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Molecular Cloning and Characterization of Type 2 Metallothionein cDNA from *Codonopsis lanceolata* (S. et Z.) Trautv

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ABSTRACT : A class I type 2 metallothionein (*CMet2*) cDNA from taproot of *Codonopsis lanceolata* was isolated and characterized. A *CMet2* cDNA was 572 nucleotides long and had an open reading frame of 234 bp with a deduced amino acid sequence of 78 residues (pI = 4.99). The deduced amino acid sequence of *CMet2* matched to the previously reported type 2 metallothionein-like protein genes and showed 74% identity with that of *G. max* (BAD18377) and *C. arietinum* (CAA65009). Expression of *CMet2* by the RT-PCR was increased at 1 hr after cadmium and hydrogen peroxide treatment, respectively.

Key words : Abiotic stress, cadmium, *Codonopsis lanceolata*, metallothionein, RT-PCR

INTRODUCTION

Metallothionein (MT) is widely distributed in animals, eukaryotic microorganisms, certain prokaryotes and plants, and is defined as low molecular weight cysteine-rich proteins that bind with heavy metals (Robinson *et al.*, 1993). The molecules were found to play an important role in heavy metal tolerance and detoxification of plants, animals and microorganisms. Plant MTs and MT-like proteins are sub-divided into three classes and several types, according to the location and distribution of the cysteine clusters in protein sequences (Kortba *et al.*, 1999; Rauser, 1999; Cobbett, 2000)

Despite the confirmation of the presence of MT genes in various plants, their expression and functions in plants are still unclear. In sight of their metal-binding capacity, it has been suggested that MTs may play a role in the homoeostasis of essential metal ions and the detoxification of heavy metals, such as Cd^{2+} or Hg^{2+} (Hamer, 1986). However, MTs have now been known to be implicated in a wide range of biological processes relating to normal development and both biotic and abiotic responses, some but not all of which obviously involve metal sequestration (Riordan & Vallee, 1991).

The expression of MT-like genes by plants grown in excess heavy metals is different as to plant species-, tissue- and metal-specific. In *Brassica juncea*, the level of type 2 MT-like mRNA decreased in response to elevated external Cu^{2+}, but increased when exposed to Zn^{2+} (Snowden *et al.*, 1995). The expression of plant MTs was also detected in response to various stress conditions, such as heat shock and glucose starvation in rice (Hsieh *et al.*, 1995), leaf senescence in *Brassica* (Buchanan-Wollaston, 1994), wounding and viral infection in *Nicotiana* (Choi *et al.*, 1996), and fruit development in kiwi fruit (Ledger & Gardner, 1994). The functions of plant MT-like genes appear to be diversified. The majority of the plant systems used for MT genes expression studies, however, were non-tolerant to heavy metals. The possible role for MTs in high-level metal tolerance remains elusive. The present study therefore, aims to provide further information on the MT-like gene present in *C. lnceolata* genome, as well as, its expression and functions.

MATERIALS AND METHODS

RNA purification and cDNA library construction

Total RNA was isolated from 3-year taproot of *C. lanceolata* using aqueous phenol extraction procedure as described by Morris *et al.* (1990). The taproot was frozen and ground in liquid nitrogen prior to extraction of RNA. Poly $(A)^+$ RNA was isolated by oligo (dT) cellulose column using the Poly(A) Quick mRNA isolation kit (Stratagene, USA). A commercial cDNA synthesis kit was used to construct library according to the manufacture's instruction manual (Clontech, AUS). To produce single-stranded cDNA appropriate for directional cloning 5 μg of poly $(A)^+$ RNA was primed with an

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oligo (dT) primer. Double-stranded cDNA was produced using RNase H and *E. coli* DNA polymerase. After ds cDNA synthesis by primer extension, cDNA was digested with *Sfi* I and then size-fractionated through a Chroma spin-400 column. Size-selected cDNA was ligated into λTriplEx2 vector and was packaged *in vitro* using Gigapack III Gold Packaging Extract kits (Stratagene, USA). Fractions containing cDNA greater than 500 bp were recovered and this library was amplified once to yield a final titre of 2×10^9 pfu ml^{-1}.

