Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 140: 153–162. 2010

Enhanced tolerance to methyl viologen-induced oxidative stress and high temperature in transgenic potato plants overexpressing the *CuZnSOD*, *APX* and *NDPK2* genes

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Received 26 March 2010;
revised 2 June 2010

doi:10.1111/j.1399-3054.2010.01392.x

Oxidative stress is a major threat for plants exposed to various environmental stresses. Previous studies found that transgenic potato plants expressing both copper zinc superoxide dismutase (*CuZnSOD*) and ascorbate peroxidase (*APX*) (referred to as SSA plants), or nucleoside diphosphate kinase 2 (*NDPK2*) (SN plants), showed enhanced tolerance to methyl viologen (MV)-induced oxidative stress and high temperature. This study aimed to develop transgenic plants that were more tolerant of oxidative stress by introducing the *NDPK2* gene into SSA potato plants under the control of an oxidative stress-inducible peroxidase (*SWPA2*) promoter to create SSAN plants. SSAN leaf discs and whole plants showed enhanced tolerance to MV, as compared to SSA, SN or non-transgenic (NT) plants. SSAN plants sprayed with 400 μ*M* MV exhibited about 53 and 83% less visible damage than did SSA and SN plants, respectively. The expression levels of the *CuZnSOD, APX* and *NDPK2* genes in SSAN plants following MV treatment correlated well with MV tolerance. SOD, APX, NDPK and catalase antioxidant enzyme activities were also increased in MV-treated SSAN plants. In addition, SSAN plants were more tolerant to high temperature stress at 42°C, exhibiting a 6.2% reduction in photosynthetic activity as compared to plants grown at 25°C. In contrast, the photosynthetic activities of SN and SSA plants decreased by 50 and 18%, respectively. These results indicate that the simultaneous overexpression of *CuZnSOD, APX* and *NDPK2* is more effective than single or double transgene expression for developing plants with enhanced tolerance to various environmental stresses.

Introduction

Plants are frequently exposed to diverse environmental stresses, including salt, drought and extreme temperature. Exposure to such abiotic stresses can give rise to the excessive accumulation of reactive oxygen species (ROS) in plant cells (Asada 1999, Foyer et al. 1994). ROS are potentially harmful, as they can cause oxidative damage in cellular structures and functions. Plants have several defense mechanisms involving antioxidative enzymes

Abbreviations – APX, ascorbate peroxidase; CAT, catalase; Chl, chlorophyll; *CuZnSOD*, copper zinc superoxide dismutase; FeSOD, iron superoxide dismutase; MAPK, mitogen-activated protein kinase; MS, Murashige and Skoog; MV, methyl viologen; NBT, nitro blue tetrazolium; NDPK, nucleoside diphosphate kinase; NT, non-transgenic; POD, peroxidase; PSII, photosystem II; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase.

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and low molecular weight antioxidants that protect them against oxidative stress caused by excess ROS accumulation.

Among antioxidative enzymes, superoxide dismutase (SOD) and ascorbate peroxidase (APX) play key roles in intracellular ROS scavenging. SOD, the first enzyme in the ROS detoxifying process, converts superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2). APX reduces H_2O_2 to water using ascorbic acid as a specific electron donor. Chloroplasts, the major cellular component of photosynthetic plant tissue, are highly exposed to ROS damage, which is frequently generated by the reaction of chloroplast O_2 and electrons that escape from the photosynthetic electron transfer system (Foyer et al. 1994). To cope with oxidative damage, chloroplasts employ ROS-scavenging enzymes such as copper zinc SOD (CuZn-SOD) and APX (Kwon et al. 2002). The role of CuZnSOD and APX at the onset of oxidative stress in chloroplasts has been extensively characterized (Asada 1999).

