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ORIGINAL ARTICLE

Functional analysis of *Triticum durum* type 1 metallothionein gene (dMT) in response to varying levels of cadmium

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Abstract The effect of varying levels of cadmium and correlated changes on the expression level of a type 1 metallothionein gene (*dMT*) were investigated in *Triticum durum* cv. Balçalı-85. Increasing the cadmium concentration resulted in a decrease in the dry weights of roots and shoots, and the effect was stronger in roots. Roots also showed a higher capacity to accumulate cadmium. Southern blot analyses revealed that the *dMT* gene, delineated by two exons and a non-coding intron region, exists at a single locus in the *T. durum* genome. Changes in *dMT* gene expression during cadmium exposure were monitored by two approaches. Northern blot analyses showed that the transcript level in roots increased upon treatment with increasing cadmium, which was quantified by qRT-PCR as 4.5 fold of the base level at 10 µM Cd. These results show a positive correlation between cadmium exposure and expression of *dMT* gene in durum wheat, and will provide a basis for studies on the role of type 1 metallothioneins in cadmium response.

Keywords Cadmium · Gene expression · Metallothionein · *Triticum durum*

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Introduction

Metallothioneins (MTs) are defined as a superfamily of low molecular mass (6–7 kDa) cysteine-rich proteins with a high capacity for binding metal ions. MTs are found in a large variety of organisms including plants, animals, fungi, and even in some prokaryotes (Freisinger 2008; Cobbett and Goldsbrough 2002, Vašák and Kägi 1994).

MT superfamily of proteins was classified into 15 families according to the phylogenetic relationships and the distribution patterns of Cys residues in the amino acid sequences (Binz and Kagi 1999). Family 15 represents the plant MTs and has been further subdivided by Cobbett and Goldsbrough (2002) into four types (1, 2, 3 and 4) depending on the localization of Cys residues and the Cys-devoid linker regions which are characteristic for plant MTs. Types 1 through 3 contain 2 Cys-rich regions in the N- and C-terminal domains and a 30–45 residue long linker, whereas in type 4 the Cys-rich sequences form 3 regions separated by linkers. The long linker regions observed in plant MTs are in contrast to those from vertebrate origin which consist of only 2–10 amino acids. Plant MTs differ further from their mammalian counterparts in terms of the variable position of Cys residues and their lower Cys content (Leszczyszyn et al. 2013).

Gene expression studies show abundance of MT mRNAs in different plant tissues. MT transcripts are detected in roots, stems, leaves, flowers, fruits and seeds under different conditions (Rauser 1999). Although there appears to be no strict pattern that applies to all plants, some level of organ specificity and developmental dependence has been reported for expression of the different types. Type 1 genes were reported to be expressed at high levels in roots, whereas type 2 gene expression was higher in leaves. Type 3 expression was detected in leaves or in

ripening fruits and expression of type 4 MT was showed to be restricted to developing seeds (Guo et al. 2003; Cobbett and Goldsbrough 2002). The variable arrangement of Cys residues in the sequences of plant MTs contrasts with the strictly conserved arrangement in all vertebrate MTs and suggests more diverse functions in plants. Studies on the function have been generally limited to analyses of gene expression in different species and tissues under stress conditions, and a large number of factors including presence of metals, hormones, heat shock, wounding, senescence, virus infection and other stress conditions have been shown to influence gene expression (Guo et al. 2003; Butt et al. 1998; Hsieh et al. 1995; Zhou and Goldsbrough 1994). It has also been reported that plant MT genes are expressed as part of a general stress response under different environmental stimuli, including sucrose starvation, heat shock and abscisic acid (Hsieh et al. 1996), drought (Quan et al. 2008, Yang et al. 2009; Xue et al. 2009), H_2O_2 (Brkljacic et al. 2004; Zhou et al. 2005; Kim et al. 2014), wounding (Choi et al. 1996; Butt et al. 1998), salt stress (Jin et al. 2006; Quan et al. 2008, Xue et al. 2009), indole-3-acetic acid, and ethylene (Cho et al. 2006), and low temperature (Cho et al. 2006; Xue et al. 2009). Involvement of plant MTs in processes such as seed development (Brkljacic et al. 2004; Zhou et al. 2005), root development (Yuan et al. 2008), embryogenesis (Reynolds and Crawford 1996; Chatthai et al. 1997), fruit ripening (Davies and Robinson 2000), and leaf and stem senescence (Chen et al. 2003; Guo et al. 2003; Navabpour et al. 2003) have also been shown. Moreover, there are studies showing that plant MTs may have a protective function during oxidative damage (Akashi et al. 2004; Wong et al. 2004; Xue et al. 2009). Related with metal ion response, MTs are believed to play a dual role. They appear to participate in response mechanisms to heavy metals including cadmium (Cd) and mercury (Hg) and also take part in maintaining the homoeostasis of essential metals copper (Cu) and zinc (Zn) (Lee et al. 2004; Zimeri et al. 2005; Palmiter 1998; Klaassen et al. 1999; Freisinger 2008).

