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Environment

Metallothioneins from a Hyperaccumulating Plant *Prosopis juliflora* Show Difference in Heavy Metal Accumulation in Transgenic Tobacco

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Abstract

Overexpression studies in plants have demonstrated the ability of metallothioneins (MTs) to hyperaccumulate heavy metals, but comparative analysis of heavy metal accumulation by different types of MTs from the same species is largely unavailable. Our previous study on three types of MTs (*PjMT1, PjMT2, PjMT3*) from heavy metal accumulating phreatophyte *Prosopis juliflora* reported that PjMT1 showed enhanced binding to cadmium, copper and zinc than other two types in *E. coli*. The present study, an extension of the previous work, compares the ability of *PjMT1* and *PjMT2* to impart heavy metal tolerance in transgenic tobacco plants. *PjMT1* and *PjMT2* were cloned separately in plant transformation vector under constitutive promoter along with GUS screening marker and transformed into *Nicotiana tabacum.* When subjected to 0.3 mM $CdSO_4$, both *PjMT1* and *PjMT2* expressing transgenic plants demonstrated better survival and higher accumulation of Cd than wild type plants. Atomic absorption spectrometry revealed *PjMT1* and *PjMT2* transformed tobacco plants exhibiting nine fold and five fold higher Cd accumulation respectively, in comparison to non-transgenic plants. Measurement of chlorophyll degradation upon cadmium stress also indicated more chlorophyll retention in *PjMT1* and *PjMT2* transgenics compared to wild type plants. The results of the study identify *PjMT1* as a better candidate gene for phytoremediation of cadmium.

Highlights

- Two metallothionein genes from *Prosopis juliflora*, *PjMT1* and *PjMT2* were cloned separately in pCAMBIA 1301 and transformed into *Nicotiana tabacum.*
- When tested for cadmium accumulation and tolerance, *PjMT1* transformants showed better performance than *PjMT2* counterparts.

Abbreviations: MTs-metallothioneins; AAS-atomic absorption spectrometry; GUS-ß-glucuronidase; cys-cysteine.

Keywords: *Prosopis juliflora;* metallothioneins; overexpression; tobacco.

Cadmium (Cd) is one of the most toxic metals contaminating the soil and water thereby reducing the productivity of plants. Cadmium disrupts the photosynthetic rate, respiratory and nitrogen metabolism, causing poor growth and low biomass (Dixit *et al.,* 2001). It also causes reduction in the shoot length, size and number of the leaves (Prasann *et al.,* 2012). At the cellular level, Cd produces oxidative stress by generating free radicals and reactive oxygen species (ROS). ROS cause unspecific oxidation of proteins and membrane lipids or may cause DNA injury leading to cell death. Several plant species including *Amaranthus viridis* and *Spinachea oleracea* that grow in metal contaminated soil are able to accumulate these heavy metals in their aerial parts and act as bioindicators of heavy metal pollution in the soil (Pal *et al.,* 2013; Prasann *et al.,* 2013). Plants that can survive on metalliferous soils with abnormally high concentrations of some of the heavy metals are called hyperaccumulators (Reeves, 1992).

Prosopis juliflora commonly known as mesquite is the one of the most economically and ecologically important tree species in arid and semi-arid zones of the world. Originally from central and/or South America, the mesquite is now pantropically introduced and well established, often as a weed. *P. juliflora* grows well in heavy metal laden industrial sites and is considered a hyperaccumulator (Senthilkumar *et al.,* 2005). Plants acquire heavy metal tolerance through various mechanisms like compartmentalization, sequestration, chelation or exclusion. Of all the detoxification mechanisms, chelation of heavy metals is very effective and is achieved using proteins like phytochelatins (PCs) and metallothioneins (MTs) (Zhou and Goldsbrough, 1994; Murphy *et al.,* 1997).

Metallothioneins (MTs) have been a focus of the plant metal tolerance research. MTs are small molecular weight peptides (4-8 kDa) rich in cysteine residues (25-33%) that are directly encoded by the MT gene. MTs typically contain two metal-binding, cysteine-rich domains that give these metalloproteins a dumbbell conformation. The large number of cysteine residues in MTs binds a variety of metals by mercaptide bonds. MTs can effectively sequester several metal ions, most notably Cu^{2+} and Zn^{2+} under normal physiological conditions, and more toxic metals like Cd^{2+} or Hg^{+} following exposure. There are also numerous indications that MTs are involved in responses to oxidative stress, possibly by scavenging peroxyl and free hydroxyl radicals (Mir *et al.,* 2004).

