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Expression of Catalase (CAT) and Ascorbate Peroxidase (APX) in *MuSI* Transgenic Tobacco under Cadmium Stress

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The *MuSI* is known as a multiple stress resistant gene with several lines. A previous study using RT-PCR showed that the expression of *MuSI* gene in tobacco plant induced its tolerance to Cd stress. This study was conducted to examine the enhanced Cd tolerance of the *MuSI* transgenic tobacco plant through germination test and to understand the role of the involved antioxidant enzymes for the exhibited tolerance. Germination rate of *MuSI* transgenic tobacco was more than 10% higher than that of wild-type tobacco, and seedlings of *MuSI* transgenic tobacco grew up to 1.6 times larger and greener than seedlings of wild-type tobacco at 200 and 300 μM Cd. From the third to the fifth day, CAT activities at 100 and 200 μM Cd and APX activities at 100, 200 and 300 μM Cd of *MuSI* transgenic tobacco were up to two times higher than those of wild-type tobacco. *MuSI* gene is shown to enhance the activities of antioxidant enzymes resulting in higher tolerance to oxidative stress compared with the control plant.

Key words: *MuSI* transgenic tobacco, Cadmium resistance, Catalase (CAT), Ascorbate Peroxidase (APX)

Introduction

Soil contamination by human beings goes back as far as the Bronze Age (2500 B.C.) (Kabata-Pendias and Mukherjee, 2007). Early industrial activities such as mining and smelting of metalliferous ores, brick and pipe manufacturing as well as power generation and agricultural practices significantly contributed to soil contamination (Adriano, 1986; Cherian and Oliveira, 2005). Remediation of contaminated soil and water is a popular subject for both the scientific and the business communities. Phytoremediation is a promising soil remediation technique among many remediation techniques involving chemical, physical, biological and thermal means. One of the disadvantages of phyto-remediation is the small amount of biomass of most hyperaccumulators. A plant with greater biomass and deep root system would be ideal for phytoremediation. As such plants are rare, scientists try to create new plants with the help of genetic engineering.

The *MuSI* is a gene with tolerance to a number of stresses including dehydration, salt, heavy metal, oxidation, and plant hormones. Several of its lines are extracted from sweet potatoes (*Ipomoea batatas* L. cv. Yulmi) (Seo et al., 2010). A previous study using RT-PCR showed that the expression of *MuSI* gene in tobacco plant induced its tolerance to cadmium stress. Seo et al. (2010) also showed that the seedlings of *MuSI* transgenic tobacco germinated and grew better than the seedlings of wild-type tobacco at MS medium containing 50 and 100 μM Cd. Furthermore, in an experiment examining Cd tolerance of *MuSI* transgenic tobacco with a hydroponic system containing Cd, *MuSI* transgenic tobacco showed resistance to cadmium at 200 μM Cd (Kim, 2010). Damages appeared in leaves of the wild-type tobacco induced by Cd toxicity continued through whole growth period showing chlorosis and withering, while *MuSI* transgenic tobacco was recovering from the Cd damage 10 days after Cd exposure.

When plants were exposed to cadmium toxicity, superoxide radicals generated in cells, and they were deformed to H_2O_2 by superoxide dismutase (SOD) (Dixit et al., 2001; Schùtzendùble and Polle 2002; Markovska et al., 2009). H_2O_2 is removed by CAT in peroxisomes and by the ascorbate-glutathione cycle where APX reduces it to H_2O (Dixit et al., 2001; Mittler, 2002; Schùtzendùble et al., 2001; Vitoria et al. 2000). The objectives of this study were to determine

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enhanced Cd tolerance of *MuSI* transgenic tobacco through germination test and to understand the role of antioxidant enzymes for the Cd tolerance through hydroponic study.

Materials and Methods

Germination test This experiment was conducted with *MuSI* transgenic tobacco seeds and wild-type tobacco (control) seeds. MS mediums containing Cd at four different levels of concentration (0, 100, 200 and 300 μM) were prepared, and all four treatments were quintuplicated. Each treated medium was added in the petri dish (Φ10 cm), and 20 seeds were placed in the medium with even distribution. Then the petri dish was sealed with plastic wrap, and placed in the incubator at 25℃. The germinated seeds were counted 10 days after sowing. The growth parameters such as shoot/root length were determined for both group of tobacco plants 20 days after sowing.