Nucleotide sequencing and sequence analysis

pTriplEx2 phagemids were converted from λTriplEx2 library in *E. coli* strain BM25.8. Phagemids containing inserts were selected by blue and white color screening on IPTG/X-GAL/ampicillin plates. Single-run partial sequencing of such randomly selected cDNA clone was performed. The 5' ends of the cDNA inserts were sequenced using sequencing primer by an automatic DNA sequencer (ABI prism 3700 DNA sequencer, Perkin-Elmer, USA) according to the thermal cycling protocol of the BigDye Terminator Cycle Sequencing kit. Sequences were edited to remove the vector sequence, poly A tails and ambiguous regions. Bacterial genomic DNA sequences were identified by BLASTN comparisons against the GenBank non-redundant databases. Sequences shorter than 100 bases were discarded. The individual ESTs were searched against the GenBank nr database using a BLASTX algorithm. A pTriplEx phagemid for metallothionein cDNA was excised from the λTriplEx2 and used as templates for sequence analysis. The cDNA insert was sequenced using the 5' and 3' sequencing primer by an automatic DNA sequencer (ABI prism 3700, USA). Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi, Japan). Comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm of Altschul *et al.* (1990).

Plant materials and abiotic stress treatments

Three-year old *C. lanceolata* grown at field were used. Leaves attached in the upper region cut with a scalpel. Cut leaves were soaked in 1 mM H_2O_2 for the oxidative stress or in 100 uM cadmium ($Cd\cdot 5/2H_2O$, Sigma) during 30 min, 1 hr, 2 hr, and 4 hr, respectively. After the stress treatments, treated leaves were immediately frozen in liquid nitrogen and stored at −80℃.

Total RNA preparation

Total RNA was isolated from the stress treated *C. lanceolata* leaves using the method of guanidine isothiocyanate (TRIzol, Gibco BRL). The tissue was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Extraction reagent was added 1 ml and ground more than 3 min. The mixture was centrifuged for 5 min at 4. The supernatant was removed to a new tube, added 0.2 mℓ chloroform, and vigorously mixed. Following centrifugation, total RNA was precipitated with isopropanol. The pellet was washed once with 75% ethanol, dried in vacuum for 3 min and dissolved in DEPC treated DDW. The total RNA solution was stored at −80℃ for the RT-PCR analysis.

Quantitative RT-PCR analysis

To analyze the gene expression of *CMet2* gene against the oxidative and heavy metal stresses, we employed the quantitative RT-PCR. A pair of gene-specific primers of the *CMet2* gene was designed and used for RT-PCR analysis. Specific primers included the following: (*CMet2*-forward) 5'-TCT TGC GGA GGA AAC TGT GGG T-3'; (*CMet2*-reverse) 5'-CCT AGT CGG GCT AGG GCA TAG G-3'. As a control, we used a pair of the specific primers to *C. lanceolata* actin gene (*CAct*), 5'-CGA GAA GAG CTA CGA GCT ACC CGA TGG-3' (forward) and 5'-CTC GGT GCT AGG GCA GTG ATC TCT TTG CT-3' (reverse). Ten microgram of total RNA was used for the RT-PCR analysis, according to the method of Takakura *et al.* (2000). The PCR cycles for quantitative RT-PCR numbered 30 for the *CMet2* and the *PAct* genes. RT-PCR products were run on 1.5% (W/V) agarose gel in 0.5× TAE buffer and then photograped for the expression analysis.

RESULTS AND DISCUSSION

EST (expressed sequence tags) analysis was performed with a full length cDNA library prepared with the tap root of *C. lanceolata.* The 5' ends of randomly selected cDNA inserts were sequenced using the 5' sequencing primer and then comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm (Altschul *et al.*, 1990). A Class I type 2 metallothionein-like protein homolog of analyzed EST clones was isolated and full sequenced and named with *CMet2.*