Plant MTs have been sub-divided into three classes (class I, II and III) based on the arrangement of cysteine residues (Zhou *et al.,* 2006). Our previous study has identified and characterized three different types of class I metallothioneins (*PjMT1, PjMT2, PjMT3*) from *Prosopis juliflora* (Usha *et al.,* 2009). When the heavy metal binding capacity of three MTs were compared using fusion proteins in *E. coli*, PjMT1 showed higher sequestration of cadmium, copper and zinc as compared to type 2 and type 3 MT. In this study we further analyzed and compared the ability of *PjMT1* and *PjMT2* to sequester cadmium in transgenic tobacco plants.

Materials and Methods

To overexpress *PjMT1* and *PjMT2* in tobacco (*Nicotiana tabacum* cv. petit havana), the cDNA were cloned in the binary vector pCAMBIA 1301+35S CaMV. The recombinant vectors, *pCPjMT1* and *pCPjMT2* were transformed into *Agrobacterium tumefaciens* strain LBA4404 as per Mozo and Hooykaas (1992) and the positive colonies were selected on kanamycin (50 mg/ml) and rifampicin (10 mg/ml) YEP agar plates. The transformed colonies were screened by PCR using *Pj MT1* and *PjMT2* - specific forward and reverse primers. Tobacco leaf discs were transformed with recombinant *Agrobacterium* harbouring *pCPjMT1* or *pCPjMT2* by co-cultivation (Horsch *et al.,* 1985). The transformed plants were selected using hygromycin (25mg/ml) as the selection marker. Transformation of plants was confirmed by staining the leaf discs with β-glucouronidase (GUS) (Jefferson *et al.,* 1986).

Total genomic DNA was isolated from GUS-positive tobacco plants according to Murray and Thompson (1980). The incorporation of *PjMT1* and *PjMT2* cDNA into the tobacco genome was confirmed by PCR amplification from tobacco genomic DNA using gene specific primers (PjMT1 55 UTR F, PjMT1 33 UTR R; PjMT2 55 UTR F, PjMT2 UTR R). (PCR conditions -1 min pre-amplification at 94 ºC, 30 PCR cycles for 30 seconds at 94 °C, 40 seconds at 58 ºC, 1.30 min at 72 ºC, and a final extension of 10 min at 72 ºC). Stable transformation of *PjMT1* and *PjMT2* into the tobacco genome was confirmed with Southern blot analysis. Genomic DNA (20 µg) isolated from tobacco (control and PCR positive plants) was digested with *Eco* RI to release a 1 kb DNA fragment containing the gene of interest and electrophoresed on 1% agarose gel. It was then transferred to nylon membrane and hybridization was carried out with radio-labeled 33 UTR region of *PjMT1* or

PjMT2 cDNA as probe. To analyze the expression of integrated genes in tobacco, total RNA isolated from the control and transgenic tobacco plants were subjected to Northern hybridization using *PjMT1* or *PjMT2* 33 UTR region as probe.

The transgenic and control plants were subjected to cadmium stress and the extent of chlorophyll degradation was measured using the protocol by Arnon (1949). Sixty days old healthy and fully expanded leaves from control and transgenic plants were briefly washed with sterile distilled water and leaf discs (2 cm diameter) were punched out and floated in water containing different concentrations of $CdSO_4$ (1 mM, 5 mM, 10 mM) in continuous light at 26 ± 2 °C until chlorophyll degradation was prominently visible. Leaf discs were ground in 1 ml of pre-chilled 80 % acetone. The crude extract was centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to another tube. The remaining pellet was re-extracted with 1 ml of pre-chilled 80 % acetone and centrifuged again at the same conditions and this was repeated until clear chlorophyll free debris was obtained. The extract was made to a final volume of 10 ml with 80 % acetone. The absorbance of the solution was measured at 645 and 663 nm and the amount of chlorophyll was calculated using the following formula: μg of chlorophyll/g tissue = 20.2 (A645) + 8.02 (A663) /W × C, where W- Fresh weight of tissue and C - Dilution factor.