Previously, durum wheat cv. Balçalı-85 was identified as a Cd-tolerant genotype (Cebeci et al. 2008), and cloning and overexpression of a MT gene, dMT, from cv. Balçalı-85 in *E. coli* was already reported (Bilecen et al. 2005). The dMT gene has two exons and one intron and codes for a type 1 MT, *T. durum* MT (dMT) with six Cys residues in each of the N- and C-terminal domains and a 42 residue long hinge region. It was shown that synthesis of the recombinant dMT in bacteria resulted in tolerance to higher levels of Cd in the growth medium compared to controls. In this study Cd-dependent expression of dMT in durum wheat Balçalı-85 was evaluated. We showed that dMT gene exists as a single copy in the durum genome and its expression increases in the root tissue with increasing concentration of cadmium in the growth environment. We argue that dMT expression is induced by cadmium and discuss the results in relation to heavy metal detoxification in *T. durum*.

Materials and methods

Plant growth conditions and cadmium treatments

Triticum durum cv. Balçalı-85 seeds were surface sterilized with 1% (w/v) $Ca(ClO)_2$ for 10 min and then rinsed with distilled H_2O. Seeds were germinated in the dark for 5 days in perlite moistened with saturated $CaSO_4$ solution at room temperature before transferred to 2.5 l plastic pots containing continually aerated nutrient solution composed of the following macro and micronutrients; 0.88 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM KCl, 100 µM Fe-EDTA, 1.0 µM H_3BO_3, 1.0 µM $ZnSO_4$, 1.0 µM $MnSO_4$, 0.2 µM $CuSO_4$, and 0.02 µM $(NH_4)_6Mo_7O_{24}$ (Ozturk et al. 2003). Plants were allowed to grow for 7 days in a growth chamber under controlled conditions with a light/dark regime of 16/8 h, temperature 24/22 °C, relative humidity 60/70%, and photon flux density of 600–700 µmol m^{-2} s^{-1} with renewing nutrient solution every 3 days. After 7 days of growth, nutrient solution was supplemented with 0, 2, 5, 10 and 20 µM Cd in the form of $CdSO_4$ with renewing every 3 days. Cd concentrations was choosed according to an unpublished data, *T. durum* cv. Balçalı-85 showed a Cd uptake saturation situation with 10 µM Cd application. Three different sets of 5 pots were prepared for each Cd concentration including the control plants that were kept under the same conditions without supplemented Cd. After 7 days following Cd treatment, control and stress plants were harvested at the same time, and roots and shoots were collected separately. Roots were first rinsed with 2 mM $CaCl_2$ for about 15 min to remove surface absorbed Cd and rinses with distilled H_2O. 0.2 g of roots and shoots were taken from each pot and immediately frozen in liquid nitrogen and stored at − 80 °C for RNA extraction.

Total RNA isolation from plants

Total RNA were isolated from 0.2 g frozen tissue using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically and samples were stored at − 80 °C.

