and its expression in *E. coli*

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ABSTRACT

Halostachys caspica is a short shrub distributed in the semi-arid and saline–alkali area, which evolved various mechanisms for modulating salt and metal level. In the present study, a Type 2 metallothionein (*HcMT*) gene was cloned from the salt induced suppression subtractive hybridization (SSH) cDNA library of *H.caspica*. Quantitative real time PCR (qRT-PCR) analysis indicated that *HcMT* gene was up-regulated under the stress of Cu^{2+}, Zn^{2+} and Cd^{2+}, and the tolerance of *E. coli* strain harboring with the recombinant *HcMT* (pET-32a-*HcMT*) to Cu^{2+}, Zn^{2+} and Cd^{2+} was enhanced compared to strain with control vector (pET-32a). Moreover, the purified TrxA-HcMT fusion protein from *E. coli* cells grown in the presence of 0.3 mM $CuSO_4$, 0.3 mM $ZnSO_4$, or 0.1 mM $CdCl_2$ could bind more metal ions than TrxA alone. The predicted 3D structure showed that HcMT could form a single metal–thiolate cluster, which confers the ability to bind five divalent metal ions through fourteen cysteine residues. These data indicate that *HcMT* may be involved in processes of metal tolerance in *H. caspica* and could be employed as a potential candidate for heavy metal phytoremediation.

1. Introduction

Heavy metal contamination is a concern of environmental issues. Heavy metal contaminates soil then on to the food chain which does harm to human or other creatures (Gall et al., 2015). Physical, chemical and biological methods are available to remediate soil contaminated by heavy metals (Cao et al., 2002). Phytoremediation is a technology that uses green plants to clean up environmental pollution. It is an economical and effective, environmental protection and sustainable development approach. Usually, plants can respond to the heavy metal stress by expressing metallothioneins (MTs) to protect themselves (Gu et al., 2015; Kim et al., 2014).

MTs is a kind of low molecular weight, rich in cysteine, which has the ability to bind metal ions (Fernandez et al., 2012; Nevrtalova et al., 2014; Rauser, 1999). Since being purified from horse kidney for the first time (Margoshes and Vallee, 1957), MTs have been found in many living organisms (Huang and Wang, 2010). Based on the structural characteristics, MTs can be divided into three classes: Class I MTs contain 20 conserved cysteine residues that are widely found in vertebrates, and class II MTs are mainly found in plants, fungi and invertebrates. Class III MTs mainly exist in plants, which are nontranslationally synthesized polypeptides composed of some repeating units of γ-Glu-Cys (Rauser, 1999). According to the location of cysteine residues, Class II MT proteins in plant can be further divided into four types (Cobbett and Goldsbrough, 2002; Freisinger, 2011). Type 1 contains only Cys-x-Cys motif, and Type 2 contains a Cys-Cys and a Cys-x-x-Cys pair in the N-terminal domain 1. Type 3 has the same Cys arrangement in domain 1 with the Type 2, but the number of Cys in domain 2 increased to 9. The six Cys residues located at the C-terminus in Type 1, Type 2, and Type 3 are arranged in a highly conserved pattern (CxCxxxCxCxxCxC) (Freisinger, 2011). The Type 4 genes from a variety of fruit and rice have a truncated domain 1, with four Cys, and a domain 2 with Cys arranged as in Types 1 and 2 (Ledger and Gardner, 1994). The unique arrangement of Cys residues of plant MTs is very different from that of mammalian MTs with highly conserved sequence. The diversity of gene family of plant MT suggests that they may have different sequence and function (Freisinger, 2011).

Expression of metallothionein genes is regulated by abiotic stress including metals and plays an important role in metal detoxification (Singh et al., 2011; Usha et al., 2009). It has been reported that the expression of Type 2 *MT* genes in leaves was enhanced under Cu^{2+}, Zn^{2+} and Cd^{2+} treatment (Choi et al., 1996), some were not affected by the metal ions (Foley and Singh, 1994) and some expression was down regulated by Cu^{2+} and Cd^{2+} (Hsieh et al., 1996). In addition, some MTs expressed in *E. coli* showed metal-binding ability, suggesting that MTs had the function of detoxification of heavy metals (Chaturvedi et al., 2012; Sauge-Merle et al., 2012).

