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Expression of metallothionein type 2 and 3 genes in *Prosopis glandulosa* leaves treated with copper

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ABSTRACT. For a better understanding of the strategies that are used by *Prosopis glandulosa* in heavy metal tolerance, the present study evaluated the gene expression of three metallothioneins (MTs; *PgMt2-1*, *PgMt2*, and *PgMt*3) in plants exposed to sub-lethal concentrations of copper. The *PgMt2-1*, *PgMt2*, and *PgMt3* sequences were homologous to the MT type 2 (isoform 1), Mt2, and Mt3 sequences of other plant species found in GenBank. A reverse transcriptase-polymerase chain reaction showed that treatment with 100 mM Cu^{2+} induced a significant increase in *PgMt2* and *PgMt3* expression during the first 4 h of exposure compared to that of *PgMt2-1*. However, after 8 h of exposure, the expression levels of *PgMt2* and *PgMt3* were significantly lower than those of *PgMt2-1*. *PgMt* transcript levels only increased significantly during the first hour after exposure to copper, suggesting that *PgMt*s could play a key role in the plant's detoxification mechanism. However,

additional studies are required to confirm MTs as a mechanism of heavy metal tolerance and accumulation in this species.

Key words: Metallothionein; *Prosopis glandulosa*; Copper; Tolerance; Gene expression; RT-PCR

INTRODUCTION

Heavy metals in soils can severely affect ecosystem health because of their acute and chronic toxic effects in the food chain (McLaughlin et al., 2000; Castro-Larragoitia et al., 2013). Both chemical and physical remediation methods have been employed to clean soils in different ecosystems (Machado et al., 2010); however, because of their relatively high costs, these methods may not be available in many countries, and they require a large amount of energy and are not able to completely remove heavy metals. Phytoremediation is a cost-effective alternative for the treatment of contaminated soils (Alkorta et al., 2004). Many plant species can grow in heavy metal-polluted environments (Gonzalez-Mendoza and Zapata-Perez, 2008), including species of the genus *Prosopis* L., such as *P. glandulosa* Torr. and *P. juliflora* (Sw.) DC. These species grow in northern Mexico, where they have formed forest extensions known as mezquitales that form part of the desert ecosystem (Carevic, 2016). Previous studies have reported that *Prosopis* species are particularly resistant to heavy metals, even before any visible signs of toxicity are apparent (Senthilkumar et al., 2005; Varun et al., 2011). Previous studies of *P. glandulosa* have mainly focused on whether it can germinate under different copper concentrations, and the effects of this metal on photochemical efficiency, cellular viability, and the total phenolic content of leaves (Michel-López et al., 2014). Metallothionein (MT) genes are involved in copper homeostasis and tolerance (Dabrowska et al., 2013), and have been grouped into four different types: MT1, MT2, MT3, and MT4, based on the distribution of cysteine residues (Xia et al., 2012). MT1 genes are expressed in roots, whereas MT2 genes are expressed in aerial tissues (leaves and shoots); MT3 genes are expressed in leaves and fruit tissues, and MT4 genes in developing seeds (Guo et al., 2003). A previous study demonstrated that *P. juliflora* plants treated with different copper concentrations develop an exclusion strategy that favors the retention of metals in roots, suggesting efficient metal uptake and an accumulation mechanism in the roots (Michel-López et al., 2016). However, the molecular basis of the tolerance mechanism is unknown. A few studies on changes in the expressions of genes that encode MTs have been conducted (mainly in *P. juliflora*), but there have been few studies on *P. glandulosa* (Usha et al., 2014). Therefore, to gain a better understanding of the strategies that are used by *P. glandulosa* in heavy metal tolerance, the present study aimed to elucidate the molecular mechanism by which *P. glandulosa* tolerates heavy metal exposure, by measuring MT gene expression in plants exposed to sub-lethal concentrations of copper.

MATERIAL AND METHODS

Seed collection and germination

Wild *P. glandulosa* seeds were collected from a native population in the Mexicali valley, Baja California, Mexico (32°24'6.8394"N, 115°11'51"E). The native population was within a protected area, and the responsible authority (National Forestry Commission of

Mexico) authorized the seed collection. The seeds (500 g) were collected from a representative population sample of 100 plants and transported to the laboratory. One hundred seeds were disinfected with 1% NaOCl (Clorox) for 5 min, and then washed with deionized sterile water. The seeds were then scarified with 5% H_2SO_4 for 10 min, washed thoroughly, and germinated in sterilized sand (121°C for 2 h over two consecutive days). Seedlings were cultivated in a greenhouse with a 12-h light:dark photoperiod (>350 $mmol \cdot m^{-2} \cdot s^{-1}$ photon flux density), 60% relative air humidity, and 30/32°C day/night temperatures. The seedlings were irrigated daily with water, and fertilized with Hoagland solution every other week, according to Michel-López et al. (2016).