Cadmium concentration and content

Roots and shoots were dried at 70 °C to determine dry matter and Cd concentration. Dried root and shoot samples

were ground and approximately 0.3 g ground sample washed at 500 V for 12 h followed by dissolving in 3.3% HNO_3 (v/v) for determination of Cd content. The concentration of Cd was measured by inductively coupled argon plasma optical emission spectroscopy (ICP-OES, Varian, Australia) at 214.439 nm emission wavelength. The Cd content was calculated by multiplying the dry weight values of roots or shoots with the Cd concentration values. Three blank reagents were similarly processed and used to derive the detection limit of 0.02 ng/g for the analytical application. Each sample was analyzed as duplicate and the replicate samples indicated that the error did not exceed 7%. The recovery tests, which involved the addition of a known amount of inorganic cadmium before digestion, yielded 96 ± 4% on average.

Southern blot analysis of *dMT* gene

Genomic DNA were isolated from leaves using modified CTAB method (Murray and Thompson 1980). Southern blot analysis was carried out by standart protocols as described by Sambrook et al. (1989). Fifteen µg genomic DNA were digested with restriction enzymes *Eco*RI or *Bam*HI, and separated on a 1% agarose gel. DNA was transferred by downward capillary blotting onto Nytran SPC nylon membrane (Whatman). The 228 bp cDNA probe for *dMT* was synthesized from 2 µg total RNA using the *Omniscript* Reverse Transcription kit (*Qiagen*, USA) with specific *dMT* gene primers of 5′-ATGTCTTGCAACTGTGGA-3′ for upstream and 5′-TTAACAGTTGCAGGGGTT-3′ for downstream with the PCR condition: 1 min at 95 °C, 1 min at 53.5 °C and 1 min at 72 °C for 40 cycles, followed by a final extension of 10 min at 72 °C. The membrane was hybridized overnight at 60 °C with the cDNA probe labeled with ^{32}P-dATP by 5′-end labeling. For labeling reaction 2 µg cDNA and 6000 Ci/mmol ^{32}P γ-ATP isotope and T4 polynucleotide kinase enzyme were used. After hybridization, blots were washed twice in low stringency buffer; 2× SSC, 0.1% SDS (2–5 ml/cm^2) for 15 min at 60 °C followed by high stringency washes with 1× SSC, 0.1% SDS for 15 min at again 60 °C. Finally, the blot was exposed to Kodak BioMax MS film for 16 h to 2 days at − 80 °C with Cronex intensifying screens.

Northern blot analysis

Northern blot analysis was carried out by standart protocols as described by Sambrook et al. (1989). Ten µg of total RNA from root tissue were size separated by electrophoresis through denaturing 0.7 M formaldehyde/1.5% (w/v) agarose gel, transferred to SPC nylon membrane and fixed by baking the filter at 80 °C in a vacuum oven for 2 h. Two µg *dMT* cDNA were labeled with Prime-It II Random Primer Labeling Kit (Stratagene) with ^{32}P α-dCTP. Hybridization was performed using ULTRAhyb hybridization buffer (Ambion) according to the manufacturer's instructions. Hybridization and washing temperatures were 55 °C. Membrane was then washed with 2× SSC, 0.1% SDS at 55 °C for 5 min, which was followed by two successive washes using 0.1× SSC, 0.1% SDS at 55 °C for 15 min each. Membrane was then exposed to X-ray film at − 80 °C for 1 day.

Quantification of the gene expression using quantitative real-time PCR

Gene expression levels were quantified using realtime PCR. Total RNA (1.5 µg) were used for first strand cDNA synthesis with the *Omniscript* Reverse Transcription kit (Qiagen USA) according to manufacturer's instructions, and diluted to 300 ng/µl. One µl of this cDNA was amplified with 1 µM of specific primers of 5′-ATGTCTTGCAACTGTGGA-3′ for upstream and 5′-TTAACAGTTGCAGGGGTT-3′ for downstream in a total of 20 µl volume using SYBR green PCR master mix (Applied Biosystems) with Icycler Multicolor Realtime PCR Detection Systems (BioRad Laboratories) with the following conditions: denaturation step at 95 °C for 3 min, 50 cycles at 95 °C for 30 s, 51 °C for 30 s, 72 °C for 45 s, and a final extension step at 55 °C for 10 s. The quantification was performed according to Muller et al. (2002) using actin (GenBank accession AY663392, forward: 5′-GGATCTCACGGACTCCCTCAT-3′/reverse: 5′-CGGCTGAGGTTGTGAAGGA-3′) as reference and three independent PCR results with acceptable efficiency were averaged.