To study cadmium accumulation by the *PjMT1* and *PjMT2* transformed transgenic plants, two month old control and transformed plants were placed in ½ MS nutrient solution containing 0.3 mM $CdSO_4$ (Usha *et al.*, 2009). Leaves from the plants were harvested after 3 days and dried in a hot air oven at 105 ºC overnight. The dried tissues were crushed to a fine powder and to 0.1 gm of the leaf powder 10 ml nitric acid was added. This mixture was heated on a hotplate at 120 ºC for 5 min. The yellow colored solution was allowed to cool to room temperature. 4 ml of 70 % perchloric acid was added to this and heated again until the yellow solution turned colorless. The volume was made up to 15 ml using HPLC grade water and diluted further prior to estimating metal concentration.

Standard solutions covering a range of 0.2, 0.4, 0.6, 0.8, 1.0 ppm were prepared by diluting stock solutions (1000 ppm, Merck) to appropriate concentrations using HPLC water. Standard solutions were aspirated into the flame in AAS to obtain a linear calibration curve. Samples were then aspirated into the flame to determine the metal content. Metal content in the samples is given in mg/L. Metal content present in 1 g dry weight of the tissues was determined using the following formula.

Element μg/g dry weight = C × V × DF/W

where C, Concentration of the element as given by AAS; V, Volume of the digested sample;

DF, Dilution Factor= diluted solution volume in ml/ml aliquot taken for dilution and W, weight of the leaf tissue taken for digestion.

Results and Discussion

Seven *PjMT1* (lines, 1, 3, 4, 8, 11, 12, 17) and *PjMT2* (lines, 1, 4, 6, 7, 9, 13, 14) transgenic plants that showed blue coloration in their leaves with X-Gluc staining were taken for PCR screening. Genomic DNA was isolated from three lines and PCR amplification of *PjMT1* and *PjMT2* cDNA was performed using respective 52 and 32 UTR forward and reverse primers. All the plants analyzed were PCR positive.

Northern analysis was performed from *PjMT1* (1, 3 and 17) and *PjMT2* transformed lines (1, 4, and 6) to check the level of transgene expression in different lines. Northern hybridization confirmed the expression of the transgene in all the lines, (Fig. 1c) and (Fig.1d). Two lines 3 and 17 from *PjMT1* plants and lines 4 and 6 from *PjMT2* transformants were taken for further experiments. Southern hybridization for these lines confirmed the stable integration of *PjMT1* (Fig. 1a) and *PjMT2* cDNA (Fig. 1b) into tobacco genome.

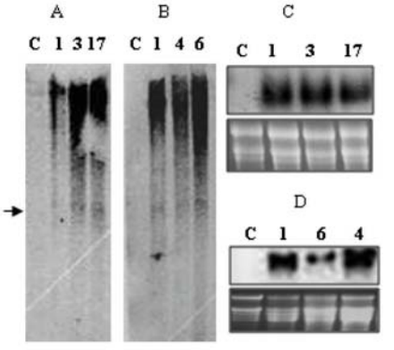

Fig. 1: Screening of *PjMT1* transformed tobacco plants: Genomic DNA from control and transgenic plants were digested with *Eco* RI and Southern hybridization was performed with radio-labeled 33 UTR of *PjMT1* cDNA (A) and *PjMT2* cDNA (B). Total RNA from control and transgenic plants were blotted onto a nylon membrane and Northern hybridization was performed with radio-labeled 33 UTR of *PjMT1* cDNA (C) and *PjMT2* cDNA (D).