Exposure to heavy metals

Fifteen one-month-old *P. glandulosa* plants (N = 4) were randomly selected and transferred to individual plastic containers that contained 100 mL modified quarter-strength Hoagland solution, pH 5.5, which had been prepared with 100 mM copper sulfate ($CuSO_4 \cdot 5H_2O$). Control plants (N = 4) were transferred to plastic containers with 100 mL Hoagland solution without heavy metals. Treated and control plants were exposed to their respective solutions for 12 h under hydroponic conditions. The aerial parts were collected at 4, 8, and 12 h, and stored at -80°C for molecular analyses. All treatments were performed in quadruplicate.

Extraction of total RNA and cDNA synthesis

RNA extraction was based on the phenol-chloroform method as described by Michel-López et al. (2013), with minor modifications. Approximately 200 mg of biomass was ground with 0.2 mL extraction buffer, before 0.1 mL extract was mixed with 0.1 mL phenol:chloroform (1:1) and incubated at 65°C for 5 min. After incubation, the mixture was centrifuged at 11,000 *g* at 4°C for 5 min. The supernatant was collected and transferred to a tube, and a 1-fold volume of cold isopropanol was added and mixed thoroughly at -20°C for 30 min to precipitate the total RNA. The mixture was centrifuged at 11,000 *g* for 10 min at 4°C, then the pellet was re-suspended in 0.05 mL diethylpyrocarbonate-treated water. The purity and concentration of the RNA samples were spectrophotometrically determined at 260 and 280 nm, respectively, as described by Gonzalez-Mendoza et al. (2008). cDNA was synthesized using 1 µL SuperScript™ II Reverse Transcriptase (50 U/µL) according to the manufacturer's instructions (Invitrogen, USA), and stored at -20°C.

Semi-quantitative polymerase chain reaction (PCR) analysis of metallothionein (*MTs*) gene expression

Differential transcript abundance of three different types of metallothionein [type 2 (isoform 1), type 2, and type 3] in the areal tissues of plants treated with copper were further quantified by reverse transcriptase-PCR, as described by Gonzalez-Mendoza et al. (2007). Degenerate primers were designed for the amplification of gene fragments coding for MT2 isoform 1 (*PgMt2-1*), MT2 (*PgMt2*), and MT3 (*PgMt3*), and were conserved regions of MT sequences obtained from different plants. *β-actin* was used as an internal control (Table 1).

Table 1. Sequences of primers used in the study.

Primer name	Primer sequence	Annealing temperature (°C)	Product size (bp)
PgMt2-1	F: 5'-GCTGCGGAAGCAAGATG-3'	62	533
	R: 5'-GATGGACCTTTATGGACAACA-3'		
PgMt2	F: 5'-CAAGTGCGGCTCTGGAT-3'	62	443
	R: 5'-GCTTTCATACAACACCCTCAA-3'		
PgMt3	F: 5'-GAAAGGCAACGGCTATG-3'	62	460
	R: 5'-TGGATGTGCCTCGTGTAT-3'		
Actin	F:5'-GGATGGGTCAGAAGGATG-3'	60	500
	R: 5'-CGTCCACTGGCATACAGA-3'		

PCRs were conducted using the following protocol: 94°C for 3 min (1 cycle), 60°C for 40 s, and 72°C for 40 s (35 cycles). The PCR products were separated by electrophoresis on 1.3% (w/v) agarose gel stained with ethidium bromide, and visualized under ultraviolet light. Changes in gene expression levels were evaluated by a semi-quantitative analysis of band densities, using the image analysis software ImageJ version 1.33 (http://rsb.info.nih.gov/ij).

PCR product sequencing and phylogenetic analysis

Three PCR products that were 533, 443, and 460 bp long were obtained using the primers *PgMt2* and *PgMt3*. The PCR products were purified using a QIAEX® II gel extraction kit (Qiagen, USA) and sequenced by GENEWIZ Inc. (South Plainfield, NJ, USA). Virtual translations of the cDNA sequences from *PgMt2*, *PgMt2-1*, and *PgMt3* were analyzed by the BLAST program to identify similarities in the GenBank database. The *PgMt* sequences previously obtained were compared with other DNA sequences using BLAST. Sequences with a high similarity were recovered from GenBank, and a phylogenetic neighbor-joining tree that included the obtained isolates and their closest relatives was constructed using MEGA 6.0 (Tamura et al., 2013).

