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Improving Zinc Content and Antioxidant Activity in Transgenic Tomato Plants with Expression of Mouse Metallothionein-I by *mt-I* Gene

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Metallothioneins (MTs), as a family of low-molecular-weight, cysteine-rich, and metal-binding proteins, show potential for utilization in functional food. Tomato plants were transformed with gene constructs that contained *mt-I* encoding the mouse MT-I, similar in sense orientation with the constitutively active double 35S promoter from cauliflower mosaic virus. Three independent transformants, which had copies of the gene in their genomes, were obtained. In these transgenic lines, high-level expression of MT-I, high zinc content, and some antioxidant enzyme activities were detected in leaves. The average zinc content in transgenic tomato leaves was 32.7 mg/100 g FW, which about 1.6 times higher than that in wild-type. The superoxide dismutase activity was also higher (68.6, 66.9, and 66.1 U/g FW in the three transformants) than that in wild-type (57.4 U/g FW). In particular, the levels of superoxide free radical scanvenging in the three transformants were 14.2%, 14.6%, and 13.7%, respectively, which about 1.5 times higher than that in control (5.6%). Transgenic MT tomato may potentially be used as an antioxidant and for zinc supplementation.

KEYWORDS: Metallothionein; *Lycopersicon esculentum*; zinc; free radical; transgenic plant

INTRODUCTION

Tomatoes (*Lycopersicon esculentum*) are an important food in many parts of the world, with an attractive color and flavor and high nutrient content, and they are used both fresh and processed. High zinc content and high oxidant activity are highly desirable in foods. Zinc plays an important role in human development; nutritional zinc deficiency is a problem in many developing countries, especially among children (*1*).

Metallothioneins (MTs) are a family of low-molecular-weight (6–7 kDa), cysteine-rich, and metal-binding proteins, widely distributed in animals, plants, and microorganismd (*2*). They may be involved in the maintenance of trace metal homeostasis and detoxification of heavy metals in a wide variety of organisms (*3*). Through formation of thiol bonds by cysteine residues, MT may regulate essential intracellular zinc ion (Zn^{2+}) and copper ion (Cu^{2+}) availability for homeostatic reactions such as nucleic acid metabolism, protein synthesis, and other processes involved in tissue growth and regeneration (*4*, *5*), associated with cleavage of radicals and antiradiation and oxidation effects, hormone regulation, adaptation to stimuli, and also having a good effect in cancer therapy (*6*).

MTs are found in animal liver, induced by heavy metal or fermentation of mutant microorganisms. Transgenic plants with MT genes might be important in the future. Some transgenic plants with increased levels of MT have been produced through expression of foreign genes, such as tobacco (*7*), lettuce (*8*), and medlar (*9*). Foods with high content of MTs have potential as functional foods. However, little is known about the antioxidant activity, besides detoxification of heavy metal in transgenic MT plants.

In this study, we showed that expression of the *mt-I* gene from mouse, which encoded MT, could modulate MT synthesis ability in tomato plant, with resultant increases in levels of zinc content, superoxide dismutase (SOD) activity, and superoxide free radical scanvenging ability.

MATERIALS AND METHODS

MT Expression Vector. Plant expression vector pGPTVd35s-MT was constructed by Zhang et al. (*10*). It has resistant marker gene *NPT II* and selection marker *bar* gene (**Figure 1**). *Agrobacterium tumefaciens* EHA 105, was obtained from the Food Biotechnology Laboratory, China Agricultural University, was transformed with the resultant plasmid. *Bam*HI, *Xba*I, and *Sac*I were bought from Progema Co., a DIG CDP-Star kit was bought from Boehringer Mannheim (Mannheim, Germany), and polyclonal antibody MT-1H obtained from rabbit antiserum bought from Santa Cruz Biotechnology (Santa Cruz, CA).

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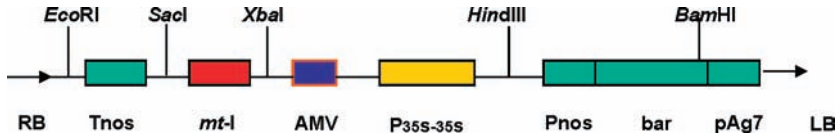

Figure 1. Structure of plant expression vector pGPTVd35s-MT. The construct carries the left and right borders (LB, RB) of the T DNA that demarcates the sequences normally incorporated into the plant genomic DNA via agrobacterium-mediated transformation. The *mt-I* gene lies downstream of the double CaMV 35S promoter and AMV enhancer and is followed by the nopline synthase gene (*nos*) terminator. This sequence is the plant's preferred sequence. The bar gene provides PPT resistance on the transformed plants.