Protein kinases also control the expression of many target genes including antioxidant genes by signal transduction cascades (Kobayashi et al. 2004, Moon et al. 2003, Tena et al. 2001). Plant nucleoside diphosphate kinase (NDPK) is an ubiquitous housekeeping enzyme that maintains the intracellular levels of all nucleoside triphosphates in biosynthesis, except that of ATP. The NDPK2 protein plays a significant role in signal transduction pathways involved in oxidative stress; thus, it can regulate the expression of genes involved in antioxidant such as SOD, APX, catalase (CAT), thioredoxin, thioredoxin reductase and glutathione S-transferase and protective functions (Moon et al. 2003, Yang et al. 2003).

We previously isolated the oxidative stress-inducible *SWPA2* peroxidase (POD) promoter from sweetpotato cell cultures and characterized its function in transgenic tobacco plants in response to environmental challenges including oxidative stress (Kim et al. 2003). In addition, transgenic plants expressing both *CuZnSOD* and *APX* in chloroplasts under the control of the *SWPA2* promoter (referred to as SSA plants) showed enhanced tolerance to methyl viologen (MV)-induced oxidative stress and other abiotic stresses (Lee et al. 2007, Lim et al. 2007, Tang et al. 2006). Also, transgenic plants expressing *Arabidopsis NDPK2* under the control of the *SWPA2* promoter (referred to as SN plants) showed increased tolerance to MV, salt and temperature stress (Kim et al. 2009, Tang et al. 2008). The development of transgenic plants with enhanced tolerance to multiple environmental stresses may entail the expression of several antioxidative enzymes in a gene-stacking manner. In this study, we show that the simultaneous expression of *CuZnSOD*, *APX* and *NDPK2* under the control of the *SWPA2* promoter in transgenic potato plants confers greater tolerance to MV-induced oxidative stress and to high temperature.

Materials and methods

Plant materials and transformation

Transgenic potato (*Solanum tuberosum* cv. Atlantic) plants simultaneously expressing *CuZnSOD*, *APX* and *NDPK2* were generated by the transformation of SSA plants (Tang et al. 2004, 2006) with a vector harboring the *Arabidopsis NDPK2* gene under the control of the *SWPA2* promoter using the hygromycin phosphotransferase (*hpt*) gene as a selectable marker (Fig. 1). Transgenic potato plants selected with hygromycin (25 mg l^{-1}) on MS (Murashige and Skoog 1962) agar medium were grown at 25°C with a 16-h photoperiod.

Ion leakage analysis of leaf discs

Ion leakage was detected according to the method of Bowler et al. (1991), with modifications. Fifteen leaf discs (1.0-cm diameter) from fully expanded leaves of plants grown for 2 months were floated on a solution containing 0.4% (w/v) sorbitol and 3 μ*M* MV, placed in the dark for 12 h to allow diffusion of the MV into

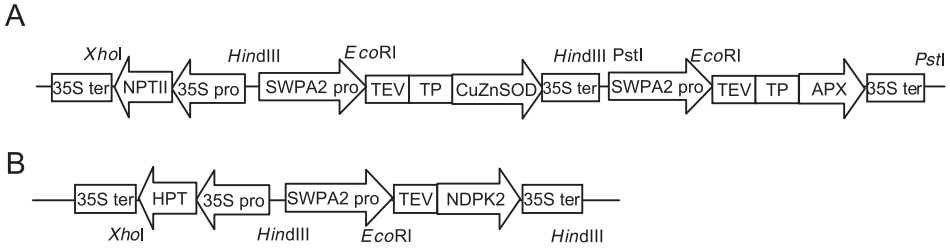

Fig. 1. Schematic presentation of the pSSA-K (A) and pSN-H (B) expression vector. SWPA2 Pro, sweetpotato peroxidase promoter; TEV, tobacco etch virus untranslated region; TP, chloroplast-targeted transit peptide; CuZnSOD, cassava copper–zinc superoxide dismutase; APX, pea ascorbate peroxidase; NDPK2, Arabidopsis nucleoside diphosphate kinase 2; 35S ter, CaMV 35S terminator; 35S Pro, cauliflower mosaic virus 35S promoter; NPTII, neomycin phosphotransferase; HPT, hygromycin phosphotransferase.

the leaf discs and then transferred to continuous light (150 μmol m^{-2} s^{-1}) at 25°C. The loss of cytoplasmic solutes following MV treatment based on the electrical conductance of the solution was measured with an ion conductivity meter (model 455C; Istek Co., Seoul, Korea) and compared with the total conductivity of the solution following tissue destruction.