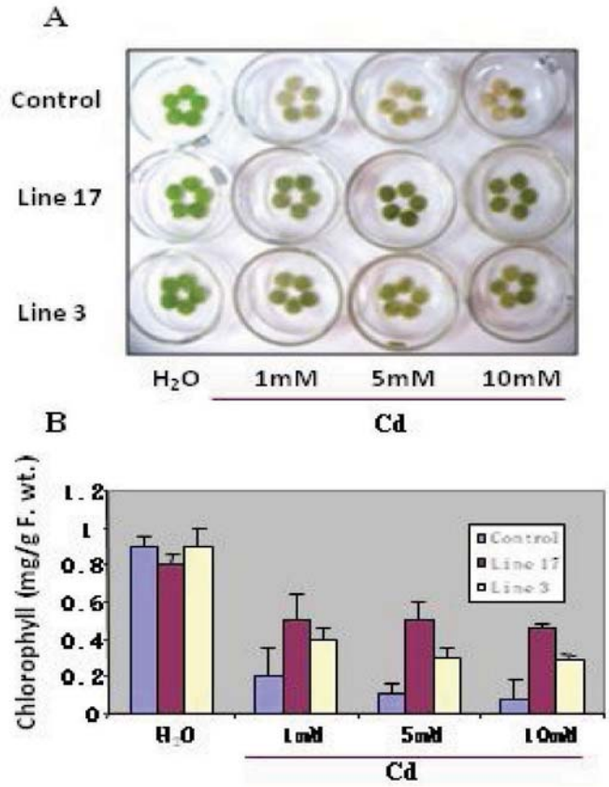

Figure 2a

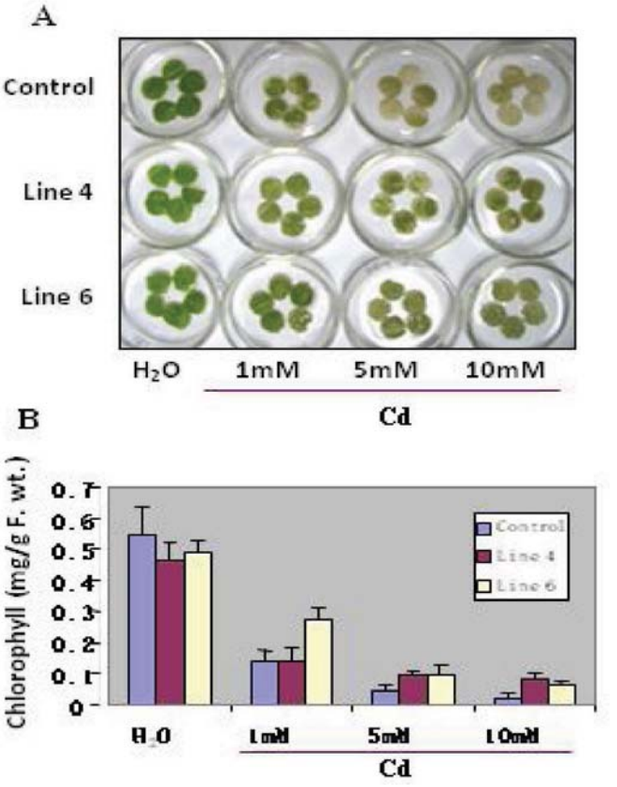

Figure 2b

Fig. 2: Leaf Disc Assay to show tolerance of *PjMT1* (2A) and *PjMT2* (2B) transformants against cadmium. Phenotypic differences and chlorophyll content (mg/g fresh weight) from Cd treated leaf discs of WT and transgenic plants after incubation in 1, 5 and 10 mM solutions of $CdSO_4$ for 3 days are shown. Discs floated in water served as the experimental control.

To corroborate that *PjMT1* conferred greater heavy metal tolerance than *PjMT2* not only in *E. coli* (Usha *et al.*, 2009) but also in plants, leaf discs of transgenic plants were floated in 0, 1, 5 and 10 mM cadmium containing solutions for 3 days. Bleaching of leaf discs was seen prominently after 3 days in $CdSO_4$. Incubation in $CdSO_4$ showed an early bleaching of wild type leaf discs compared to those from transgenic plants. In cadmium solution transgenic plants showed greater chlorophyll retention compared to control wild type plants. Of the *PjMT1* and *PjMT2* transgenic plants, line 17 and line 4 respectively, showed better performance than other lines. After three days under 10 mM cadmium stress, *PjMT1* (line 17) transformed leaf discs possessed 4.2 times higher chlorophyll content (Fig. 2a) while *PjMT2* (line 4) transformed leaf discs possessed only 1.7 times higher chlorophyll than the wild type plants (Fig. 2b). This showed that *PjMT1* imparted greater cadmium tolerance to transgenic tobacco plants than *PjMT2*.

Cadmium content in the leaves of both the control and transgenic plants treated with 0.3 mM $CdSO_4$ were estimated using flame atomic absorption spectrometry. Overexpression of *PjMT1* and *PjMT2* increased the accumulation of cadmium in transgenic tobacco as compared to control plants. Among *PjMT1* and *PjMT2* plants, cadmium content was high in *PjMT1* plants (about 9 fold higher than wild type plants) than *PjMT2* plants (about 5 fold higher than wild type plants) (Fig.3). Our observations establish a positive relationship between the overexpression of *PjMT* genes and heavy metal tolerance and accumulation in transgenic plants.

