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Cyamopsis tetragonoloba type 1 metallothionein (*CtMT1*) gene is upregulated under drought stress and its protein product has an additional C-X-C motif and unique metal binding pattern

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ABSTRACT

Metallothioneins (MTs) are involved in cellular homeostasis of essential metal ions and detoxification of nonessential metal ions. We report here the identification of four MT genes, *CtMT1*, *CtMT2*, *CtMT3* and *CtMT4*, encoding CtMT1, CtMT2, CtMT3 and CtMT4 proteins, respectively, from the industrial guar crop. The primary structures of last three proteins were similar to those of respective MT proteins of other plants but the CtMT1 protein primary structure was different from the other plant MT1 proteins in having an additional C-X-C motif. The four MT genes showed tissue specific expression patterns suggesting their specific roles in different tissues. High expression of *CtMT1* gene was observed in roots and nodules whereas *CtMT2* and *CtMT3* genes showed high expression in leaves. The expression of *CtMT4* gene was high in seeds. The qRT-PCR studies revealed upregulation in expression of *CtMT1* gene under drought stress. Recombinant CtMT1 protein was produced in *E. coli* Rosetta cells and purified by metal affinity chromatography. The purified protein showed antioxidant property and the order of its metal ion binding affinities was $Cu^{2+} > Zn^{2+} > Fe^{2+} > Cd^{2+}$. This information about CtMT1 protein is expected to be useful in understanding its role in drought tolerance and other physiological processes of guar.

1. Introduction

Metallothioneins (MTs) are a family of ubiquitous, small cytosolic proteins having low molecular weights (M_r < 10 kDa), high cysteine (Cys/C) contents, and metal binding abilities. The first classification system divided MTs into three classes, viz., I, II and III [1]. The class I contains MTs which are homologous to mammalian MTs whereas the class II includes MTs which are highly distant to mammalian MTs. The class I and II MTs are direct gene encoded polypeptides. The enzymatically (non-translationally) synthesized Cys-rich peptides, named as phytochelatins, are included in Class III [1]. According to the current (second) classification system, the class I and class II MTs are classified into 15 families whereas the class III MTs are classified as Family 99, a special MT-family [2]. The plant MTs have been placed into family 15 and further classified into four subfamilies i.e., type 1 (MT1), type 2 (MT2), type 3 (MT3) and type 4 (MT4) based on the distribution patterns of 'Cys' amino acid residues in the N- and C-terminal regions [2,3]. Each of the MT types 1, 2 and 3 contains two Cys-rich domains, one at the N-terminal region and the other at the C-terminal region. The N-terminal Cys-rich domains of types 1, 2 and 3 MTs have 6, 8 and 4 'Cys' amino acid residues, respectively whereas the each of the C-terminal Cys-rich domains of these three MT types contain 6 'Cys' amino acid residues. The two Cys-rich domains are separated by a large central Cys-free spacer region of 30–45 amino acids. The type 4 MT (MT4) has three Cys-rich domains containing 6, 6, and 5 'Cys' amino acid residues and these domains are separated by 10–20 amino acids [3–5]. These 'Cys' amino acid residues, generally distributed as 'C-X-C', 'C-X-X-C' and 'C-C' clusters, are known as metal binding motifs [3,5]. The variants of plant MTs with atypical organization of 'Cys' residues or the replacement of conserved amino acids with other amino acids in Cys-rich domains at N- or C-terminals have been observed in *Ipomoea batatas* [6], *Thlaspi caerulescens* [7] and *Prosopis juliflora* [8].

The genes encoding MTs have been identified in several plants including *Triticum aestivum*, *Vicia faba*, *Brassica napus*, *Arabidopsis thaliana*, *Oryza sativa*, *Nicotiana glutinosa*, *Lycopersicon esculentum* [5]. The expression analysis of the four types of MT genes revealed that each type has a typical temporal and tissue-specific expression profile [8–10]. Plant MTs have been found to play an important role in transport, sequestration and cellular homeostasis of essential metal ions [3,7,11,12] and detoxification of non-essential metal ions [3,13]. Studies of Yuan et al. [14] in *O. sativa* have indicated the role of plant MTs in seed germination and root development. Ren et al. [15] reported the role of MTs in seed development in *A. thaliana*. The plant MTs also appear to

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be involved in tolerance to various stresses. Yang et al. [16] reported the involvement of OsMT1a, a type 1 metallothionein, in drought tolerance in rice. The cotton metallothionein GhMT3a has been found to increase drought tolerance ability of transgenic tobacco plants [17].

Guar (*Cyamopsis tetragonoloba*, L. Taub.) is an important industrial crop and is cultivated in the arid and semiarid regions of India, Pakistan, United States, Sudan, Brazil and Australia. Traditionally, a vegetable, fodder and green manure crop, it has now attained the status of an industrial crop because of the presence of galactomannan gum (guar gum) in its seeds. Guar gum is a commercial gum used in several industries. In addition, guar gum because of its medicinal uses is an important pharmaceutical product [18,19]. Guar being a legume is a nitrogen-fixing crop and is well known for its tolerance to stresses such as drought, heat and salinity. Despite the economic importance of guar, very little work has been done on the identification, functions and regulation of the genes involved in the growth and development and, stress tolerance in this important crop. The plant MTs have not only the type-specific, but also the species-specific expression patterns [20,21] and the variants of plant MTs may have altered and efficient function [7,22]. As per our knowledge, genes encoding MTs have not been identified in guar so far. Thus, it would be very important to get the information about the arrangement and distribution of 'Cys' residues in MTs of this drought tolerant crop and, the expression profiles of MTs under natural and drought stress. Information related to their metal binding affinities would also be helpful to understand their functions. In view of this, the present work was undertaken to identify and study the expression analysis of MTs along with their metal binding and ROS scavenging ability in guar crop.