Transformation of Tomato. Tomato (*Lycopersicon esculentum* cv. Yinfeng; Beijing Agricultural Technical Extension Center, Beijing, China) was transformed by agrobacterium-mediated gene transfer by a leaf-disc inoculation technique (*11*). Shoots regenerated on MS medium (*12*) supplemented with 1.0 mg/L *trans*-zeatin, 0.5 mg/L indoleacetic acid, 1.0 mg/L phosphinothricin (PPT), and 500 mg/ L carbenicillin (Cb) were transferred to rooting medium (MS medium supplemented with 50 mg/L kanamycin and 300 mg/L cefotaxime). Rooted transformants were grown to maturity in garden soil in a growth chamber under the white light from fluorescent lamps with 16 h of light daily at 25 °C. The transgenic plants showed the same fertility as non-transgenic plants. Selected transgenic plants were grown in garden soil (1 kg per pot) in a growth chamber as described above.

Polymerase Chain Reaction Analysis, Southern Blotting, and Northern Blotting. The extraction of plant DNA used in PCR was based on the procudure described by Shi et al. (*13*). The length of expected PCR amplification product is 200 bp. The forward and reverse primers used were 5′-GCTCTAGAAACAATGGACCCAAACTGCTCC-3′ and 3′-CGAGCTCGGATCCTCAGGCACAGCAAGTGCA-5′, respectively. Reaction conditions were as follows: initial PCR activation (95 °C, 3 min) was followed by 30 amplification cycles (denaturing, 94 °C, 1 min; annealing, 60 °C, 1 min; and extension, 72 °C, 1 min) and a final extension step at 72 °C for 10 min.

Total DNA for Southern hybridization was prepared from entire transgenic tomato and non-transgenic tomato leaves according to the CTAB method (*14*). After digestion of the DNA with appropriate endonuclease (*Xba*I and *Sac*I) and separation on a 1.2% agarose gel, Southern hybridization was performed with a DIG High-Prime Labeling and DIG CDP-Star kit, with the DIG-labeled *mt-I* gene from mouse as probe.

Total RNA (25μg) from transformed plants for Northern blotting was separated on a 1.2% formaldehyde/agarose gel, followed by Northern transfer and hybridization with DIG-labeled*mt-I* probe.

Western Blotting Analysis. Fresh leaves (0.5 g) were frozen in liquid nitrogen and then ground thoroughly in a mortar with 0.5 mL of buffer (50 mmol/L Tris-HCl, pH 7.5; 50 mmol/L NaCl; 400 mmol/L sucrose; 5 mmol/L EDTA; 1 mmol/L DTT), boiled 3 min, and centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was recovered, and the protein in it was isolated using 18% SDS–PAGE and then analyzed following the instruction manual, *Molecular Cloning* (*15*). The polyclonal antibody MT-1H (Santa Cruz Biotechnology) was antiserum from rabbit immunized by mouse liver MT.

Zinc Treatment and Zinc Content Analysis in Plants. When tomato plants grew 3–4 leaves, 0.2% $ZnSO_4 \cdot 7H_2O$ was sprayed every 7 days, three times, in the afternoon. Leaves at the same position were picked up from transgenic tomato and non-transgenic tomato and then digested with HNO_3 (*16*). The concentration of zinc was measured by polarography (*17*).

SOD Activity and Superoxide Anion Scavenging Rate. SOD was extracted from 1 g of frozen leaf ground in 5 mL of potassium phosphate buffer (pH 7.8) and then centrifuged at 12000*g* for 1 min. SOD activity was assayed by measuring its ability to inhibit the nitroblue tetrazolium (NBT) photochemical reduction, using the method described by Dhindsa et al. (*18*). One unit (1 U) of SOD was considered to be the amount of enzyme that inhibited NBT reduction by 50%. The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 63 μM NBT, 1.3 μM riboflavin, 1 μM EDTA, and 100 μL of enzyme extract. The tubes were placed under light for 10 min. The absorbance by the reaction mixture was read at 560 nm.