Statistical analysis

Differences in normalized *PgMt2 and PgMt3* transcript abundance levels between the treatments were analyzed using the Kruskal-Wallis test (StatSoft version 5.5). Differences were considered significant if $P < 0.05$. The data are presented as means ± SDs.

RESULTS

The neighbor-joining tree illustrates the relationships between different types of MT sequences in *P. glandulosa* and other plants (Figure 1). Three different MT sequences were identified in *P. glandulosa*, and were designated *PgMt2-1* (MT type 2, isoform 1) *PgMt2* (MT type 2), and *PgMt3* (MT type 3). The sequences were deposited in GenBank with the accession numbers KJ957829 (*PgMt2-1*), KJ949044 (*PgMt2*), and KJ949045 (*PgMt3*).

PCR product sequencing

The *PgMt2-1* sequence was homologous with the MT type 2 (isoform 1) sequences of *P. juliflora* (97%), *Leucaena leucocephala* (80%), and *Fagus sylvatica* (73%), which had the accession numbers ACC77566, AGL79967, and CAA10232, respectively (Figure 2).

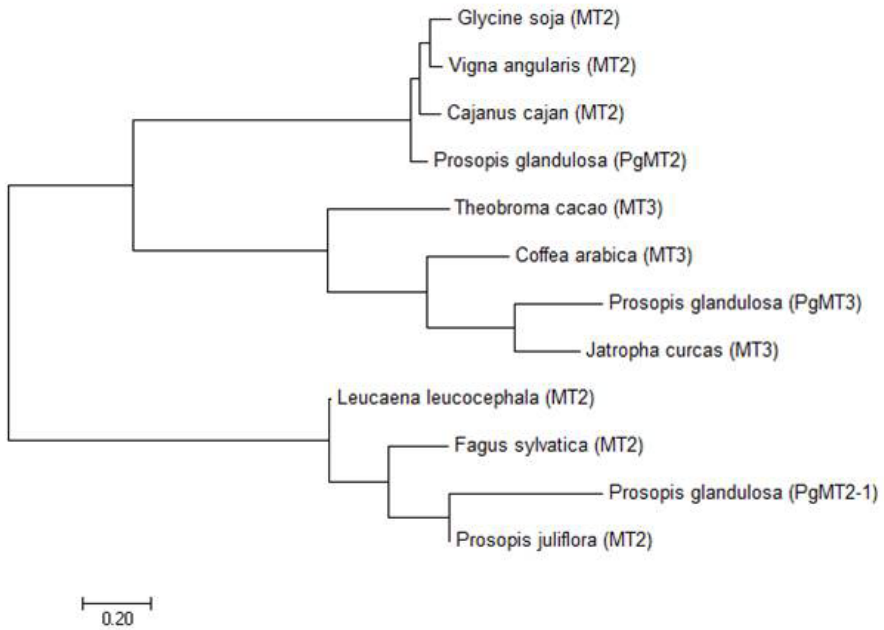

Figure 1. Phylogenetic tree based on metallothionein (MT) sequences of *Prosopis glandulosa* and other plants, showing a close relationship between the three MTs and their nearest relatives. Only values greater than 90% are shown. The scale represents the percentage sequence divergence.

