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Screening of Cd tolerant genotypes and isolation of metallothionein genes in alfalfa (*Medicago sativa* L.)

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ABSTRACT

In order to evaluate Cd tolerance in wide-ranging sources of alfalfa (*Medicago sativa*) and to identify Cd tolerant genotypes which may potentially be useful for restoring Cd-contaminated environments, thirty-six accessions of alfalfa were screened under hydroponic culture. Our results showed that the relative root growth rate varied from 0.48 to 1.0, which indicated that different alfalfa accessions had various responses to Cd stress. The candidate fragments derived from differentially expressed metallothionein (MT) genes were cloned from leaves of two Cd tolerant genotypes, YE and LZ. DNA sequence and the deduced protein sequence showed that *MsMT2a* and *MsMT2b* had high similarity to those in leguminous plants. DDRT-PCR analysis showed that *MsMT2a* expressed in both YE and LZ plants under control and Cd stress treatment, but *MsMT2b* only expressed under Cd stress treatment. This suggested that *MsMT2a* was universally expressed in leaves of alfalfa but expression of *MsMT2b* was Cadmium (Cd) inducible.

1. Introduction

Cadmium (Cd) is a highly toxic element to plant growth in the environment (Redondo-Gómez et al., 2010). It is taken up by the roots via essential metal transporters (Cohen et al., 1998; Pence et al., 2000) and partly translocated to the shoot after a prolonged exposure. Cd^{2+} decreases growth rate by affecting various aspects of plant physiology in different plant species, such as reducing carbon assimilation (Krupa and Baszynski, 1995), increasing oxidative stress (Dong et al., 2006; Lin et al., 2007; Romero-Puertas et al., 2004; Schützendübel et al., 2001; Wang et al., 2011a), interfering with sulfur assimilation and glutathione metabolism (David et al., 2005), wilting (Perfus-Barbeoch et al., 2002) and absorption of nutritional elements (Gardea-Torresdey et al., 2004a). Plants have evolved many adaptive mechanisms to cope with heavy metal stress, including governing uptake of heavy metal ions, detoxification by chelation, intracellular sequestration and cellular homeostasis to minimize the damage from exposure to nonessential metal ions. It has been demonstrated that plant detoxification response proceeds through synthesis of some heavy metal binding compounds, such as phytochelatin (PC) (Cobbett, 2000; Kneer and Zenk, 1997) involving an increase in the expression of γ-glutamylcysteine synthetase (Sun et al., 2005), metallothionein (MT) (Klaassen et al., 1999), and finally Cd^{2+} sequestration in less-sensitive subcellular compartments, such as the cell wall and the vacuole (Hinkle et al., 1987; Martinoia et al., 1993). Some of these detoxification elements have been identified at molecular level (Clemens, 2001). Membrane transporters also play an important role in Cd homeostasis, and many genes are involved, such as *ZIP* (ZRT IRT related proteins) family, natural resistance associated macrophage proteins (Nramp) and P_{1B}-type ATPase family which were discovered and cloned in recent years (Eren and Arguello, 2004).

The accumulation of Cd within soil, sediments, and the aquatic environment is of concern because following uptake by plants or other life forms, Cd can be passed on throughout the food chain. It is therefore important to develop methods of rehabilitating Cd polluted soils. Conventional approaches for clean-up of contaminated sites are not only costly and inefficient, but also cause other damage to the environment (Gardea-Torresdey et al., 2004b). Phytoremediation has gained more attention in recent times as an alternative technology for environmental restoration in these contaminated sites with Cd (Dewez et al., 2005; Hou et al., 2007; Ji et al., 2011; Sun et al., 2009). Alfalfa (*Medicago sativa* L.) is one of the most important crop plants in the world. According to its rapid growth, high biomass and suitability for nutrient stress habitats (Wang et al., 2011b), alfalfa can be used for the removal of

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heavy metals (Patterson, 1977). Recent reports about alfalfa genetic linkage map construction based on various molecular markers indicate that it is a good way to locate genes related to abiotic stress, such as Al tolerant genes (Choi et al., 2004; Narasimhamoorthy et al., 2007b; Sledge et al., 2002). So any Cd tolerant genes identified in *M. sativa* might be used to improve Cd tolerance of cultivated alfalfa either by cross breeding or genetic transformation. High throughput DNA homology searches for the same or similar Cd tolerance genes in alfalfa germplasm collections are therefore important, yet remains little examined.