Measurement of superoxide anion scavenging activity of transgenic tomato and non-transgenic tomato leaf extracts was based on the method described by Liu et al. (*19*), with slight modification. Superoxide radicals are generated in phenazine methosulfate (PMS)–NADH systems by oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 mM) solution, 1 mL of NADH (78 mM) solution, and 1 mL of sample solution of tomato leaf extracts in water. The reaction was started by adding 1 mL of PMS solution (10 mM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. L-Ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ inhibition} = 100 - [(A_1/A_0) \times 100]$$

where A_0 was the absorbance of the control and A_1 was the absorbance of plant extracts of transgenic or non-transgenic tomato (*20*).

Statistical Analysis. Data were analyzed for significant differences by one-way analysis of variance (ANOVA) using the statistical software SPSS 11.5 for Windows (SPSS Inc., Chicago, IL). Significant effects were performed by employing Duncan's multiple comparison procedure, and differences at the 5% level were considered significant. The data presented are the means from three replications.

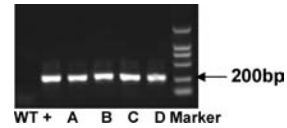

Figure 2. PCR amplification analysis of *mt-I* from transformed plants A–D. Genomic DNA was isolated from wild-type (WT) and transformed plants A–D. A 5 ng sample of pGPTVd35s-MT (containing *mt-I* plasmid) was used as a positive control (+).

RESULTS

Selection of Transgenic Plants. The plant expression vector pGPTVd35s-MT (**Figure 1**), with an inserted *mt-I* gene, was used to produce tomato transformants by agrobacterium-mediated transformation. Eight transformant plants were regenerated on selective MS medium, supplemented with 1.0 mg/L PPT and 500 mg/L Cb. Four independent transformants with strong growth trends, designated as A, B, C, and D, were selected for PCR analysis. All four transformants showed positive PCR results (**Figure 2**). The genomic DNA from transgenic and wild-type tomato plants' young leaves was digested with *Xba*I and *Sac*I and analyzed by Southern hybridization (**Figure 3**). Bands specific for the *mt-I* gene were detected in the analysis of transgenic A–D, but not of wild-type. Only fragments of 200 bp were observed in all *Xba*I and *Sac*I lanes.

Detection of *mt-I* mRNA in transformants was performed by Northern blotting analysis. Total RNA (25 μg) from transformed plants A–D was separated by 1.2% formaldehyde/agarose gel, and then the RNA was transferred and hybridized. Transformants A–C showed positive hybridization results, while D showed a negative result (**Figure 3**).

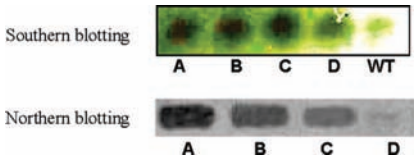

Figure 3. Integration of *mt-I* gene in transformants, analyzed by Southern and Northern blotting detection. Genomic DNA (10 μg) from wild-type (WT) and transformed plants A–D was restricted by *Xba*I and *Sac*I and hybridized with DIG-labeled *mt-I* probe for Southern blotting analysis. Total RNA (25 μg) from transformed plants A–D was separated by 1.2% formaldehyde/agarose gel, followed by Northern transfer and hybridization with DIG-labeled *mt-I* probe for Northern blotting analysis.

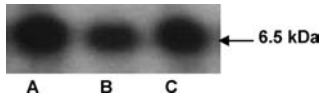

Figure 4. Western blotting detection of MT protein expression in transformed plants. Total soluble protein samples (50 mg) from the three transformed plants, A–C, were separated on 15% SDS–PAGE, transferred to poly(vinylidene difluoride) membrane, and developed with rabbit MT antibody.

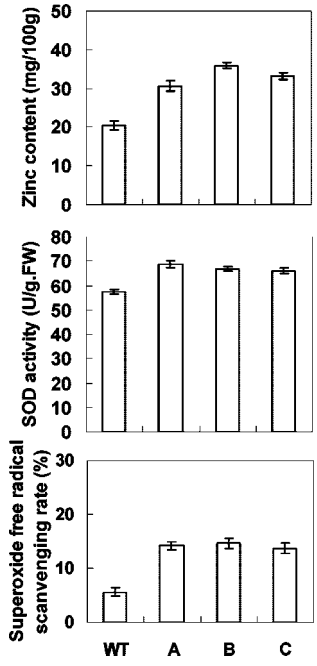

Figure 5. Zinc content, SOD activities, and superoxide free radical scanvenging rate of tomato leaf from wild-type (WT) and transformed plants A–C. Values are means ± SD ($n = 3$).