Results

Plant growth and cadmium accumulation in Balçalı-85

The gradually increasing effects of 2, 5, 10 and 20 µM of Cd in the growth medium on the growth of 1 week old seedlings of Balcali-85 can be seen in Fig. 1. A dose dependent inhibition of growth and a decrease in dry weight of roots and shoots were observed. As can be seen from the analysis in Fig. 2, increasing the Cd supply from 0 to 5 µM resulted in a 40% decrease in the shoot dry weight; but no further change was observed at higher concentrations. The root dry weight, on the other hand, showed a steady decrease between 0 and 20 µM Cd in the environment with a total decrease of 60% compared to control plant at the highest concentration.

Fig. 1 Cd application results in biomass loss in roots and shoots of wheat cultivar Balçalı-85. Plants were treated with Cd at indicated concentrations on the 7th day of growth. The photograph was taken after 7 days of exposure

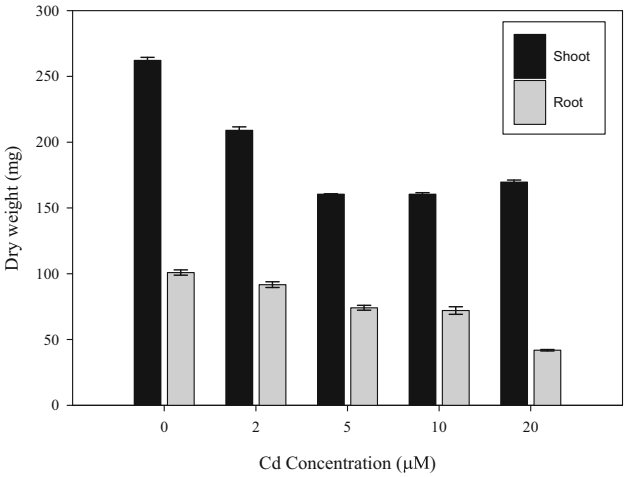

Fig. 2 Effect of increasing Cd application on shoot and root dry weight. The data represent mean ± SD of three independent experiments

Dose dependent Cd uptake was also evaluated. As expected the amount of Cd uptake both in roots and shoots increased with increasing concentrations and accumulation monitored as mg/kg tissue, is shown in Figs. 3a, b, respectively. When the scales of the graphs are compared, 2300 mg/kg Cd accumulation in roots at 20 µM exposure is significantly higher than that of shoots, which is at about 30 mg/kg tissue. The steady increase in roots from 350 to 2300 mg/kg is in contrast to the saturation observed in shoots where the Cd level roughly doubled from 2 µM exposure to 10 µM and remained almost the same with higher exposure. Because of this observation, we further analyzed the effect of Cd specifically in root tissues.