CMet2 gene was 572 nucleotides long and possessed an open reading frame of 234 bp with 69 bp 5'-untranslated region (5'UTR) and 266 3' UTR (Fig. 1). BLASTX database searches with the *CMet2* sequence gave metallothioneins and metallothionein-like proteins from various organism as the top 100 best matches. The *CMet2* cDNA encodes a protein of 89 amino acids (MW 7.8 kDa) with pI 4.99 (Fig. 1). The deduced amino acid residues of *CMet2* contained the cysteine-rich regions typical of plant type 2 MT-like proteins, including the presence of C-C, C-X-C, C-X-X-C motifs at amino-terminus and carboxy-terminus. The deduced amino acid sequence of

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GGTTCATATAGGATATAATATTCTTTGAAGTTCTTTCTCTGTGTGAAAGAAAAGAAAAGG   60
AAAAAGAAAATGTCTTGCTGTGGAGGAAACTGTGGGTGTGGATCTGGCTGCAAGTGCGGC  120
        M  S  C  C  G  G  N  C  G  C  G  S  G  C  K  C  G       17

AGTGGGTGTGGAGGGTGCAAGATGTACCCAGACATGAGCTACACAGAGTCAGAGAGCACC  180
S  G  C  G  G  C  K  M  Y  P  D  M  S  Y  T  E  S  E  S  T     37

ACCGCTGAGACCCTCATTCTTGGTGTTGCACCCAAATCCAAGACCATCATGTACTGTGAA  240
T  A  E  T  L  I  L  G  V  A  P  K  S  K  T  I  M  Y  C  E     57

GGATCAGAGAATGGAGGATGCAAGTGTGGGGCCAACTGCACCTGCGACCCATGCACCTGC  300
G  S  E  N  G  G  C  K  C  G  A  N  C  T  C  D  P  C  T  C     77

AAATGATGAGTGGACTAGCCCCCCAAGCAGAGATGGATCTGATCTGGAGTAGGATCATGG  360
K  *                                                           78

ATGGGTGATTCTAAATTAGAAAAAAATAAATCAAATAGGTTAGTGTGATGGGAGGTAATT  420
AGGGGGTTTAATAATGGTGTAACTGAAACCCCTATGCCCTAGCCCGACTAGGTCTTGTTA  480
ATAAGTAAAAGAAGTGACTATTGTGTTGAAGTTGCTTTTTTTTTGTGTGTGTGTGTGTGTG  540
TGTGTGTGTGGCAGTTAGTTGCCTTGCTTTTG                               572
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Fig. 1. Nucleotide and deduced amino acid sequence with the open reading frame from 70 to 306. The positions of nucleotides are shown on the left and the positions of amino acids under the below. Asterisk shows the termination codon. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequencing Database under the accession number AY833717.

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Codonopsis    MSCCGGNCGCGSGCKCGSGCGGCKMYPDMSYTESESTTAETLILGVAPKSK------TIMY 55
Poa           MSCCGGSCGCGSGCKCGNGCGGCKMYPGM---DEGLTTSQTLIMGVAPSSKPSFEDDAAA 57
Brassica      MSCCGGNCGCGSGCKCGNGCGGCKMYPDLG-FSGELTTTETFVFGVAPTMKN----QHEAS 56
Pringlea      MSCCGGNCGCGSGCKC-VGCGGCKMYPDLG-FSGETTTTETLVLGVAPAMNS----QYEAS 55
Cicer         MSCCGGNCGCGSSSCKCGSGCGGCKMYPDMS-YTEQ-TTSETLVMGVASGKTQ----FEGAE 55
Vigna         MSCCGGNCGCGSSSCKCGSGCGGCKMYPDLS-YTEQ-TTTETLVMGVAPVKAQ----FEGAE 55
Glycine       MSCCGGNCGCGSSSCKCGNGCGGCKMYPDLS-YTES-TTTETLVMGVAPVKAQ----FESAE 55
Lycopersicon  MSCCGGNCGCGSSSCKCGNGCGGCKMYPDMS-YTESSTTTETLVLGVGPEKTS----FGAME 56
Plantago      MSCCNGNCGCGSGCKCGSGCGGCKMYPDLSYSEASTTVSVSSVLGLAPKVTYFE----GSE 57
Avicennia     MSCCGGNCGCGAGCKCGNGCGGCNMYPDLGYSEA-TAPAEALVLGVAP-LKFYE----GAE 55
              ****.*.****:.***  *****:***.:      : : : ::*..