Metallothioneins (MTs) is one of the best-characterized heavy metal-binding ligands in plant cells. It is valuable to explore the expression pattern of these two genes in response to heavy metal stresses and characterize their functions by transferring them into plants. The spatial and temporal expression patterns of MT isoforms in other plants have been investigated in rice (Xu et al., 2007), *Arabidopsis* (Guo et al., 2003), watermelon (Akashi et al., 2004), tomato (Giritch et al., 1998), and garlic (Zhang et al., 2006). In *Arabidopsis* control plants, *MT1a* expression was localized in leaf trichomes and in the vascular tissue in leaves, roots, flowers, and germinating embryos, and *MT1a* was also observed in the leaf mesophyll and in vascular tissue of developing siliques and seeds in copper-treated plants. In contrast, *MT2a* was expressed primarily in the trichomes of both untreated and copper-treated plants (García-Hernández et al., 1998). Two full-length cDNAs (Y459 and G14) encoding MT-like proteins were isolated from leaves of sweet potato (*Ipomoea batatas*), and semi-quantitative RT-PCR showed that Y459 was expressed in significant quantity in roots and stems, but was much less in leaves, in contrast, relatively constant gene expression levels were found for G14 in all tissues or treatments analyzed (Chen et al., 2003).

The overall goal of this research was to evaluate Cd tolerance in wide-ranging sources of alfalfa accessions and to screen for the Cd tolerant genotypes which may potentially be useful for restoring Cd-contaminated sites. The aim of this study is to 1) investigate the ability of alfalfa seedlings to grow in Hoagland solution containing Cd and screen for the Cd tolerant genotypes, 2) clone the coding region of differentially expressed Cd-MT genes under Cd stress in alfalfa accessions and analyze their characteristics.

2. Materials and methods

2.1. Plant materials

Thirty-six alfalfa (*M. sativa* L.) accessions were used in this study (Table 1). These accessions were obtained from the Grassland Research Institute, Chinese Academy of Agricultural Science at Hohhot, Inner Mongolia.

2.2. Hydroponics procedure

Alfalfa seeds were surface sterilized by dipping in 75% (v/v) ethanol for 30 s after a 1.5 h soak under running water. Disinfected seeds were rinsed with sterile distilled water for 3–5 times and soaked in 0.1% $HgCl_2$ for 5 min, then rinsed with sterile distilled water 3–5 times. After that, the seeds were placed in sterile petri dishes with filter paper for 24 h in darkness at 25 °C. Germinated seedlings with root 1.0 cm long were transferred to a hydroponics system in a growth room and grown under 16 h light/8 h dark regime at a constant temperature of 25 °C. The light intensity at the shelf height was 230 μmol m^{-2} s^{-1} using fluorescent lamps. The hydroponic system (Australian Center for Plant Functional Genomics, Australia) consisted of 20 plastic vessels with 25-L capacity, in which Hoagland modified medium plus Cd treatment or without Cd was pumped up by a small aquarium air pump for 15 min, twice an hour. The Hoagland medium was modified to contain 5 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 92 μM H_3BO_3, 18 μM $MnCl_2 \cdot 4H_2O$, 1.6 μM $ZnSO_4 \cdot 7H_2O$, 0.6 μM $CuSO_4 \cdot 5H_2O$, 0.7 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 59 μM Fe-EDTA. The media were maintained at pH 6.3 by adding 1 M HCl as needed throughout the experiment. An appropriate cadmium concentration for screening was estimated by measuring the relative root growth response of 50 randomly chosen alfalfa seedlings to 0, 5, 10, 15, 20, 25, 50, 100 mg L^{-1} $CdCl_2$ in hydroponics as described above.

The effects of $CdCl_2$ stress on biomass production and growth parameters were determined after 2 weeks of growth with and without Cd. Each harvested plant was removed and the shoot length, root length, shoot biomass and root biomass were measured. Relative shoot (or root) growth rate was determined with the ratio of shoot (or root) biomass of Cd treatment/shoot (or root) biomass of control treatment.