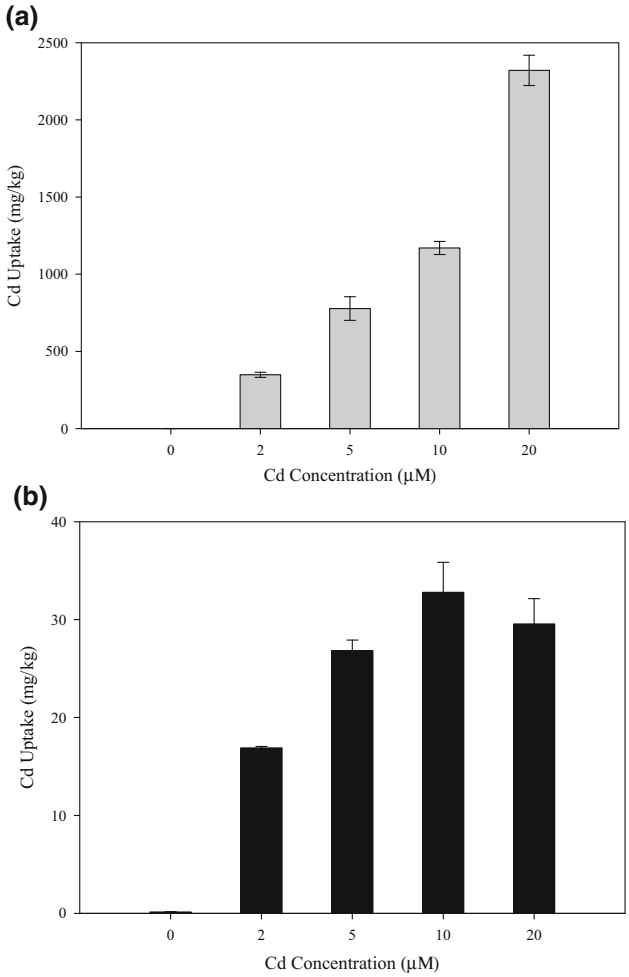

Fig. 3 Effect of increasing Cd application on root (**a**) and on shoot (**b**) Cd uptake. The data represent mean ± SD of 3 independent experiments

Determination of the copy number of dMT gene

To investigate the copy number of *dMT*, genomic DNA of *T. durum* cv. Balçalı-85 was digested with a rare cutter, *Eco*RI, and a frequent cutter, *Bam*HI, both of which were selected not to cut the *dMT* cDNA or intron region. The digested genomic DNA samples were then hybridized with radioactively labeled *dMT* cDNA. As can be seen in Fig. 4, Southern blot analysis resulted in a single band suggesting that *dMT* gene exists as a single locus in the genome of *T. durum*.

Change in dMT expression in response to environmental Cd

Quantitative RT-PCR was performed to analyze the change in the levels of *dMT* gene in the root tissue in response to increasing levels of Cd in the growth medium. The normalization was performed according to Muller et al. (2002)

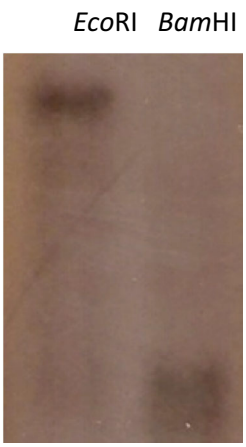

Fig. 4 Southern blot analysis of the *T. durum dMT*. Fifteen µg genomic DNA was digested with *Eco*RI, *Bam*HI and hybridized with ^{32}P γ-ATP-labelled *dMT* cDNA

using the expression of actin gene (GenBank accession AY663392).

The induction pattern of *dMT* in the root tissue was analyzed and as shown in Fig. 5, a significant increase compared to the control plants was observed in expression amount in parallel to the level of Cd after 7 days of exposure. In particular, transcript levels increased dramatically by about 15% from control to 2 µM and 50% when the exposure was increased from 5 to 10 µM. At 20 µM the level of *dMT* mRNA dropped approximately to that of 5 µM.

Further analysis of a possible correlation between Cd exposure and *dMT* expression was carried out by Northern blot analysis. Total RNA was isolated from the root tissue and hybridized with a radioactively labeled *dMT* cDNA probe. The increase observed in the intensity of the *dMT* band with increasing Cd concentration as shown in Fig. 6 confirms that the transcript level of *dMT* changes parallel to the increasing level of Cd exposure. Contrary to the qRT-PCR results, there was no decrease, but rather an increase, in the transcript level of *dMT* at 20 µM Cd.

