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Expression of the seed-specific metallothionein *mt4a* in plant vegetative tissues increases Cu and Zn tolerance

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ABSTRACT

The aim of this work was to express the seed-specific metallothionein 4 (*mt4a*) gene in *Arabidopsis thaliana* vegetative tissues, in order to study its effect on plant Cu and Zn tolerance and accumulation. In that way, *mt4a* gene was expressed under the regulation of the 35S CaMV promoter in *A. thaliana* plants. Expression of *mt4a* gene in vegetative tissues at different developmental stages conferred increased plant tolerance towards Cu and Zn, as concluded from root length measurement and shoot biomass determination. However, our results suggest that expression of *mt4a* gene in vegetative tissues of *A. thaliana* plants leads to a slight increase in plant Cu accumulation and do not increase Zn content in the plants.

1. Introduction

Metallothioneins (MTs) are metal-binding cysteine-rich polypeptides encoded by a family of genes which are widespread throughout the animal and the plant kingdoms. Several highly divergent MTs have been identified in cyanobacteria, protist, algae and higher plants [1]. These MTs differ dramatically from mammalian MTs in sequences, charge and total cysteine content. The plant and algal MTs exhibit beneficial metal binding and induction properties that may protect these organisms from elevated levels of toxic heavy metals (such as Cd or Hg) and also affect for example the homeostasis of Cu and Zn, essential micronutrients for a range of plant physiological processes [1].

The small *Arabidopsis* genome contains nine genes encoding MTs [2]. In angiosperms, MTs can be classified into four types based on the conserved positions of Cys residues [1]. Expression of all MT genes has been shown to be induced by Cu, although they have distinct function in heavy metal homeostasis, especially for Cu [3]. Moreover, expression of MT genes correlates closely with Cu tolerance among *Arabidopsis* ecotypes [4]. The divergence of plant MT protein sequences and the complex expression patterns of MTs suggest that the functions of MTs may not be limited to Cu detoxification. It has been reported that the four classes of MTs are differentially stabilized by element ions and may have different metal binding specificities in plants [2]. MTs in general can function as metal chelators of Cu and Zn in vivo [5]. In particular, MT1 class isovariants are required to protect *Arabidopsis* plants from the toxic effects of Cd and possibly As [2]. In addition, type-2 MTs are involved in reactive oxygen species scavenging and signalling in rice in response to pathogen attack [6].

It has been proposed that plant MTs have distinct, although sometimes overlapping, expression patterns [7], probably correlated with distinct functions in heavy metal homeostasis, especially for Cu [3]. *MT1a* and *MT2b* are expressed in the phloem, being probably involved in the distribution of Cu via the phloem; *MT2a* and *MT3* are expressed predominantly in mesophyll cells in young leaves and root tips, being proposed to chaperone excess metals in these tissues. Type-4 MTs are specifically expressed in seeds. MT4 proteins may function in pollen embryogenesis and preparing seed tissues for desiccation [8,9]. These seed specific MTs may also provide a mechanism for storing Zn and other metals that are required for growth after germination [8,9].

Attempts have been made to increase the level of different MTs (mainly 1 and 2 types) in *Escherichia coli*, yeast and plants. In most cases, an increase of tolerance to Cu and some other metals has been reported, while, in particular cases, an increase in metal accumulation has also been described [5,10–12]. Concerning the seed specific type-4 MTs, recent results indicate that these proteins are more effective than other *Arabidopsis* MTs in providing

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protection against Zn toxicity and enhancing Zn accumulation in yeast, suggesting that type-4 MT protein may have a greater capacity to bind Zn ions compared to others MT isoforms [5].

The aim of this work was to express the seed specific *mt4a* gene in *Arabidopsis thaliana* vegetative tissues in order to study its effect on plant Cu and Zn tolerance and accumulation. The analysis has been performed at three different plant developmental stages: seed germination, 7–14-day-old seedlings and 1-month-old mature plants.

2. Materials and methods

2.1. Isolation and cloning of mt4a sequence for constitutive expression in plants

The open reading frame encoding MT4a protein was amplified using *Pfu* polymerase from *A. thaliana* genomic DNA with the forward primer MT4X (5′-GGTCTAGACAAAAAATGGCAGACA-CAGGC-3′) and the reverse primer MT4B (5′-GGGGATCCATAAG-GATTTACAAACTCTCG-3′). In order to facilitate the cloning of the PCR product, primers contain restriction sites for XbaI and BamHI, respectively (underlined in the sequence). The amplified *mt4a* gene was cloned under the control of the CaMV 35S promoter in XbaI and BamHI sites of the plant binary vector *pPZPY112* [13]. This construct was electroporated into *Agrobacterium tumefaciens* EHA105 [14].

