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Research Article

Resistance of SOD2-transgenic petunia line to oxidative stress

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Abstract SOD2-transgenic T_3 petunia line (A2-36-2-1-1-35) was treated with different levels of methyl viologen (MV) to determine its resistance to oxidative stress. Four (4) levels of MV (0, 100, 200, and 400 μM) were applied. The SOD2-transgenic T_3 petunia line exhibited a very significant oxidative stress resistance at the highest MV concentration (400 μM) treatment compared to non-transgenic plant. RNA and protein expression of SOD2 transgene and higher parenchyma cell density in the transgenic petunias exhibiting resistance to oxidative stress proves its contribution to the expression of its resistance to oxidative stress.

Keywords transgenic petunia, oxidative stress, resistance, methyl viologen (MV)

Introduction

It is necessary to develop new cultivar resistant to environmental stresses such as rainfall, humidity, and air pollution for petunia (*Petunia hybrida* L.), since it is one of the promising hedge garden flowers planted along the roads in Korea together with some pansy plants during spring to late summer. However, the difficulty to develop new cultivar resistant to abiotic stress through conventional breeding technique has led to the use of genetic transfer method to further improve plant resistance to abiotic stresses (Fang et al. 2002; Moon et al. 2003; Tang et al. 2004a, 2004b, 2007; Kim et al. 2005). Notably, McKersie et al. (1996) and Wang et al. (2005) reported that the transgenic MnSOD alfalfa and rice exhibited reduction of injury from water deficit and drought, respectively. In previous study, it was reported that SOD2 transgene inherited and expressed stably in T_1 and T_2 lines of SOD2 (MnSOD)-transgenic petunia plants (Lee et al. 2009a, 2009b). Meanwhile, success of the gene transfer to enhance resistance to abiotic stress needs to identify resistance of transgenic plants to oxidative stress. Many researchers had identified transgenic plants resistance to oxidative stress using methyl viologen (MV, paraquat) (Kim et al. 2005; Kwon et al. 2002; Lee et al. 2007; Lim et al. 2007; Moon et al. 2003; Tang et al. 2004, 2008), which induces treatment of superoxide anion (O_2^-), singlet oxygen, as well as hydroxyl and peroxy radicals (Kim et al. 1986; Suntres 2002). Thus, this study was conducted to determine the resistance of SOD2-transgenic T_3 petunia line to oxidative stress using different MV treatment concentrations.

Materials and methods

Plant materials

SOD2 transgenic (T_3 and T_4 lines) plants obtained from Wongyo A2-36 (purebred line of petunia) were used as plant materials. Only one (1) copy transfer of the transgene into SOD2-transgenic primary plants (T_0) was identified through Southern analysis in our previous study (Lee et al. 2009a). Likewise, integration of the SOD2 gene in the petunia genome at a single locus was identified through segregation ratio (1:0) of the transgene in T_1 and T_2 plants (Lee et al. 2009b).

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Treatment of MV on whole plant

The transgenic T_3 line seedlings grown for twenty days after being sown on plastic box with cultural medium (400 g) mixed with perlite (SungHyun Perlite Co., South Korea) and commercial compost, were transplanted to plastic pot (15 cm) with previous culture medium. After cultivation for 15 days in the greenhouse, about 3.75 mL of MV concentrations (0, 100, 200, 400 μM) dissolved in 20% acetone solution supplemented with 0.1% (w/v) Tween 20 was sprayed on the transgenic T_3 line (4 plants per treatment) using a sprayer. No damage was observed in the plants with the applications of 20% acetone solution. Each treatment was repeated three times. Injury of the transgenic plants was visually observed with a 0~100% rating table (0: no damage, 100: completely killed) by three researchers.

Western analysis

Two days after treatment of MV, total soluble proteins were extracted from leaves of the transgenic T_3 line plants treated with MV concentrations at 0, 100, 200, and 400 μM using distilled water. Twenty (20) μg of protein quantified through Bradford assay was mixed with 5X SDS-PAGE gel loading buffer and separated on 12.5% SDS polyacrylamide gel. The electrophoresed proteins were blotted onto a PVDF membrane with transfer buffer (Tris 25 mM, glycine 192 mM, SDS 0.1%, pH 8.3) by using a semidry electroblotter (Bio-Rad, U.S.A.). The membrane blot was incubated first in anti-SOD2 polyclonal antibody solution at a 1:500 dilution. After primary antibody incubation, the membrane was incubated again in alkaline phosphotase-conjugated goat anti-rabbit IgG (H+L) (KPL, U.S.A.) solution at a 1:2500 dilution, and then visualized by color metric detection (Promega, U.S.A.).

Northern analysis

Total RNA was isolated from leaves (100 mg) of the transgenic lines (T_3 and T_4) with the RNeasy Plant Mini Kit (QIAGEN, Germany) following the manufacturer's recommendations. T_4 line came from self-pollination of T_3 line which showed outstanding resistance to oxidative stress due to treatment of MV 400 μM based on the preceding experiment (MV treatment on whole plant). Quality and concentration evaluation of the isolated RNA were done by gel electrophoresis (1%, RNase-free) and spectrophotometer (260/280 nm).