Methyl viologen and high-temperature treatment of whole plants

MV (0, 200, 300 or 400 μ*M*) dissolved in 0.1% (w/v) Tween 20 solution was sprayed on the leaves of 6-week-old plants using a spray booth (Model SB-6; DeVries Manufacturing, Hollandale, MN). MV solution (70 ml) was applied to five potato plants using an 8001 VS type nozzle, 0.5 inch s^{-1}, 0.22 MPa. Tests for visible damage caused by MV were repeated in triplicate. Leaf damage caused by MV was evaluated 5 days after treatment and expressed as percentage (0% indicates no damage; 100% means fully damaged leaves).

For high temperature stress, 6-week-old potato plants growing at 25°C were transferred to 42°C for 24 h in a growth chamber. The treated plants were then transferred to normal conditions (25°C, 100 μmol m^{-2} s^{-1}) for recovery.

Photosynthetic efficiency and total chlorophyll content measurement

The photosystem II (PSII) photosynthetic efficiency of MV-treated potato leaves was estimated by chlorophyll (Chl) fluorescence determination of photochemical yield (Fv/Fm), which represents the maximal yield of the photochemical PSII reaction, by using a portable Chl fluorescence meter (Handy PEA, Hansatech, King's Lynn, UK). Chl content was measured with 0.1 g fresh weight leaf materials that were quickly frozen in liquid nitrogen and then extracted with 2 ml of methanol. Samples were centrifuged at 12 000 *g* for 15 min at 4°C, and Chl in supernatants was analyzed using a spectrophotometer according to Porra et al. (1989).

RT-PCR analysis

Total RNA was isolated from 300 μ*M* MV-treated potato leaves with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated extensively with RNase-free DNase I. RT-PCR amplification was conducted using an RT-PCR kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. Total RNA (1 μg) was utilized to generate first-strand cDNA using MMLV reverse transcriptase. PCR primers used for PCR of transgenes (CuZnSOD, APX, NDPK2) in SSAN plants and potato endogenous SOD, APX and CAT were represented in Table 1. The actin gene was also amplified as an internal standard using actin gene-specific primers.

Table 1. Primer sets used for PCR analysis of transgenes and endogenous genes in NT and transgenic potato plants.

Gene	Forward	Reverse
Transgene		
CuZnSOD	atggtgaaggctgaagctgttctt	ctatcctcgcaaaccaataccg
APX	atgggaaaatcttacccaactgtta	ttaggcttcagcaaatccaagctc
NDPK2	gttggccgcatttcgtcctca	ccacttgcatagctcgccctc
Endogenous gene		
FeSOD	tgctgaaaatcctcttgttttggg	ttcggttttctcgctaagcagg
CuZnSOD	tggaagagctgttgttgttcatgc	aaggctcagccgctccaataaa
APX	tttccgcctccttgttgagaaa	tccaacaactccagcgagcttt
CAT	caagaaaatgttcgcgttgtgc	ttgatgatgttcttcaagcaccaaa
Actin	tggactctggtgatggtgtgc	cctccaatccaaacactgta