Hydroponic culture Seedlings of *MuSI* transgenic tobacco and wild-type tobacco were cultured in a nutrient solution prepared according to Yamazaki's method (Yamazaki, 1982). The nutrient solution consisted of macro element (NO_3-N, NH_4-N, P, K, Ca, Mg and SO_4-S) and micro element (Fe, Cu, B, Mn, Zn and Mo), and was maintained at pH 5.8 and EC 1.2 dS m^{-1} during the cultivating period.

Prior to the exposure to Cd, each seedling was transplanted to separate hydroponic containers and was acclimatized for five days. After the acclimation, the nutrient solution in each container was changed with another nutrient solution containing Cd. These new nutrient solutions contained four different levels of Cd concentration at 0, 100, 200 and 300 μM. Then the seedlings were grown for seven days with replacement of the Cd solution twice (three days interval) using deep flow technique (DFT).

Antioxidant enzymes (CAT and APX) The method by Sawada et al. (2008) was used to observe the activities of CAT and APX. The leaf tissues (0.2 g FW) of *MuSI* transgenic tobacco and wild-type tobacco influenced by Cd exposure were ground with a mortar and pestle under chilled condition in homogenization buffer solution containing 25 mM potassium phosphate (pH 7.8), 1 mM ascorbic acid, 0.4 mM ethylene diamine tetra-acetic acid (EDTA) and 2% (w/v) polyvinylpoly-pyrrilidone. An aliquot (1.5 mL) of the homogenized mixture was centrifuged at 15,000 g for 20 min at 4℃. The supernatant was used for analyzing activities of CAT (EC 1.11.1.6) and APX (EC 1.11.1.11). The protein concentration of samples were measured by the Bradford's method (1976).

For the estimation of CAT activity, 0.1 ml of extraction solution was added to 1.9 ml of reaction buffer containing 50 mM potassium phosphate (pH 7.0) and 10 mM H_2O_2. And decomposition of H_2O_2 was determined at 240 nm for 1 min (Aebi, 1984).

For the estimation of APX activity, 0.1 ml of extraction solution was added to 1.88 ml of reaction buffer containing 25 mM potassium phosphate (pH 7.0), 0.25 mM ascorbic acid, 0.1 mM EDTA and 0.02 ml of 10 mM H_2O_2. And a decreased amount of the ascorbic acid was determined at 340 nm for 1 min (Nakano and Asada, 1981). The activities of CAT and APX and protein concentration of each sample were measured with a UV-visible spectro-photometer (UV-2500, Shimadzu, Japan).

Results and Discussion

Germination rate No differences in germination rate was observed between wild-type tobacco and *MuSI* transgenic tobacco in MS mediums with Cd levels of 0 and 100 μM. However, the germination rate of *MuSI*

Table 1. The component of nutrient solution according to Yamazaki's method.

Macro-element	NO_3-N	NH_4-N	P	K	Ca	Mg	SO_4-S
	---------- $cmol_c\ kg^{-1}$ ----------						
	6.0	0.5	1.5	4.0	2.0	1.0	1.0
Micro-element	Fe	Cu	B	Mn	Zn	Mo	
	---------- $mg\ kg^{-1}$ ----------						
	2	0.01	0.2	0.2	0.02	0.05	

Table 2. Germination rate of *MuSI* transgenic tobacco and wild-type tobacco at 10 days after germination on MS medium containing Cd. Values are expressed as the average ± standard deviation of five media from each treatment.

(unit: %)

Treatment level (μM)	0	100	200	300
Wild-type tobacco	90.7 ± 3.4	91.0 ± 1.9	70.0 ± 3.5	72.0 ± 3.4
MuSI transgenic tobacco	91.0 ± 3.2	90.0 ± 4.2	81.0 ± 4.3	88.0 ± 2.0

Table 3. Length of seedling shoot of *MuSI* transgenic tobacco and wild-type tobacco on MS medium containing Cd after one month from germination. Values are expressed as the average ± standard deviation of eight seedlings from each treatment.

