Journal of Hazardous Materials 233–234 (2012) 65–71

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Overexpression of *Elsholtzia haichowensis* metallothionein 1 (EhMT1) in tobacco plants enhances copper tolerance and accumulation in root cytoplasm and decreases hydrogen peroxide production

Yan Xia[a,b], Ying Qi[c], Yuxiang Yuan[a], Guiping Wang[a], Jin Cui[a], Yahua Chen[a], Hongsheng Zhang[d], Zhenguo Shen[a,*]

[a] College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, PR China
[b] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, PR China
[c] Yunnan Academy of Tobacco Agricultural Sciences and China Tobacco Breeding Research (Southern) Center, Yuxi 653100, PR China
[d] State Key Laboratory of Crop Genetics & Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, PR China

HIGHLIGHTS

- Transgenic tobacco overexpressing *EhMT1* showed higher tolerance to Cu.
- Plants expressing *EhMT1* accumulated more Cu in soluble fraction of roots.
- The study provides the first cytochemical evidence of MT decreasing H_2O_2 accumulation.

ARTICLE INFO

Article history:
Received 30 March 2012
Received in revised form 8 May 2012
Accepted 25 June 2012
Available online 2 July 2012

Keywords:
Copper tolerance
EhMT1 overexpression
Oxidative stress
Peroxidase
Transgenic tobacco

ABSTRACT

To evaluate the functional roles of metallothionein (MT) in copper tolerance, we generated transgenic tobacco plants overexpressing *EhMT1* from the Cu-accumulator *Elsholtzia haichowensis* Sun. Overexpression of *EhMT1* in tobacco plants imparted increased copper (Cu) tolerance based on seedling dry biomass when compared to wild-type plants. Plants expressing *EhMT1* accumulated more Cu in roots, which was mainly attributable to an increase of the soluble fraction. Levels of lipid peroxidation and production of hydrogen peroxide were lower in roots of transgenic tobacco than in wild-type plants. EhMT1 was suggested to bind Cu in the cytoplasm, thereby decreasing activity of free Cu^{2+} ions and blocking Cu^{2+} from interacting with cytoplasmic components, which in turn decreases the production of reactive oxygen species. In addition, our results also indicate that *EhMT1*-overexpressing tobacco has a more efficient antioxidant system, with improved peroxidase activity to better cope with oxidative stress.

1. Introduction

Copper (Cu) is an essential micronutrient associated with proteins and enzymes involved in electron transfer and redox reactions in plants and algae [1]. In agricultural soils, Cu tends to accumulate due to industrial waste dispersal, sewage sludge application, and the excessive use of fertilizers and pesticides. Excess Cu is toxic to plants and interferes with important biochemical and physiological processes such as photosynthesis, respiration, and mineral uptake [1–3]. Being a redox-active metal, Cu also can catalyze the generation of harmful reactive oxygen species (ROS) and subsequently cause lipid peroxidation, membrane damage, and enzyme inactivation. To control metal homeostasis and redox status, plants have several mechanisms of metal tolerance, including exclusion, compartmentalization, and binding to organic ligands such as organic acids, phytochelatins, and metallothioneins (MTs) [3,4]. Recent experiments also showed a clear relationship between metal stress and redox homeostasis and antioxidant capacity [5].

The MTs are a class of low-molecular-weight, cysteine (cys)-rich, heavy metal-binding proteins widely distributed in animals, plants, and microorganisms [2]. Plant MTs, which have two cys-rich domains, can be grouped into four types, MT1, MT2, MT3, and MT4, based on the distribution of cys residues. The different types of MT exhibit different expression in plant tissues during development and possibly have different functions. Type 1 MT genes are predominantly expressed in roots, whereas type 2 are in aerial tissues, including leaves, stems, and flowers; type 3 are expressed in ripening fruit tissues, and type 4 in seeds. The expression of plant MT genes has been shown to be regulated by heavy metals, hormone treatment, heat shock, cold, and osmotic stress [6].

* Corresponding author. Tel.: +86 25 84396391; fax: +86 25 84396325.
E-mail address: zgshen@njau.edu.cn (Z. Shen).

0304-3894/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jhazmat.2012.06.047

The effect of metals on the expression of plant MTs varies with the species, tissue, and MT type. Increasing evidence suggests that plant MTs play a role in maintaining the homeostasis of essential metal ions, detoxifying Cu, Zn and Cd, and scavenging ROS [2,7–11]. In some plant species, Cu tolerance is associated with constitutively enhanced transcript levels of a type 2 MT [12–14].

Although it has been difficult to study MT proteins in planta because of high level of proteolysis associated to native protein purification, several plants MTs have been expressed in microbial hosts or overexpressed in planta to examine the metal-binding properties of MT proteins and their ability to provide metal tolerance [2,15]. When expressed in *Escherichia coli* and *Arabidopsis*, a pea MT-like gene, $PsMT_A$, increased Cu tolerance and accumulation [16]. Foley et al. [17] observed the ability of MT1 from *Vicia faba* to bind Cu and cadmium (Cd) in *E. coli*. The convincing evidence is also derived from loss-of-function studies, where changes in metal tolerance and accumulation have been observed. *Arabidopsis* MT1 family knock-down lines with reduced MT1a and MT1c levels are hypersensitive to Cd and accumulate less Cd and Zn in leaves than WT plants [18]. The lack of MT1a plants showed a 30% decrease in Cu accumulation in the roots [19]. Gene expression analyses and functional testing in yeast also revealed a role for MTs in Cu homeostasis and detoxification [7,20–22]. Lee et al. [23] reported that the Cd detoxification mechanism of AtMT2a and AtMT3 may not include sequestration into vacuoles or other organelles, but does involve reduction of ROS in Cd-treated cells. Xue et al. [11] reported that recombinant GhMT3a (*Gossypium hirsutum*) protein could bind metal ions and scavenge ROS. Transgenic yeast overexpressing *ChlMT1* from *Chloris virgata* or *GhMT3a* from *G. hirsutum* showed high tolerance to ROS stress [9,11]. Compared to the control, accumulation of hydrogen peroxide (H_2O_2) in transgenic plants overexpressing *BrMT1* from *Brassica rapa* [24], *cgMT1* from *Casuarina glauca* [25], or *GhMT3a* from cotton also decreased [11]. Compared to control glutathione-*S*-transferase (GST) proteins, GST–MT fusion proteins displayed higher antioxidant activity against ROS [11,26].