Enzyme assays

For analysis of SOD, APX, NDPK, POD and CAT activities, total soluble protein was extracted from MV-treated (300 μ*M*) leaves of potato plants, and protein concentrations were determined with the Bio-Rad protein assay kit (Bradford 1976). SOD activity was measured according to the method of McCord and Fridovich (1969) using xanthine, xanthine oxidase and cytochrome *c*. One unit of SOD was defined as the amount of enzyme that inhibits the rate of ferricytochrome *c* reduction by 50%. APX activity was assayed according to the method of Nakano and Asada (1981), using ascorbic acid as a substrate. Ascorbate oxidation was initiated by H_2O_2, and the decrease at 290 nm was monitored for 1.5 min. One unit of APX activity was defined as the amount of enzyme necessary to oxidize 1 μ*M* ascorbate. NDPK activity was measured using the coupled reaction method with lactate dehydrogenase and pyruvate kinase (Tang et al. 2008, Yano et al. 1995). NDPK activity was calculated based on the reduction at 340 nm following a decrease in NADH. One unit of enzyme activity was defined as 1 μmol ADP production per minute. CAT activity was assayed according to the method of Aebi (1984). The activity was determined by the decrease at 240 nm for 1 min as a result of H_2O_2 consumption.

Native PAGE

Native PAGE of SOD, APX and CAT were performed on a 7.5% gel at 120 V at 4°C (Beauchamp and Fridovich 1971). For SOD activity staining, the gel was incubated in the dark for 30 min in staining buffer [50 m*M* potassium phosphate buffer, pH 7.8, 0.026 m*M* riboflavin,

0.25 m*M* nitro blue tetrazolium (NBT), 0.2% TEMED] and then exposed to a light box until the SOD activity bands became visible. The SOD isozymes were differentiated by incubating the gel for 20 min in 50 m*M* potassium phosphate buffer (pH 7.8) containing either 3 m*M* KCN or 5 m*M* H_2O_2 before staining for activity. CuZnSODs were inhibited by KCN and H_2O_2; iron superoxide dismutase (FeSODs) were resistant to KCN but were inactivated by H_2O_2; MnSODs were resistant to both inhibitors (Beauchamp and Fridovich 1971). APX isozymes were detected by equilibrating the gels in a solution composed of 50 m*M* sodium phosphate buffer (pH 7.0) containing 2 m*M* ascorbate for 30 min, and then 50 m*M* sodium phosphate buffer (pH 7.0) containing 4 m*M* ascorbate and 2 m*M* H_2O_2 for 20 min. After washing with 50 m*M* sodium phosphate buffer (pH 7.0) for 1 min, gels were submerged in a solution of 50 m*M* sodium phosphate buffer (pH 7.8) with 28 m*M* TEMED and 1.25 m*M* NBT for 10 min at room temperature (Mittler and Zilinskas 1994). CAT isozymes were detected by native gel in a solution composed of 50 m*M* potassium phosphate buffer (pH 7.0) containing horseradish POD (50 $\mu g\,ml^{-1}$) for 45 min. H_2O_2 was then added to a concentration of 5 m*M* and were soaked for 10 min. The gels were then rapidly rinsed twice with water and were placed into 0.5 $mg\,ml^{-1}$ of diaminobenzidine (DAB) in the phosphate buffer until staining was completed (Clar et al. 1984).

Statistical analysis

Data were analyzed with the Statistical Package for the Social Sciences (SPSS 12). Means were compared using Duncan's multiple range test at $P = 0.05$.

Results

Generation of transgenic potato plants

Transgenic potato plants simultaneously expressing the *CuZnSOD*, *APX* and *NDPK2* genes were generated by the transformation of SSA plants with the *NDPK2* gene under the control of the *SWPA2* promoter (Fig. 1). Fourteen independent transgenic lines were established and propagated in a growth chamber. Genomic integration and the copy numbers of the constructs in transformed plants were determined by PCR with *CuZnSOD*, *APX*, *NDPK2* and *HPT* gene-specific primers (data not shown), then analyzed further by Southern blot analysis using a *NDPK2*-specific probe (data not shown). Among the 14 transgenic lines,6 had an insertion of a single copy, and the others had multiple copies. SSAN1 and SSAN2 lines showed single copy of NDPK2 gene and SSAN3 plant had three copies.