Halostachys caspica, a dwarf shrub grown in saline–alkaline area, can endure high salt concentration up to 700 mM (Guan et al., 2010). This extremely salt tolerant species has evolved various mechanisms for modulating salt and metal level. Though metallothioneins have been

Abbreviations: qRT-PCR, quantitative real time PCR; HcMT, *Halostachys caspica* metallothionein; IPTG, isopropyl β-D-thiogalactoside; OD, optical density; Trx, thioredoxin protein.

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reported from some plant species under heavy metal stress, there are few reports on the regulation of salt induced halophyte metallothionein, especially in *H. caspica*.

In the present study, the metallothionein gene (*HcMT*) was cloned and characterized based on the SSH cDNA library of *H. caspica* under salt stress constructed in our laboratory (Liu et al., 2012). Biological information analysis showed that HcMT shared high homology with some plant Type 2 MT proteins (Chaturvedi et al., 2012), which can be induced under heavy metal stress (Choi et al., 1996). Therefore, we carry out the study on gene expression and the binding of heavy metal. Expectations based on these results, further study of transgenic plants, the transgenic plants overexpressing metallothionein, for the removal of heavy metals irons from soil contaminated by heavy metals. So the expression pattern of *HcMT* mRNA in response to metal ions stress by qRT-PCR in *H. caspica* and the heavy metal tolerance of recombinant *E. coli* strain with *HcMT* gene were studied. Moreover, the metal-binding ability of the recombinant protein TrxA-HcMT was assayed. This work may shed some light on the roles of HcMT in response to metal stress in *H. caspica* and lay the foundation for development of transgenic plants for phytoremediation.

2. Materials and methods

2.1. Plant growth and heavy metal treatment

H. caspica seeds were collected in the Gurbantunggut Desert in Xinjiang in northwestern China and were germinated on wet filter paper saturated with distilled water. Ten days later, the seedlings were transferred to compost soil (3:1 vermiculite:perlite), watered with 1/4 MS solution (Murashige and Skoog, 1962), and grown under laboratory conditions with dark/light cycle of 8/16 h at 26 °C and 20–30% relative humidity for 2 months. Salt stress was conducted by treating the plants with 1/4 MS nutrient solution containing 600 mM NaCl for 24 h. For heavy metal treatment, 1/4 MS solution containing different metals ions (100 mM $CuSO_4$, 100 mM $ZnSO_4$, 100 mM $CdCl_2$) was used to treat plants for 6, 12, 24, 48, 72 h, respectively.

2.2. Cloning of HcMT gene

In order to clone *HcMT* gene, the assimilating branches of *H. caspica* were collected to extract total RNA after treatment with 600 mM NaCl for 24 h. Trizol reagent (Invitrogen, USA) was used to extract total RNA, and 1.0 μg of which was reverse transcribed into the first strand cDNA by M-MLV reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. The reaction was incubated at 42 °C for 60 min. Specific primers of the open reading frame (ORF) sequence of *HcMT* were designed based on the EST data base of *H. caspica* (NCBI accession no. **HS586469**; Liu et al., 2012) as: Forward: 5′-ATG TCT TGC TGT GGT GGT AAC TG-3′ and reverse: 5′-TCA TTT GCA GGT GCA AGG GTT GC-3′. PCR reaction conditions were as follows: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s; and a final extension at 72 °C for 5 min.