Only transformants with positive Northern blotting hybridization results were used in Western blotting analysis, which was performed to detect MT levels of *mt-I* expression in young leaves. A–C showed positive results (**Figure 4**), which indicated that *mt-I* gene was expressed successfully.

Assay of Zinc Content. One week after being sprayed three times with 0.2% $ZnSO_4 \cdot 7H_2O$, the leaves from transgenic and wild-type tomatoes were picked for analysis. **Figure 5** shows that the zinc content in the control was 20.4 mg/100 g FW, andin transformants A–C it was 30.6, 35.9, and 33.1 mg/100 g FW, respectively, significantly higher than that in wild-type ($p < 0.05$). The zinc contents in transformants A–C were not significantly different.

Assay of SOD Activity and Superoxide Anion Scavenging Rate. Differences in SOD activity in crude extracts were confirmed by measuring the rate of oxidation of NBT by spectrophotometry (**Figure 5**). Transgenic tomato lines A–C had significantly higher activities (68.6, 66.9, and 66.1 U/g FW), while a non-transgenic tomato plant line had lower activity (57.4 U/g FW). No difference was found among the three transgenic tomato plant lines. Superoxide anion scavenging rate was detected at a level of only 5.6% in non-transgenic tomato leaves, while transgenic lines A–C promoted superoxide anion scavenging approximately 1.5 times higher (14.2%, 14.6%, and 13.7%) than that in wild-type. The values among these three transgenic tomato leaves showed no significant difference (**Figure 5**).

DISCUSSION

MTs are a family of low-molecular-weight, cysteine-rich, metal-binding proteins and have a selective binding capacity for metals such as Cu, Zn, and Cd. Zinc is a necessary element in the growth of plants, and it is also important for trace elements supplementation in humans. Brown et al. (*1*) reported that slight zinc deficiency could cause some clinical symptoms, such as growth retardation and declining age of sexual maturity as well as reduced performance of sex glands, following some diseases such as diarrhea, pneumonia, malaria, and other infections. We developed transgenic tomato plants that expressed the *MT-I* gene from mouse for high content of MT in an effort to increase the zinc content in the plants. We obtained three independent transformants, and as shown in **Figure 5**, the levels of zinc content were much higher (about double or more) in transgenic tomato leaves than that in non-transgenic control. Similar results were found in our former experiment on lettuce (*8*), where we showed that transgenic $\beta\beta$-MT lettuce root could absorb higher levels of zinc, and specifically that the zinc content in transgenic lettuce root was 1.6 times higher than that in the root of control after spraying with $ZnSO_4 \cdot 7H_2O$.

SOD has been identified as an enzymatic protector against peroxidation reactions (*21*). It is an essential component of the antioxidative defense system in plants, and it dismutates two superoxide radicals to water and oxygen. Our results showed increased SOD activity in transgenic MT plants (**Figure 5**). It has previously been reported that SOD activity was increased under salinity (*22*), β-radiation (*23*), and heavy metal toxicity (*24*). Increase in SOD activity in response to stress appears to be probably due to de novo synthesis of the enzymic protein (*25*). Transgenic plants expressing SOD show increased tolerance toward oxidative damage caused by harsh environmental conditions. Among antioxidant enzymes, the level of SOD activity is of more relevance in maintenance of the overall defense system of plants subjected to oxidative stress (*26*). In our experiment, we found that the transgenic MT tomato plants had higher SOD activities (**Figure 5**), possibly because MT expression in the plants changed the metal content, and some metals, such as zinc ion and copper ion, are components of the catalytic active center of SOD. Other reactive oxygen species defense enzymes, such as catalase, peroxidase, and enzymes of the ascorbate/glutathione cycle, need to be analyzed in the future.

Some papers reported that MTs demonstrated antiradiation, free radical scavenging, and DNA damage protection abilities (*27–29*). The MTs in those studies were obtained from animal liver, induced by heavy metals. In this experiment, we developed transgenic tomato plants that expressed the *MT-I* gene and showed a high rate of free radical scavenging. The superoxide anion scavenging in transgenic tomato lines A, B, and C was 1.53, 1.61, and 1.45 times higher, respectively, than that in non-transgenic tomato leaves (**Figure 5**).

In conclusion, the experiments described here demonstrate that expression of the *MT-I* gene from mouse in *Lycopersicon*

esculentum increased the zinc content and elevated the SOD activity and superoxide anion scavenging rate in tomato plant leaves.

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