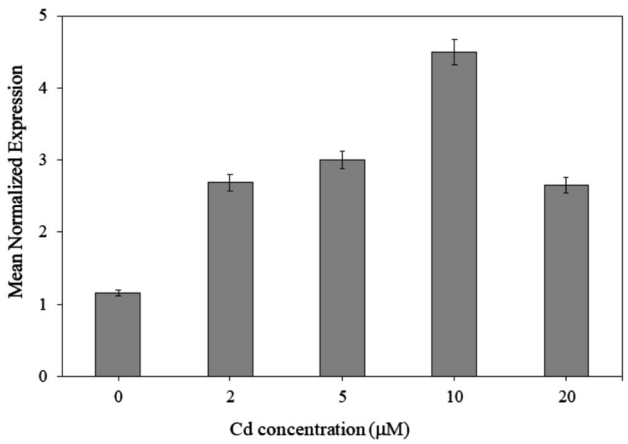

Fig. 5 Mean normalized expression of *dMT* mRNA in root tissue in control and Cd (2, 5, 10, 20 µM) exposed *T. durum* plants after 7 days of exposure

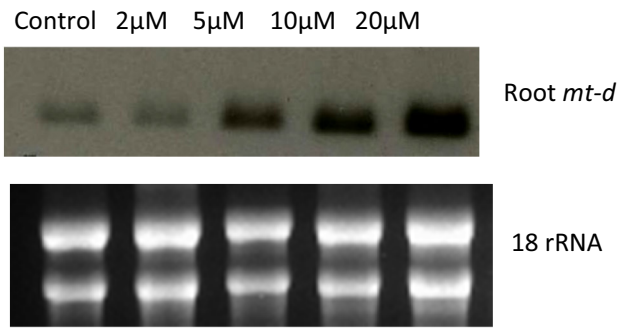

Fig. 6 Northern blot analysis of *dMT* in roots of *T. durum* treated with Cd. Total RNA was isolated from roots of wheat treated with 2, 5, 10, 20 µM $CdCl_2$. 10 µg of total RNA were loaded in each lane. Equal loading of RNA samples in each lane was confirmed by ethidium-bromide staining of rRNAs (lower panel)

Discussion

Cd is a toxic pollutant released to the environment from industrial facilities, urban traffic and phosphate fertilizers (Polle and Schützendübel 2003; Di Toppi and Gabbrielli 1999). Due to its solubility in water, it has a potential of diffusing and spreading rapidly in the soil for uptake by plants, resulting not only in toxicity in plants but also presenting a health hazard for humans by entering into the food chain. Within plant cells Cd detoxification occurs through formation of complexes with metallothioneins (MTs), glutathione and phytochelatins (Cobbett and Goldsbrough 2002; Clemens 2006; Nezhad et al. 2013). In this report, the correlation between Cd-dose dependent expressions of a type 1 MT gene, *dMT* from a Cd-tolerant durum wheat cv. Balçalı-85 has been investigated.

Results of Southern blot analysis indicated that *dMT* gene exists as a single copy in the genome of *T. durum*. Similar results were obtained for rice type 2 MT; *ricMT* (Yu et al. 1998) and in salt cress type 3 MT; *TsMT3* (Quan et al. 2008). But in some other plant species, e.g. in pea (Evans et al. 1992), maize (de Framond 1991), *Arabidopsis* (Zhou and Goldsbrough 1995), cotton (Hudspeth et al. 1996) and *Helianthus tuberosus* (Chang et al. 2004) MT genes exist as more than one copy in the genome.. In *Chloris reigate, ChlMT1* gene exists as two or three copies (Nishiuchi et al. 2007) and in *Zea mays, MZm3-4* is present as one or two copies in the genome (Charbonnel-Campaa et al. 2000). That is, there is no strict correlation between the plant species in terms of the copy number of MT genes.

The response of *T. durum* cv. Balçalı-85 cultivar to Cd stress was investigated at five different concentrations. Cd exposure resulted in significant inhibition of growth and reduction in dry weights of both shoot and root tissues (Figs. 1, 2). In general, the effect of Cd on growth was more pronounced in roots than in shoots. Similar results were reported in durum wheat by Ozturk et al. (2003),