2.3. Transcription analysis of HcMT under metal stress

RNA was extracted from the control and metal treated plants by Trizol reagent (Invitrogen, USA), after quantified with Nanodrop spectrophotometer (ND-1000, USA), total RNA was used for quantitative RT-PCR analysis according to the the instruction manual, SYBR Green (Qiagen, German) was employed and reaction was carried out with 7500 Real-Time PCR system. The qRT-PCR reaction mixture contained 10 ng of cDNA template, 1 × PCR buffer, 200 μM dNTPs, 1.25 U Taq DNA polymerase and 5 μM of each of gene specific primers (forward: 5′-CTTGCTGTGGTGGTAACTGTGGT-3′ and reverse: 5′-GAGAACGGTG GGGTTAGAAGTGT-3′) or *β-actin* primers (forward: 5′-CCAAAGGCCA ACAGAGAGAAGA-3′ and reverse: 5′-GAGACACACCATCACCAGAAT-3′). The thermal cycling conditions were as follows: 95 °C for 10 min; 40 cycles of 94 °C for 20 s, 60 °C for 30 s, and 68 °C for 40 s. The experiments were repeated in triplicate independently and the amplified product was run on 1% agarose gel to confirm expected size. The qRT-PCR data were analyzed by comparative Ct method and relative fold change in gene expression ($2^{-\Delta\Delta Ct}$) of *HcMT* in heavy metal treated plants and the control samples (without treatment) was obtained by normalizing with the internal control *β-actin* Ct values.

2.4. Expression, purification and Western blot analysis of TrxA-HcMT fusion protein

Recombinant prokaryotic expression vector pET32a-*HcMT* was constructed by inserting the *HcMT* PCR product with the following primers: Forward: 5′-CCGGAATTCATGTCTTGCTGTGGTGG-3′ (*Eco*R I site underlined) and reverse: 5′-CCGCTCGAGTCATTTGCAGGTGCAA GGG-3′ (*Xho*I site underlined), and sequenced to verify *HcMT* correctness. The *E. coli* BL21 (DE3) harboring with pET32a-*HcMT* was induced

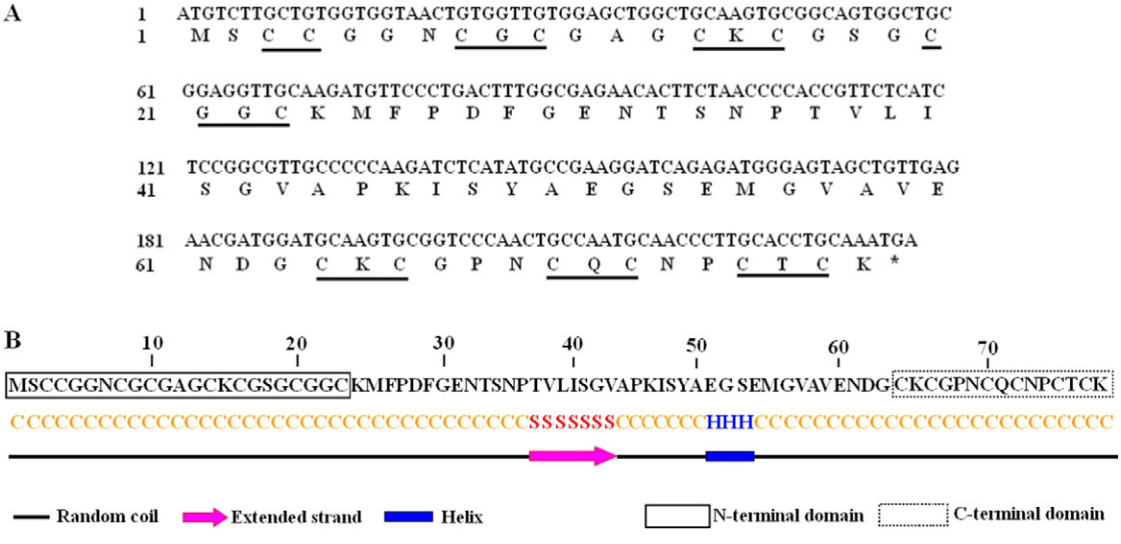

Fig. 1. Analysis of nucleotide and deduced amino acid sequence of HcMT. (A) Nucleotide and deduced amino acid sequence. 'C-C', 'C-X-C', 'C-X-X-C' motifs are underlined. (B) Secondary structure prediction by I-TASSER. Random coil is denoted by 'C'. Extended strand is denoted by 'S'. Helix is denoted by 'H'. The sequence is presented to emphasize the N-terminal domain and C-terminal domain.