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                      10        20        30        40        50        60
                       |         |         |         |         |         |
P. glandulosa         -MLLW-HQLASEATC-CGS-----KMYPDLSYT-EKTTRESLVLGVAPTKVELEGAETGV
P. juliflora          MSCSC-TNCGCGSGCGCGS-----KMYPDLSYT-EKTTRESLVLGVAPTKVELEGAETVV
L.leucocephala        MSCSCGDSCGCGSSCNCGS-----KMYPHLSYAAEKTTSESLVLGVVPTKVEFEGPEMGV
F.sylvatica           MSCCG-GNCGCGTGCKCGSGCGGCKAYPDLSYT-EKTTTETLIVGVAPQKAHSEGSEMGV
                         . .. : * ***     * **.***: **** *:*::**.* *.. **.*  *
Consensus             MSCSCG4NCGCGSGC3CGSGCGGCKMYPDLSYTAEKTTRESLVLGVAPTKVELEGAE2GV
                          70        80
                           |         |
P. glandulosa         AAENEGCKCGSNCTCDPCNCUNK_
P. juliflora          AAENEGCKCGSNCTCNPCNCUNK_
L.leucocephala        AAENEGCKCGSNCTCDPCNCS
F.sylvatica           GAENGGCKCGSNCTCDPCNCK
                      .*** **********:****
Concensus             AAENEGCKCGSNCTCDPCNC2
```

Figure 2. Sequence alignment of Mt2 (isoform 1)-like amino acids. Identical amino acids are shaded in gray and shown in the consensus line at the bottom. Dashes denote gaps introduced by the alignment program. Plant names are indicated on the left. The protein sequences used were *Prosopis glandulosa* (AIH05121), *P. juliflora* (ACC77566), *Leucaena leucocephala* (AGL79968), and *Fagus sylvatica* (CAA10232).

The BLAST analysis revealed that *PgMt2* matched closely with *Mt2* from *P. juliflora* (95%), *Glycine soja* (95%), *Cajanus cajan* (84%), and *Vigna radiata* (84%) (Figure 3).

Figure 3. Sequence alignment of Mt2-like amino acids. Identical amino acids are shaded in gray and shown in the consensus line at the bottom. Dashes denote gaps introduced by the alignment program. Plant names are indicated on the left. The protein sequences used were *Prosopis glandulosa* (AIE88422), *Glycine soja* (KHN40306), *Cajanus cajan* (KYP74286), and *Vigna. angularis* (BAD18379).

The *PgMt3* amino acid sequences were homologous with MT sequences in *Jatropha curcas* (66%), *Coffea arabica* (65%), and *Theobroma cacao* (63%) (Figure 4).

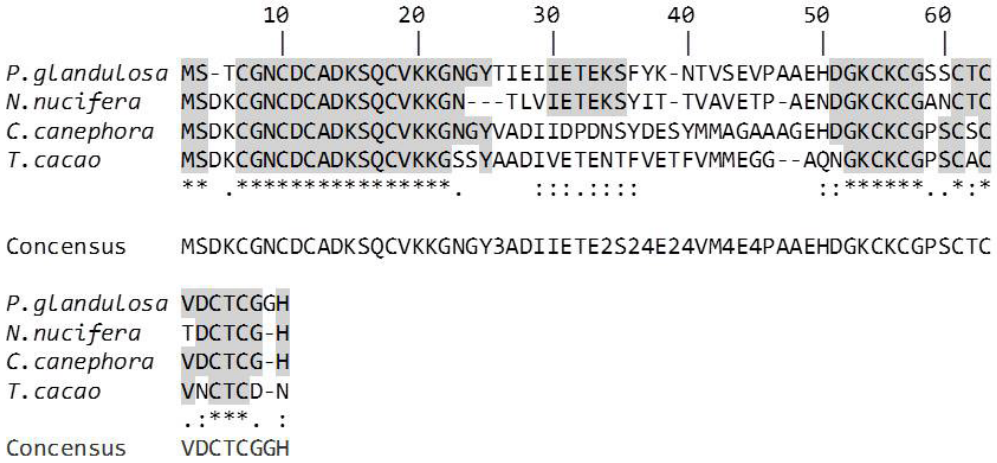

Figure 4. Sequence alignment of Mt3-like amino acids. Identical amino acids are shaded in red and shown in the consensus line at the bottom. Dashes denote gaps introduced by the alignment program. Plant names are indicated on the left. The protein sequences used were *Prosopis glandulosa* (AIE88423), *Jatropha curca* (XP_012084959), *Coffea arabica* (AGL34968), and *Theobroma cacao* (EOY13005).

PgMt gene expression in *P. glandulosa* leaves treated with Cu^{2+}