Thirty-six alfalfa accessions were screened for Cd tolerance under 25 mg L^{-1} $CdCl_2$ treatment. Forty plants of each accession were cultured for 1 month in the hydroponic system as described above, and then the 40 plants were randomly divided into two groups. One group was treated with 25 mg L^{-1} $CdCl_2$ and the other group without $CdCl_2$. The experiment was carried out for 2 weeks. The root length, shoot length, root biomass and shoot biomass were determined. All data were analyzed with statistic software and two Cd tolerant accessions YE and LZ were screened for further study below.

2.3. Cd accumulation

In order to test the ability of Cd tolerance alfalfa accessions for accumulating Cd furthermore, 80 plants of YE and LZ were cultured for 1 month in the hydroponic system as described above, then the 80 plants were randomly divided into four groups. Each group has 20 plants. Four groups were treated with 25 mg L^{-1} $CdCl_2$ for 0, 2, 4, 6 days, respectively. Leaves, shoots and roots of the plants were harvested and extensively washed with distilled water. All samples above were then oven-dried, ground to powder, ashed in a muffle furnace at 550 °C for 4 h and the residue was brought to a standard volume with 1 M HNO_3. The Cd concentration was determined by a flame atomic absorption spectrophotometer.

2.4. RNA isolation and purification

Twenty plants of YE and LZ were cultured with and without 25 mg L^{-1} $CdCl_2$ for 2 weeks in the hydroponic system. Fresh leaves from individual plants were used to extract RNA using Trizol UNIQ-10 (Sangon, Shanghai). The RNA pellets in ethanol were stored at −80 °C before being used. The total RNA of 10 μg was treated with ten

Table 1
List of alfalfa accessions in this study and their origins.

No.	Names	Code	Species	No.	Names	Code	Species
1	Gannong no. 1	GN1	*M. sativa* subsp. *sativa*	19	Tianshui	TS	*M. sativa* subsp. *sativa*
2	Gannong no. 2	GN2	*M. sativa* subsp. *sativa*	20	Dingxi	DX	*M. sativa* subsp. *sativa*
3	Gannong no. 3	GN3	*M. sativa* subsp. *sativa*	21	Qingyang	QY	*M. sativa* subsp. *sativa*
4	Zhonglan no. 1	ZL	*M. sativa* subsp. *sativa*	22	Emperor	MXW	*M. sativa* subsp. *sativa*
5	Gongnong no. 1	GO1	*M. sativa* subsp. *sativa*	23	Algonuin	GJ	*M. sativa* subsp. *sativa*
6	Gongnong no. 3	GO3	*M. sativa* subsp. *sativa*	24	Ladak	LDK	*M. sativa* subsp. *sativa*
7	Ganza no. 27	GZ27	*M. sativa* subsp. *sativa*	25	Zimuxu	ZI	*M. sativa* subsp. *sativa*
8	Shandong no. 2	SD2	*M. sativa* subsp. *sativa*	26	Jiexing	JX	*M. sativa* subsp. *falcata*
9	Huanghua	HH	*M. sativa* subsp. *falcata*	27	Indian	YDA	*M. sativa* subsp. *sativa*
10	Huangyangzhen	HYZ	*M. sativa* subsp. *sativa*	28	Yidali	YDL	*M. sativa* subsp. *sativa*
11	Yemuxu	YE	*M. sativa* subsp. *falcata*	29	Zahua	ZH	*M. sativa* var. *media*
12	Hetian	HT	*M. sativa* subsp. *sativa*	30	Meiguo	MEG	*M. sativa* subsp. *sativa*
13	Fugang	FG	*M. sativa* subsp. *sativa*	31	Graze401	MG	*M. sativa* subsp. *sativa*
14	Xiongyue	XY	*M. sativa* subsp. *sativa*	32	Peru	BL	*M. sativa* subsp. *sativa*
15	Qingyang	QY	*M. sativa* subsp. *sativa*	33	Egypt	AJ	*M. sativa* subsp. *sativa*
16	Hexi	HX	*M. sativa* subsp. *sativa*	34	Giant201	JR	*M. sativa* subsp. *sativa*
17	Longdong	LD	*M. sativa* subsp. *sativa*	35	Jindera	JDR	*M. sativa* subsp. *sativa*
18	Longzhong	LZ	*M. sativa* subsp. *sativa*	36	Queen	JHH	*M. sativa* subsp. *sativa*