Paradiso et al. (2008) and Yourtchi and Bayat (2013). Cd toxicity causes reduction in the concentrations of photosynthetic pigment and carotenoids, and as a result, photosynthesis activity is restricted causing sustained growth (Rai et al. 2005). Cd also reduces growth by inhibiting cell division (Paradiso et al. 2008). Although there are several studies in the literature on the effect of Cd on wheat growth correlation of results with tissue specific changes at molecular level are scarce. Results in the current manuscript provide some clarification through demonstration of differential expression of the gene for *dMT* in roots in response to Cd. In previous physiological studies sensitive and resistant cultivars were investigated for effects metabolic enzyme activities (Kokturk 2006). In these experiments effects of Cd concentrations at 0, 0.5, 2, 10 and 30 µM on shoot and root growth, uptake and accumulation of Cd, and activity of antioxidative defense enzymes were studied in Cd-tolerant Balcalı-85 and Cd-sensitive Balcalı-2000 durum wheat (*T. durum*) cultivars. Genotypic variation was also studied in terms of activities of antioxidative enzymes including ascorbate peroxidase (AP), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT). Results did not yield consistent correlations between Cd tolerance and activities of the antioxidative enzymes.

In this study, we found that Cd was accumulated in the roots in much higher amounts than in the shoots in Balçalı-85 plants (Fig. 3). That is, roots being the first site of contact have greater capacity to accumulate Cd than shoots. Accumulation of Cd in roots serves as a defense mechanism in order to limit the toxicity and roots are more prone to Cd toxicity than shoots (Grant et al. 1998). Previous studies with durum wheat (*T. durum* cv. Creso) also reported a dose- and time-dependent accumulation of Cd in root tissues, whereas leaf Cd accumulation was not observed during 7 days of exposure to high levels of Cd (as high as 40 µM) (Paradiso et al. 2008).

The literature on the induction of expression of MT genes in response to (heavy) metal stresses in the growth environment contains contradictory reports. For instance, in wheat *(Triticum aestivum* L.) *wali1* expression was shown to be induced by 50 µM Cd in root tissues (Snowden et al. 1995). Upon exposure to different concentrations of $CdCl_2$ (50, 100, 150, 200, 250 µM) the transcript level of rice type I *rgMT* was increased strongly in roots and leaves (Jin et al. 2006). The expression of a type 1 *ZjMT* from *Ziziphus jujube* induced stress in leaves after 100 mM $CdCl_2$ (Yang et al. 2015). In another study it was reported that Cd increased MT1 gene levels in the roots, leaves and ripening fruits of tomato when applied at 10, 20, 50 ppm (Kisa et al. 2016). In this study, due to diminished growth rate and higher accumulation of Cd specifically in root tissues, the effect of Cd on the *dMT* gene transcript level was investigated only in roots of 1 week old seedlings of Balçalı-85 exposed to increasing Cd concentrations both by quantitative real-time PCR and Northern blot analyses. Although we used a lower Cd concentration (20 µM) compared to the level stated in the literature, a partial correlation with the transcript levels of *dMT* in roots and the environmental Cd concentration (Figs. 5, 6) was observed in roots suggesting participation of dMT in the heavy metal response. A dose-dependent accumulation of *dMT* expression was observed in Northern blot results (Fig. 6); however, the transcript level of *dMT* decreased slightly upon exposure to 20 µM Cd (Fig. 5). These contrasting results might be related to the different sensitivity levels of these two experimental approaches, as qRT-PCR is more sensitive to changes in the transcript amount of the target genes, yet Northern blot analysis is less sensitive and might misinterpret slight changes in gene expression.

The positive correlation between the level of cadmium exposure and *dMT* gene expression levels presented in this paper strongly supports the induction of type 1 MT genes as part of the heavy metal response in plants. What remains to be shown is whether type 1 MTs are specific for heavy metal response or whether they participate in a general stress response mechanism.

Conclusions

In this study, response of *T. durum* cv. Balçalı-85 to increasing levels of environmental Cd was investigated. It was observed that reduction in dry weight matter occurred both in roots and shoots upon increasing Cd levels. Balçalı-85 demonstrated high capacity to retain Cd specifically in roots. Southern blot analysis revealed that the *dMT* gene exists as a single copy in the *T. durum* genome. We also showed the dose-dependent correlation between *dMT* gene expression and Cd concentration. We argue that type 1 MTs in plants might have a role in root specific cadmium response and can be used in further improvement of Cd tolerance of plants through transgenic approaches.

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