Codonopsis    CEG--SENGGCKCGANCTCDPCTCK-                                    78
Poa           ATG---AENGGCKCGDNCTCNPCTCK-                                   80
Brassica      GEG-VAENDACKCGSDCKCDPCTCE-                                    80
Pringlea      GETFAAENDACKCGSDCKCNPCTCK-                                    80
Cicer         MG-FGAENDGCKCGSNCTCNPCTCK-                                    79
Vigna         MG-VAGENDGCKCGSNCSCNPCTCK-                                    79
Glycine       MG-VPAENDGCKCGANCTCNPCTCK-                                    79
Lycopersicon  MGESPVAENGCKCGSDCKCNPCTCSK                                    82
Plantago      TGVTVSENG-CKCGDNCSCNPCNCK-                                    81
Avicennia     SVEGAAENG-CKCGANCTCNPCNCK-                                    79
                 :. **** :*.*:**.*.
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Fig. 2. Comparison of the putative amino acids sequence of *CMet2* with those of metallothionein genes from other plants; *C. lanceolata* (AAV97748), *P. secunda* (AAK38824), *P. major* (CAH59436), *G. max* (BAD18377), *C. arietinum* (CAA65009), *A. marina* (AAG50080), *B. oleracea* (AAF 70556), *P. antiscorbutica* (AAP94016), *L. esculentum* (AAB04675), *V. radiata* (BAD18375). Sequence data was obtained from GeneBank listed and aligned using DDBJ ClustalW (Thompson *et al.*, 1994 and 1997) and GeneDoc (Nicholas *et al.*, 1997).

CMet2 cDNA was compared to other related class I type 2 metallothionein gene products registered previously (Fig. 2). A *CMet2* protein sequence shared the highest similarity (74%) with *G. max* and *C. arietimum, followed by* 70% with *L. esculentum*. In the phylogenetic analysis, *CMet2* was closer with type 2 metallothinein-like protein of *P. secunda* (Fig. 3).

Plants genes encoding proteins with MT domains can be

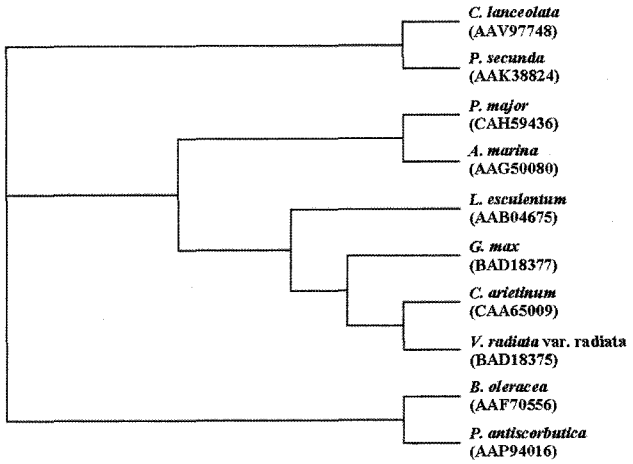

Fig. 3. Phylogeny of the class I type 2 metallothionein protein family from *C. lanceolata* and other plants. Phylogenetic analysis is based on the deduced amino acid sequences of metallothionein genes from various plant species. The branch lengths are proportional to divergence, with the scale of 0.1 representing 10% change.

subdivided into two categories (types I and II) on the basis of the arrangement of cysteine residues (Robison *et al.*, 1993). Type I MT-like genes, which include *M. guttatus*, pea (PsMT) and *A. thaliana* (MT1) (de Miranda *et al.*, 1990; Evans *et al.*, 1990; Zhou & Goldsbrough, 1994), appear to be constitutively expressed in the roots while transcripts of genes in the type II category, including soybean and *A. thaliana* (MT2) (Kawashima *et al.*, 1991; Zhou & Goldsbrough, 1994), are present predominantly in the leaves/aerial tissues. So, to investigate the expression of a *CMet2* gene related with abiotic stresses, such as cadmium and H_2O_2, we performed quantitative RT-PCR analysis using the leaf of *C. lanceolata*. Identification of the PCR product amplified with *CMet2* specific primers was confirmed by DNA sequence analysis. Expression of the actin gene isolated from *C. lanceolata* served as an internal control for quantitative RT-PCR analysis.