Elsholtzia haichowensis Sun (Lamiaceae) is a Cu-accumulator that is widely distributed on Cu mining waste and Cu-contaminated soil along the middle and lower reaches of the Yangtze River in China [27,28]. The Cu tolerance of *E. haichowensis* was reported to be based on an exclusion mechanism [29,30]. The roots of *E. haichowensis* contain much higher Cu concentrations than the shoots. In a previous study, we isolated an *MT1* gene (*EhMT1*) from *E. haichowensis* that was expressed preferentially in roots. *EhMT1* expression was markedly increased in both roots and shoots treated with Cu and H_2O_2. In this study, to better understand the physiological roles of *EhMT1* in plants, we transformed tobacco with an expression vector containing *EhMT1* cDNA and examined its growth under Cu stress. The metal accumulation and H_2O_2 production in the roots of transgenic tobacco expressing *EhMT1* were also investigated.

2. Materials and methods

2.1. Tobacco transformation and molecular characterization

The full-length *EhMT1* coding sequence was cloned into the pBI121 vector under the cauliflower mosaic virus (CaMV) 35S promoter. Recombinant *Agrobacterium tumefaciens* (EHA105) clones were selected in a medium containing 150 $\mu g\,mL^{-1}$ kanamycin. Wild-type (WT) tobacco was transformed using the leaf disk method [31]. T_0 seeds were germinated on half-strength Murashige and Skoog (MS) medium agar plates under kanamycin selection. Leaves from kanamycin-resistant seedlings were collected and the presence of the transgene was conformed by PCR with EhMT1-specific primers; the transgene expression levels determined through real-time quantitative RT-PCR on total leaf RNA using the constitutively expressed gene 18S rRNA as internal standard. Equal amount of cDNA prepared from plant materials was analyzed by quantitative real-time RT-PCR using SYBR-green fluorescence and a MyiQ Real-time PCR Detection System (Bio-Rad). The PCR protocol contained an initial 7-min incubation step at 95 °C for complete denaturation, followed by 40 cycles consisting of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The specificity of the PCR amplication was checked with a heat dissociation curve (65–95 °C) following the final cycle. The primers for EhMT1 were 5′-CAAGAAAATGTCGAGTGGA-3′ and 5′-GTGCAAGGGTTGCAGGTGCAG-3′, and for 18S rRNA were 5′-TGGGATACCTGCCAGTAGTCAT-3′ and 5′-CTGGATCCAA-TTACCAGACTCAA-3′. Data represent the means (±SD) of three independent experiments.

2.2. Determination of plant growth and metal content

The kanamycin-resistant seeds (T_2 generation of T-2 line) and WT tobacco seeds were germinated on MS medium, and then transferred to hydroponic conditions in Hoagland solution. After 2 weeks, the plants were transferred to the same nutrient solution (control) or to a similar solution supplemented with 20 μM $CuSO_4$. The solutions were aerated continuously and renewed every 2 days. Each treatment was replicated in three different vessels each containing 18 seedlings. The hydroponic culture was undertaken in a growth chamber at 28 °C/20 °C with a photoperiod of 16 h light/8 h dark.

After 7 days of exposure to 20 μM $CuSO_4$, plants were harvested and the samples dried for 15 min at 105 °C and for 24 h at 80 °C in an oven; then dry weights were measured. The dried plant samples were completely digested with extra pure grade HNO_3 and $HClO_4$ (87:13, v/v). Cu concentrations were analyzed with a flame atomic absorption spectrometer (novAA® 400; Analytik Jena, Jena, Germany). Detection limits for the AAS were as in Xia and Shen [32] and a certified standard reference material (SRM 1573a, tomato leaves) from the National Institute of Standards and Technology (Gaithersburg, MD, USA) was used as a control in the digestion and analysis. Blanks were also used for background correction and other sources of error.

2.3. Plant tissue fractionation

Plant root material was homogenized in ice-cold extraction buffer [50 mM HEPES, 1.0 mM DTT, 500 mM sucrose, 5.0 mM ascorbic acid, 1.0% (w/v) Polyclar AT (PVPP), pH 7.5]. The homogenate was sieved through a nylon cloth (100 μm mesh size) and the residue constituted the cell wall-containing fraction. The filtrate was centrifuged at 10,000 × *g* for 30 min, and the retained pellet was the organelle fraction. The supernatant was then centrifuged at 100,000 × *g* for 30 min, and the supernatant constituted the soluble fraction. All steps were performed at 4 °C. The fractions were dried and wet-digested separately, and then Cu concentrations in the digests were determined by atomic absorption spectrometry (AAS).

2.4. Determination of lipid peroxidation and H_2O_2 content in tobacco roots

Lipid peroxidation was measured as the content of total thiobarbituric acid-reactive substance (TBARS), as described in Jiang and Zhang [33]. The content of H_2O_2 in root extracts was measured by monitoring the absorbance at 390 nm of the mixtures following the method described by Loreto and Velikova [34].