(unit: cm)

Treatment level (μM)	0	100	200	300
Wild-type tobacco	2.36 ± 0.11	2.70 ± 0.10	0.85 ± 0.04	0.38 ± 0.02
MuSI transgenic tobacco	2.54 ± 0.04	2.46 ± 0.05	1.53 ± 0.04	0.55 ± 0.02

transgenic tobacco was more than 10% higher than that of wild-type tobacco in MS mediums with Cd levels of 200 and 300 μM (Table 2).

As Cd concentration increased, both tobaccos did not grow well. However, seedlings of *MuSI* transgenic tobacco grew 1.8 times (200 μM Cd) and 1.6 times (300 μM Cd) larger and greener than seedlings of wild-type tobacco after a month of sowing (Table 3; Fig. 1). This germination experiment confirmed that *MuSI* transgenic tobacco has higher tolerance to Cd than wild-type tobacco.

Activities of antioxidant enzyme In the previous hydroponic study, Kim (2010) showed both wild-type and *MuSI* transgenic tobaccos were initially damaged 2-3 days after Cd exposure. However, *MuSI* transgenic tobacco appeared to get recovered from Cd toxic symptom 10 days after Cd exposure while wild-type tobacco continued to be damaged through whole experiment period. During the one week antioxidant enzyme experiment, catalase (CAT) and ascorbate peroxidase (APX) activities of *MuSI* transgenic tobacco leaves increased more than those of wild-type tobacco leaves (Fig. 2 and Fig. 3). Between the third and the fifth day, CAT activity at 100 and 200 μM Cd and APX activity at 100, 200 and 300 μM Cd of *MuSI* transgenic tobacco were up to two times higher than those of wild-type tobacco. The experiment showed that the over-expression of *MuSI* gene enhanced antioxidant enzyme (CAT, APX) activities, which resulted in higher tolerance of *MuSI* transgenic tobacco to oxidative stress compared to the control plants (Fig. 2 and Fig. 3).

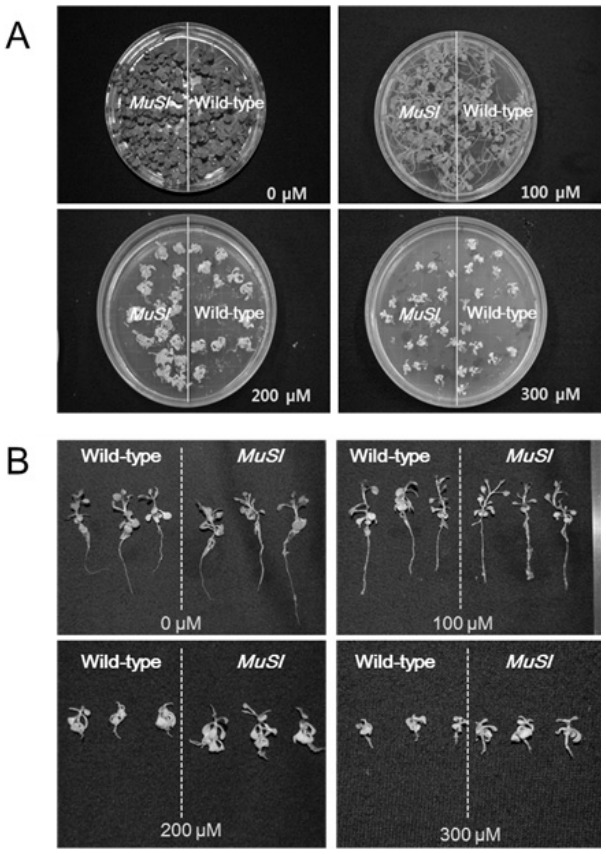

Fig. 1. Germination of *MuSI* transgenic tobacco and wild-type tobacco on MS medium containing cadmium (0, 100, 200 and 300 μM). (A) Comparison of germination for *MuSI* transgenic tobacco and wild-type according to Cd concentration at one month after germination. (B) Comparison of growth for *MuSI* transgenic tobacco and wild-type according to Cd concentration at one month after germination.