Similar results have been obtained when *BjMT2* cDNA was expressed in *Arabidopsis thaliana* under the regulation of the 35S promoter. Seedlings exhibited an increased tolerance against cadmium based on shoot growth and chlorophyll content (Zhigang *et al.*, 2006). Unlike the previous reports demonstrating increased cadmium

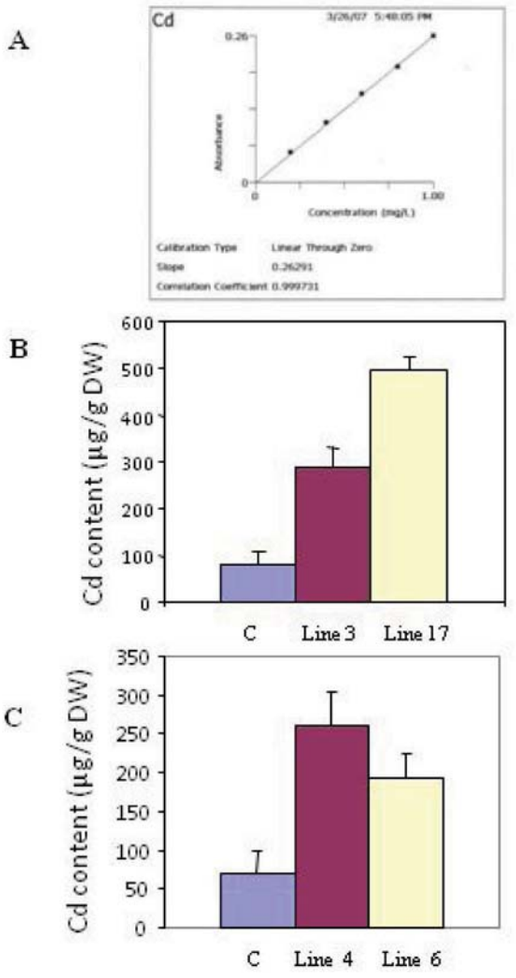

Fig. 3: Cadmium accumulation by transgenic plants. (A). Standard calibration curve for cadmium with standards in the range of 0.2, 0.4, 0.6, 0.8, 1.0 ppm. Atomic absorption spectrometric (AAS) analysis of cadmium content in the leaf tissues of wild type, *PjMT1* (B) and *PjMT2* (C) transformed transgenic plants. Two month old plants were kept in half MS nutrient solution containing 0.3 mM $CdSO_4$ for 3 days and the leaves were dried, acid digested and cadmium content measured using flame AAS.

accumulation in transgenic plants overexpressing MTs, introduction of yeast *CUP-1* gene into tobacco resulted in enhanced copper accumulation and tolerance as compared to cadmium. Transgenic *CUP-1* tobacco plants were capable of phytoextracting 2-3 times the copper as the control plants while no significant difference in Cd uptake was observed (Thomas *et al*., 2003). On the contrary, Hasegawa *et al.*, (1997) have reported that introduction of yeast *CUP-1* gene to cauliflower resulted in 16 fold higher cadmium tolerance and accumulation. Transfer of human MT-2 gene into tobacco or oil seed rape resulted in plants with enhanced Cd tolerance (Misra & Gedamu, 1989) and pea MT gene in *A. thaliana* enhanced Cu accumulation (Evans *et al*., 1992). Several studies have reported that transformation of MTs from mouse *MTI*, human *MTIA*, human *MTII*, Chinese hamster *MTII*, yeast *CUPI* and pea *psMTA* to *Nicotiana tabacum*, *Brassica* species and *A. thaliana* (Maiti *et al.*, 1988, 1989, 1991; Misra & Gedamu, 1989; Evans *et al.*, 1992) resulted in enhanced cadmium tolerance. Although several studies have reported the overexpression of MTs from different organisms in plants, this is the first report on the comparative analysis of heavy metal accumulation by different types of MTs from the same species. Our study shows that *PjMT*s can confer increased cadmium tolerance with *PjMT1* possessing greater ability than *PjMT2*. Thus, *PjMT*1 serves as better candidate gene for genetic engineering of plants for enhanced Cd phytoremediation.

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