Microscopic observation

A microscopic observation of leaf tissues of the SOD2-transgenic T_3 line plants showing symptom like necrosis was conducted seven days after spraying with MV 400 μM. Following Luft (1973), samples were prepared and stained with periodic acid staining (PAS) before viewing them with Axioskop 2 light microscope (Carl Zeiss Co., Germany).

Results and Discussion

Determination of oxidative stress resistance of SOD2-transgenic T_3 line

SOD2 transgenic T_3 line plants were treated with four (4) MV concentrations. In previous study, it was found that Wongyo A2-36 was more sensitive than any other control line (Lee and Han 2008). This means that the oxidative stress resistance of the SOD2 transgenic line from Wongyo A2-36 could positively be enhanced by over-expression of the transgene. As shown in Figure 1A to 1B, the leaf injury of the transgenic line caused by MV treatments was observed a day after the treatment and became severe thereafter. As previously reported by Kim et al. (2005) and Asada et al. (1977), generally an oxidative stress response in most plants varies according to their growth stage, younger leaf tissues were less damaged than the older ones. Minimal injury was observed in all treatments for the transgenic line compared to the control. The resistance to MV was identified at the second and fifth days as shown in Figure 1C to 1F. The transgenic line exhibited the least damage throughout the observation period. Based on these results, SOD2 gene appears to function under more extreme oxidative stress. Expectedly, the transgenic line exhibited lesser percent (%) damage/injury in all treatments compared to the control even during the 7^{th} day after treatment as shown in Table 1. Notably, the transgenic line revealed highly significant resistance to oxidative stress as shown by its minimal injury even at MV 400 μM treatment.

Microscopic observation of leaf necrosis in SOD2-transgenic T_3 line

Figure 2D shows leaf necrosis observed on the new shoots of transgenic T_3 line plants treated with MV 100 to 200 μM around 7 days after treatment. Microscopic observation revealed some variations between the leaf tissues of trans-

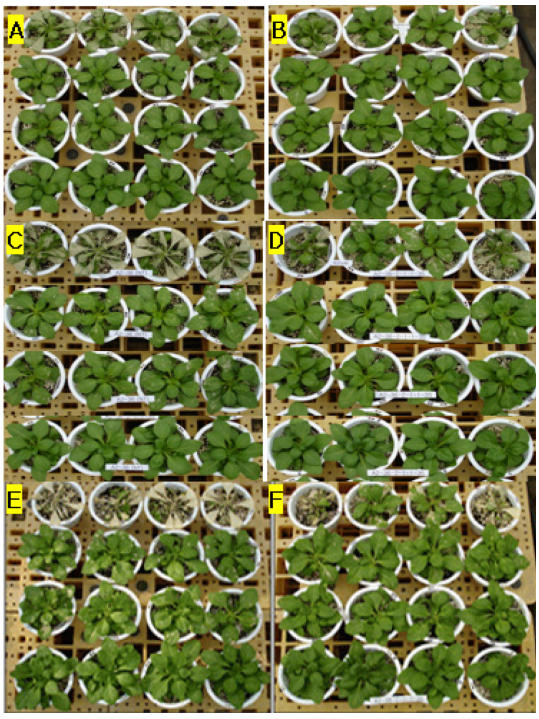

Fig. 1 Responses of SOD2-trangenic T_3 petunia line A2-36-2-1-1-35 one day (A and B), two days (C and D) and five days (E and F) after spraying different methyl viologen (MV) (paraquat) concentrations in the greenhouse. MV levels (0, 100, 200, and 400 μM) were sprayed starting from the bottom (0 μM) to the fourth row (400 μM) of the petunia lines, respectively. (A), (C), and (E) Wongyo A2-36 (non-transgenic plant); (B), (D) and (F) A2-36-2-1-1-35

genic and non-transgenic plants. Only a small number of or no cell (with chlorophyll) and normal conductive tissues were observed in the transgenic leaves treated with MV. Likewise, only few differences were observed between palisade parenchyma and spongy parenchyma, and there was little or no space between parenchyma cells, as shown in Figure 2E. The higher cell density due to this narrow space in the parenchyma cells contributed further enhancement of resistance to oxidative stress of the transgenic T_3 line plants as reported by Barth and Conklin (2003) and Konarska (2010). Meanwhile, the leaf necrosis symptom disappeared within fifteen days after treatment as shown in Figure 2F. The symptom disappearance at a reasonable period after treatment of MV suggested that the symptom is a marker of plant resistance under oxidative stress.