2. Materials and methods

2.1. Plant material, growth conditions and drought stress treatment

The seeds of *C. tetragonoloba* HG-365 variety were procured from C.C.S. Haryana Agricultural University, Hisar, India. This early maturing variety produces high galactomannan gum content in its seeds. One hundred seeds were surface sterilized by 1–2 min treatment with 0.1% (w/v) $HgCl_2$ solution. The treated seeds were soaked for 2 h in distilled water after 4 washings with sterile distilled water. A sand and soil (1:5) mixture was filled in pots. Three treated guar seeds were sown in each pot and the pots were kept in a screenhouse. The tissue samples (young leaves, mature leaves, young roots, mature roots, nodules at 27, 38 and 52 days after sowing (DAS), and developing seeds at 20, 25, 30, 35, and 40 days after flowering (DAF) were frozen in liquid nitrogen and stored at −80 °C.

When the experimental guar plants were 5 weeks old, watering was stopped till the plants showed permanent wilting. At this stage, the soil moisture content was found to be 3.5 ± 0.5%. The soil moisture content for the control plants was maintained at 10 ± 0.5% by watering. The leaf and root samples from treated and control plants were frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA isolation and cDNA synthesis

Total RNA isolation from a tissue was done using HIMEDIA® RNA-XPress reagent following the manufacturer's instructions. The DNA contamination in the isolated RNA sample was removed by RNase-free DNase I (Thermo Scientific) treatment as per manufacturer's procedure. The quality and the quantity of the RNA sample were checked by spectrophotometer (DeNovix DS-11). The quality of total RNA was also checked with the help of agarose gel (1.5%) electrophoresis. One microgram of total RNA (having absorbance ratios of A260/A280 = 1.9 to 2.08 and A260/A230 = 2.04 to 2.18) was used for cDNA synthesis using Verso cDNA synthesis kit (Thermo Scientific) following the manufacturer's protocol.

2.3. Identification of MT genes of guar

The nucleotide sequences for four types of MTs, namely, type 1, type 2, type 3 and type 4 MT genes of 20 legumes were retrieved from NCBI database. Multiple sequence alignment of sequences of each type gene was performed by Clustal Omega to identify conserved sequences. The extreme forward and reverse primer pair for each selected gene was designed manually from the conserved sequences and used in PCR to amplify the complete coding sequence (CDS) for the desired MT gene of guar. The characteristics of primer sequences used in this study are given in Table 1.

The cDNA was used as a template for PCR with specific primer pair for particular MT type (Table 1). The PCR was performed by following the protocol of 10 min at 95 °C, followed by 30 cycles of 15 s at 95 °C, 30 s at 55 °C (type 3 MT), 58 °C (type 1 MT) and 60 °C (type 2 and 4 MT) and, 45 s at 72 °C and final extension at 72 °C for 6 min. The PCR products were separated in a 1.5% agarose gel and the sequencing of these products was outsourced to Chromas Biotech. Pvt. Ltd., Bengaluru (India). The sequence data obtained from the company were confirmed by BLAST tool using NCBI database [23] and the putative CDS was translated to protein sequence using an ExPASy-translate tool (https://web.expasy.org/translate/).

2.4. In silico analysis

The translated protein sequence for each MT type of guar was searched against the non-redundant protein sequence (nr) database at NCBI (http://www.ncbi.nlm.nih.gov) using blastp. This deduced amino acid sequence for each MT type was also analyzed using ProtParam tool (https://web.expasy.org/protparam), TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/), SignalP4.1Server (http://www.cbs.dtu.dk/services/SignalP/) and InterProScan tool (https://www.ebi.ac.uk/interpro/search/sequence-search). The Multiple sequence alignment (MSA) of the deduced amino acid sequence for each MT type of guar with respective type of MTs across the plant species was performed using DNAMAN software version 9 (http://www.lynnon.com/index.html) as described in Xue et al. [17]. A phylogenetic tree was constructed based on protein sequences by N-J (neighbor-joining) method of MEGA 6 [24,25] software with 1000 replicates of bootstraps.

2.5. Amplification of type 1 MT gene on genomic DNA

Leaves were collected from 10-day-old guar plants and DNA was extracted from leaves by the CTAB method [26] with slight modifications. The quality of the extracted DNA was determined by gel electrophoresis on 0.8% agarose. The concentration and purity of the isolated DNA were determined using a DeNovix DS-11 spectrophotometer. The DNA samples having the absorption ratios of A260/A280 = 1.8 were used for PCR. PCR was performed with genomic DNA using the primer pair for *CtMT1* gene as given in Table 1. The sequencing of the PCR product was outsourced to Chromas Biotech. Pvt. Ltd., Bengaluru (India).

2.6. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR analysis was used to detect the expression of four types of MT genes in guar. cDNA synthesized from the total RNA isolated from various plant tissue samples (young leaves, mature leaves, young roots, mature roots, nodules at 27 DAS, 38 DAS and 52 DAS, and developing seeds at 20, 25, 30, 35, and 40 DAF) were used as templates to perform PCR. The ubiquitously expressed *ACT11* gene (actin 11, MF370605) (our unpublished data) was used as an internal control. Each PCR was performed by following the protocol described earlier in Materials and methods. The amplified PCR products were run on a 1.5% agarose gel and the result was documented using gel documentation system (Bio-Rad).