In plant, Cd accumulation causes reductions in photosynthesis, dimishes water and nutrient uptake (Sanita di Toppi & Gabbrielli, 1999), and induces the synthesis of phytochelatins, which bind metals in the cytosol and sequester them in the vacuole (Rauser, 1995; Mehra & Tripathi, 2000). In order to analyze the expression profiling of *CMet2* gene, leaf tissues of *C. lanceolata* were soaked in 100 uM Cd solution. Total RNA from each treated leaves was extracted separately, and performed the RT-PCR analysis (Fig. 4). The *CMet2* gene was differentially expressed with respect to different periods of exposure times. The transcription of *CMet2* gene was gradually increased from 30 min to 2 hr treatment time and the maximum accumulation was observed at 2 hr. When the

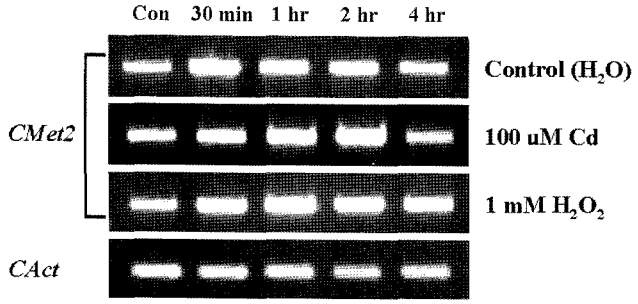

Fig. 4. Expression analysis using RT-PCR of *CMet2* under the stress of cadmium and hydrogen peroxide. Total RNA from stress-treated samples served as templates for quantitative RT-PCR with gene-specific primers. Actin gene isolated from *C. laceolata* was used for RT-PCR control.

leaves were soaked at 4 hr, the expression level of *CMet2* was decreased. The expression of plant MT-like genes was either decreased by Cu (Kawashima *et al.*, 1991; Okumura *et al.*, 1991) or enhanced by certain metals (Hsieh *et al.*, 1995; Snowden *et al.*, 1995). Some studies showed that metal exposure did not have any effects on MT-like gene expression (Lane *et al.*, 1987; Foley *et al.*, 1997). The transcript level of *CMet2* gene in *C. lanceolata* increased with the duration of Cd exposure, suggesting that the *CMet2* protein may function as one of the Cd defense mechanisms. Till now, information on the role of type 2 MTs in Cd detoxification and their induction by Cd in higher plants were scarce. Although, Giordani *et al.* (2000) reported that a type 2 MT gene isolated from *Posidonia oceanica* was induced by Cd, this seagrass tolerated high level of mercury, but not Cd.

Hydrogen peroxide (H_2O_2) is a signaling molecule which triggers, among other responses, secondary pathways and programmed cell death. We tested whether exposure to H_2O_2 had effects on the induction of *CMet2*. We used distilled water as a control. When *Codonopsis* leaves were soaked in water, the expression of *CMet2* gene was increased during 30 min to 2 hr and the maximum accumulation was observed at 1hr (Fig. 4).

The type 2 MT-like protein in *Nicotiana* by wounding and viral infection was induced (Choi *et al.*, 1996). The induction of *CMet2* in the water may cause the wounding. Cadmium is highly toxic and probably carcinogenic at low concentration. The biological effects of this metal and the mechanism of its toxicity are not yet understood. Cadmium is not redox active but contributes indirectly to oxidative stress by affecting the cellular thiol redox balance. The expression of *CMet2* gene in the *Codonopsis* leaf was induced with similar pattern at the treatment of H_2O_2 and cadmium. It may cause the contribution indirect to oxidative stress of cadmium.

We describe for the first time the isolation and characterization of class I type 2 MT-like gene in *C. lanceolata, CMet2*. Many plant systems used for MT genes expression studies were non-tolerant to heavy metals. The possible role for MTs in high-level metal tolerance and oxidative stress remains elusive. Therefore, we will continuously study further to find the relations between *CMet2* and abiotic stress and then produce the abiotic stress-tolerant transformants by re-introduction of *CMet2* into *C. lanceolata*.

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