Plants treated with 100 mM Cu^{2+} exhibited a significant increase in *PgMt2* and *PgMt3* expression ($P < 0.05$) during the first 4 h of exposure compared to that of *PgMt2-1* (Figure 5a,b). *PgMt2-1* levels in leaves of plants exposed to 100 mM Cu^{2+} significantly increased relative to those of *PgMt2* and *PgMt3* after 8 h of exposure ($P < 0.05$) (Figure 4a). After 12 h of exposure, the expression levels of *PgMt2* and *PgMt3* were significantly higher than those of *PgMt2-1*, and the transcript levels of this gene had decreased significantly ($P < 0.05$) (Figure 5a).

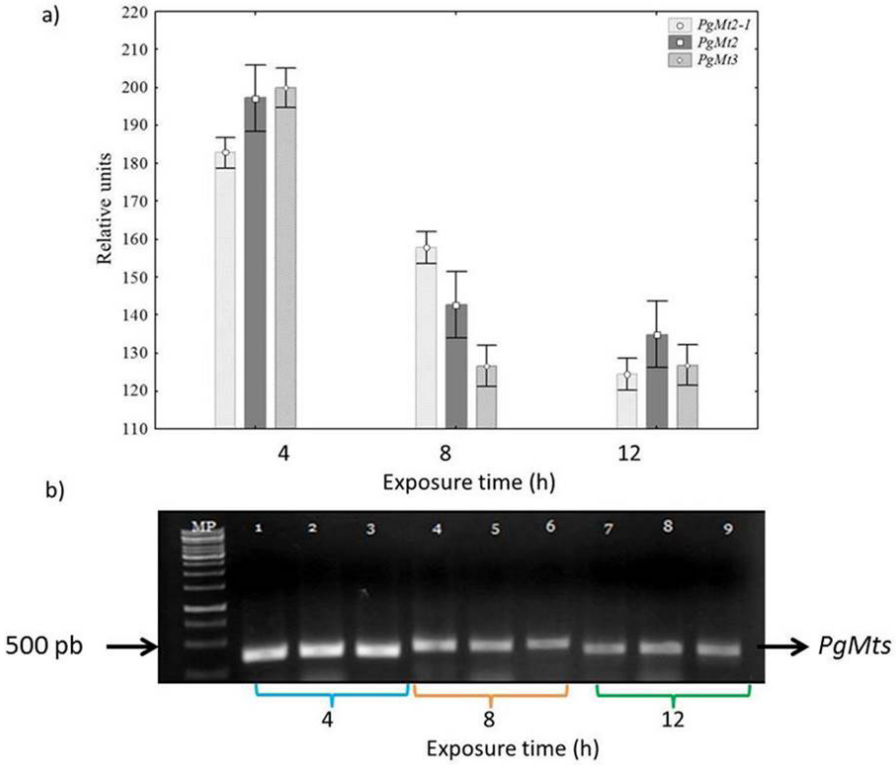

Figure 5. a. *PgMt* gene quantification expressed in arbitrary units; **b.** polymerase chain reaction products of *PgMt*s in leaves of *Prosopis glandulosa* plants treated with 100 mM Cu^{2+}. *Lanes 1*, *4*, and *7* (*PgMt2-1*); *lanes 2*, *5*, and *8* (*PgMt2*); and *lanes 3*, *6*, and *9* (*PgMt3*). All values are reported as means ± SD (N = 3).

DISCUSSION

In response to stress, plants have evolved various protection mechanisms, such as chelation, detoxification, and the activation of protective proteins (Srivastava et al., 2005). The mechanisms of metal tolerance are particularly important, because they consist of a complex network that maintains the levels of essential and non-essential metals within physiological limits (Gonzalez-Mendoza et al., 2007). MTs in plants could play an important role in metal homeostasis and protection against intracellular oxidative stress (Gonzalez-Mendoza and

Zapata-Perez, 2008). We found that *PgMt2* and *PgMt3* expression was initially induced by copper, while *PgMt2-1* was only induced after 8 h of exposure, suggesting that *PgMt* expression in response to copper exposure does not increase linearly with exposure time. Similar results have been reported in other plant species, such as *Helianthus tuberosus* and *Avicennia germinans* (Chang et al., 2004; Gonzalez-Mendoza et al., 2007). Elucidating *PgMt*s role in metal homeostasis is complicated, but the *PgMt* expression observed in this study could be explained by the existence of different MT2 isoforms in *P. glandulosa* (*PgMt2-1* and *PgMt2*) that vary in their metal-binding affinities. Usha et al. (2009) reported that two types of MT in *P. juliflora* (*PjMT1* and *PjMT2*) are induced by copper and zinc, respectively, while *PjMT3* is induced by copper, zinc, and cadmium. Usha et al. (2014) suggested that variations in *PjMT* induction during metal exposure play a specific role in heavy metal tolerance in *P. juliflora*. The present study found that *PgMt* induction could have a specific function in copper homeostasis in *P. glandulosa*, and further studies at the molecular and proteomic levels are required to confirm that *PgMt*s control the uptake, accumulation, and transport of essential metals, such as copper, in this species.

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Conflicts of interest

The authors declare no conflict of interest.

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