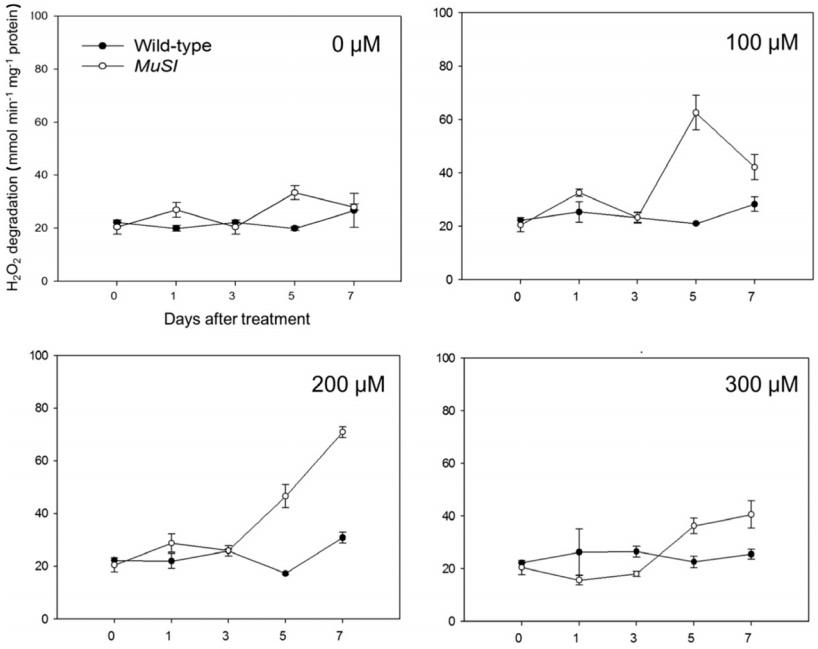

Fig. 2. Change of CAT activity in leaves of *MuSI* transgenic tobacco and wild-type tobacco treated with 0, 100, 200 and 300 μM. Values are expressed as the average ± standard deviation of three plant samples from each treatment.

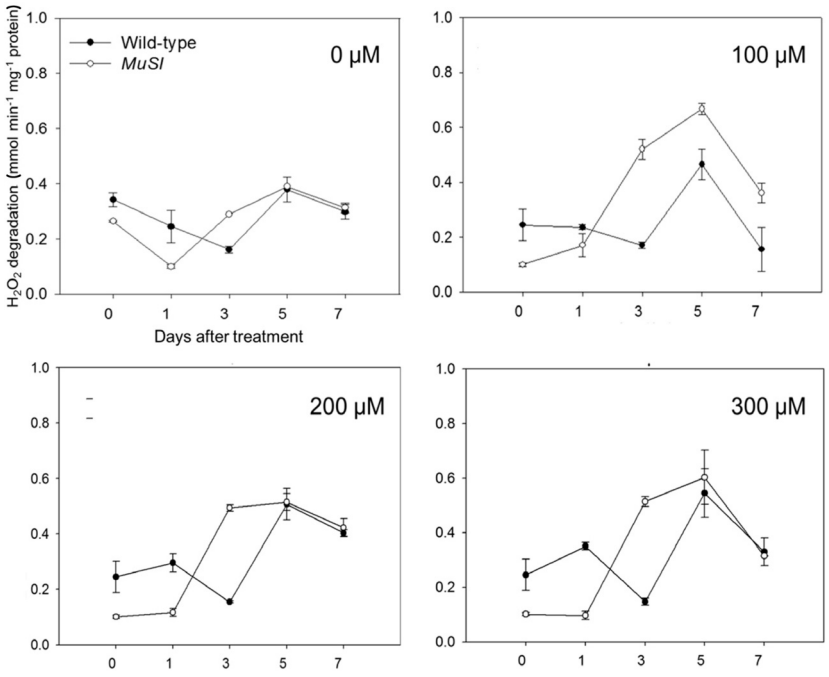

Fig. 3. Change of APX activity in leaves of *MuSI* transgenic tobacco and wild-type tobacco treated with 0, 100, 200 and 300 μM. Values are expressed as the average ± standard deviation of three plant samples from each treatment.

Conclusions

In conclusion, *MuSI* gene expressed tolerance to Cd stress. *MuSI* transgenic tobacco showed better visual growth under Cd treatment than wild-type tobacco. Also, *MuSI* transgenic tobacco induced more antioxidant enzymes such as catalase and ascorbate peroxidase compared to wild-type tobacco. The immediate mechanisms of *MuSI* transgenic plants for Cd tolerance such as phytochelatin and metallothionein should be identified through further studies. This study showed the potential of utilizing *MuSI* transgenic tobacco for phytoremediation of soils contaminated with Cd.

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