Protein synthesis and RNA expression of the transgene of SOD2-transgenic T_3 and T_4 lines

It was identified by Northern and Western blot analysis that SOD2 gene products expressed in the transgenic lines exhibiting highly significant resistance to oxidative stress compared to control. It was confirmed that the protein, which was not synthesized in the control, was fully synthesized in the leaves on the second day after being applied with the treatment of MV 400 μM (Fig. 3). Likewise, it was

Table 1 Injury (%) of SOD2-trangenic petunia T_3 line 7 days after spraying different methyl viologen (MV) (paraquat) concentrations in the greenhouse

Genotype	No. of transgenic plants	Visual injury (%)			
		MV 0 μM	MV 100 μM	MV 200 μM	MV 400 μM
A2-36-2-1-1-35	12	0 ± 0[z]	2.5 ± 2.0	4.5 ± 4.5	74.1 ± 18.1
Wongyo A2-36 (NT)	12	0 ± 0	12.5 ± 10.9	8.7 ± 4.3	98.3 ± 3.8

[z] Mean ± Standard deviation

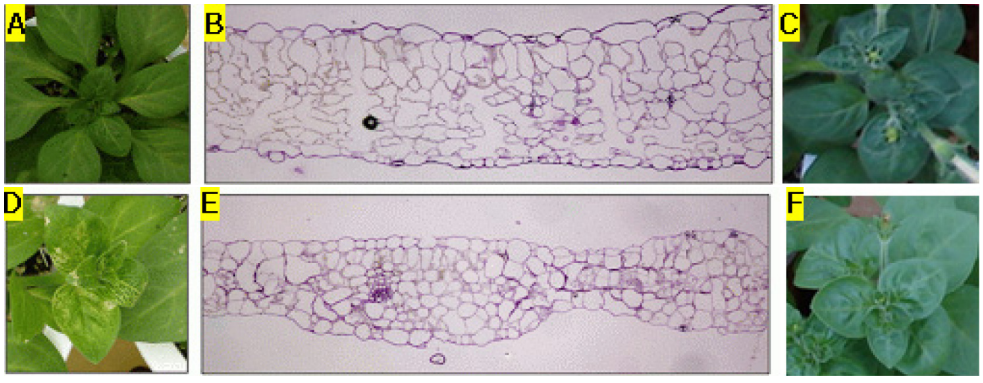

Fig. 2 Microscopic observation of non-transgenic petunia plant and SOD2-transgenic petunia T_3 plant leaf tissues showing necrosis 7 days after spraying with methyl viologen (MV) (paraquat) 100 to 200 μM. (A), (B) and (C): non-transgenic plants Wongyo A2-36; (D), (E), and (F): SOD2-transgenic petunia T_3 line A2-36-2-1-1-35 plants. (A) and (D) are non-transgenic line and A2-36-2-1-1-35 plants 7 days after treatment without and with MV, respectively. (B) and (E) are microscopic photos of (A) and (D), respectively. (C) and (F) showing plants 18 days after treatment without and with MV 400 μM, respectively

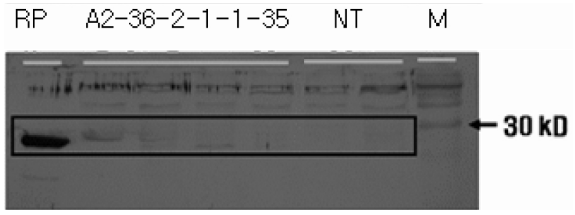

Fig. 3 Protein synthesis of transgene in SOD2-transgenic petunia T_3 line A2-36-2-1-1-35 plants 2 days after spraying methyl viologen (MV) (paraquat) 400 μM identified through Western blot analysis. RP: recombinant protein of transgene; NT: non-transgenic plants; M: protein molecular weight standards

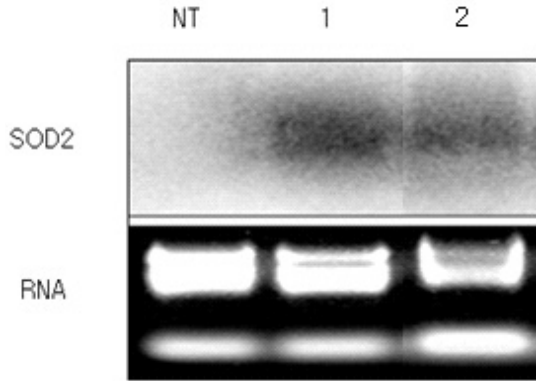

Fig. 4 Transgene expression in SOD2-transgenic petunia plants identified through Northern blot analysis. NT, non-transgenic plants; 1, A2-36-2-1-1-35 (T_3); 2, A2-36-2-1-1-35 (T_4)

revealed that the RNA of target gene in the transgenic T_3 line (not treated with MV) and the T_4 line (obtained from self-pollination of T_3 plant showing high resistance to MV 400 μM) was expressed (Fig. 4). These results confirmed that the resistance to oxidative stress in the transgenic generations was due to the activation of the SOD2 transgene.

Conclusion

This study revealed that SOD2-transgenic T_3 petunia line (A2-36-2-1-1-35) was resistant to oxidative stress induced with treatment of high MV concentration (400 μM) compared to non-transgenic plant. The expressed RNA and synthesized protein were determined. Furthermore, the RNA of the transgene was expressed even in the T_4 plants obtained from self-pollination of T_3 plants which showed high resistance even under treatment of MV 400 μM. Therefore, the SOD2-transgenic petunia line exhibiting resistance to oxidative stress is considered promising genetic material for breeding new petunia cultivar resistant to abiotic stress.

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