2.2. Plant material, growth conditions and transformation of Arabidopsis

A. thaliana ecotype Columbia plants were transformed by the floral dip method [15]. Seeds were sterilized for 8 min with 15% (v/v) household bleach containing a few drops of Tween 20, followed by three washes with sterile water. T_1 generation seedlings were germinated on plates containing one-half-strength MS medium (according to Murashige and Skoog [16]; but one-half-strength for macrosalts) supplemented with kanamycin (50 mg/l) at 21 °C under a 12-h light/12-h dark regime. Plants surviving selection were grown in a soil mix in the greenhouse and T_1 seeds were collected. The seeds were sown on MS media containing kanamycin to screen for the transgene and ten kanamycin-resistant transgenic plant lines (whose progeny segregated 3:1 for kanamycin resistance) were grown and allowed to set T_2 generation seeds (lines *mt4a-1* to *mt4a-10*). Homozygous T_3 lines were selected as described previously [17].

2.3. RNA expression analysis

RNA was isolated from 7 days old whole seedlings or roots and shoots of 30-day-old plants grown in MS plates using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Three independent RNA samples were obtained. For cDNA synthesis, 100 ng of DNase-treated total RNA (digested with DNase I recombinant RNase-free, Roche Applied Sciences) was used and reverse transcription was carried out with the gene-specific reverse primer MT4B, using the QuantiTect Rev. Transcription Kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized using MT4X and MT4B primers pair and concentration was adjusted to 75 ng/μl using a Thermo Scientific NanoDrop™ Spectrophotometer. RT-PCR was carried out using the QuantiFast Multiplex PCR Kit (Qiagen) and a TGradient termocycler (Biometra), following the manufacturer's instructions. PCR conditions consisted of an initial denaturation at 95 °C for 2 min followed by 25 cycles at 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final step at 72 °C for 10 min. Amplification of an actin cDNA (*ACT2*) was used to normalize results from different samples. Primers and conditions for *ACT2* amplification are described in Ref. [18]. Expression signals were quantified and normalized using the Multi Gauge V3.0 FUJIFILM program.

2.4. Heavy metal tolerance assays

Heavy metal tolerance was evaluated at three different plant developmental stages as follows: (a) *Seed*: To study metal effect on seed germination, homozygous T_3 seeds were surface sterilized and placed on plates containing increasing concentrations of $CuSO_4$ or $ZnCl_2$. (b) *Seedlings*: To analyze metal sensitivity of seedlings, seeds were germinated vertically on normal MS medium plates. After 3 days, seedlings were transferred to MS plates containing increasing concentrations of $CuSO_4$ or $ZnCl_2$. The highest differences between wild-type and transgenic plants were observed at 100 μM $CuSO_4$ or 200 μM $ZnCl_2$. Growth of the primary root was measured after 4 days (7-day-old seedlings) and biomass determination was estimated in 14-day-old seedlings. (c) *Mature plant*: Cu and Zn tolerance of mature plants were estimated using 7 mg of sterilized seeds grown in the greenhouse on pots containing commercial peat. After 1 week, plants were watered with a solution containing 150 μM $CuSO_4$ or 200 μM $ZnCl_2$ during 3 more weeks. Plant growth (% of viable plants) and fresh weight per plant were measured.

2.5. Heavy metal accumulation assays

To estimate metal accumulation, *Arabidopsis* plants were grown in a hydroponic system for 1 month and then watered with 150 μM Cu or 200 μM Zn during 3 days. Roots and leaves were harvested and dried and metal content in both tissues was determined by ICP-OES as described previously [19].

3. Results

3.1. Transgenic expression of mt4a in A. thaliana seedlings

In this work we have expressed the seed-specific metallothionein *mt4a* gene under the control of the CaMV 35S constitutive promoter. Semi-quantitative expression of *mt4a* gene on 7-day-old seedlings of ten transgenic lines was studied by RT-PCR. Amplification of an actin cDNA (*ACT2*) was used to normalize results from different samples. Transgenic seedlings showed different levels of *mt4a* expression (Fig. 1A). As expected, no expression was found in wild-type seedlings, in agreement with the seed specific expression of this gene reported previously [3]. *mt4a-7* and *mt4a-10* transgenic lines showed the highest levels of expression (Fig. 1B) and were chosen for further characterization.

3.2. Overexpression of the seed specific mt4a gene do not significantly increase seed germination in the presence of Cu or Zn

In order to analyze whether the overexpression of the seed specific *mt4a* gene has an effect on *A. thaliana* seed germination in the presence of Cu or Zn, transgenic and wild-type seeds were germinated in the presence of increasing concentrations of both metals. Cu concentrations ranging from 25 to 100 μM did not significantly affect seed germination (data not shown). At higher Cu concentrations (150 μM), a positive, although minor, effect of *mt4a* expression on germination was observed, especially in *mt4a-10* line, since seed germination increased around 15%. Concerning Zn effect on seed germination, no effect was observed at concentrations below 200 μM (data not shown).