Fourteen transgenic lines were utilized to evaluate the tolerance of MV-induced oxidative stress using leaf discs. MV is a typical ROS-generating redox-active compound, which has been used as a non-selective herbicide (Babbs et al. 1989). Leaf discs were incubated with 3 μM MV under illumination, and the loss of cytoplasmic solutes was followed by monitoring the electrical conductance of the solution. Ion leakage was assessed over time, and the 14 lines evidenced enhanced tolerance, as shown by lower levels of ion leakage (data not shown). Among these lines, three (SSAN1, 2 and 3) exhibiting the highest observed tolerance were selected for further characterization.

Increased tolerance of transgenic potato to MV-induced oxidative stress

SSAN1, 2 and 3 were utilized to evaluate the enhanced tolerance against MV-induced oxidative stress in leaf discs and at the whole plant level. Non-transgenic (NT), SSA (Tang et al. 2006) and SN (Tang et al. 2008) potato plants were used as experimental controls. Different extents of visible damage were observed in leaf discs from transgenic and NT plants exposed to MV-induced oxidative stress (Fig. 2A). Severe necrosis was observed in NT leaf discs in 24 h following treatment with 3 μM MV, and those of SN and SSA plants exhibited partial necrosis, whereas those of SSAN plants showed no damage. After exposure to MV, the leaf discs of SSAN plants showed relatively less membrane damage compared to those of NT, SN and SSA plants (Fig. 2B). At 48 h following 3 μM MV treatment, the leaf discs of NT plants exhibited nearly complete cellular disruption (about 82% of the maximum solute leakage), whereas those of the three SSAN plants had approximately 40% less membrane damage than did their NT counterparts.

To investigate the tolerance of transgenic plants to oxidative stress at the whole plant level, NT and transgenic plants were evaluated for visible damage 5 days after being sprayed with solutions containing 0, 200, 300 or 400 μM MV. NT plants showed significant leaf damage that correlated with MV concentration. However, transgenic plants showed reduced symptoms of damage compared to NT plants. The three SSAN plants were almost unaffected, even under high MV concentrations (Fig. 3A). NT, SN, SSA and SSAN leaves were evaluated for damage 5 days after MV treatment (Fig. 3B). SSAN plants showed higher tolerance to MV-induced oxidative stress than did NT and SN plants sprayed with 300 μM MV. After treatment with 400 μM MV, NT plants had 62% visible leaf damage, whereas SSAN plants had less than 10% visible damage, and the values for SN and SSA plants were 37 and 13%,

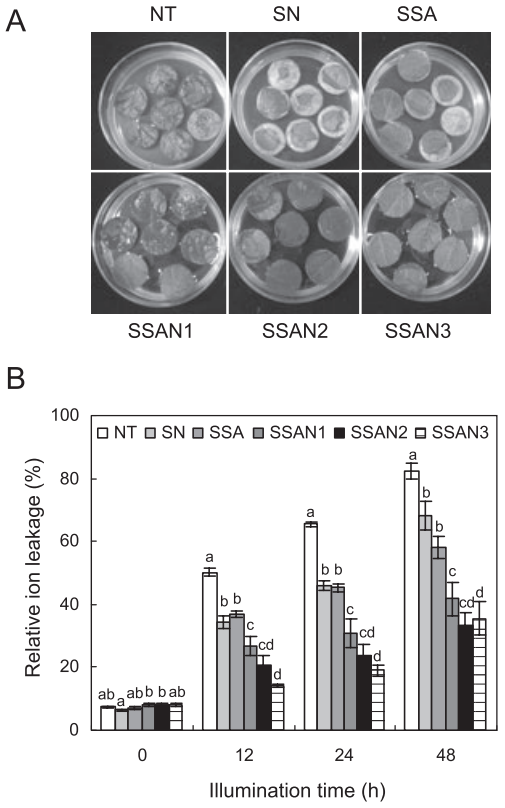

Fig. 2. Enhanced MV-mediated oxidative stress tolerance in the leaf discs of SSAN transgenic potato plants. (A) Visible differential damage of leaf discs in the NT and transgenic plants (SN, SSA and SSAN1–3) 36 h after 3 μ*M* MV treatment. (B) Analysis of cellular damage in the MV-treated leaf discs of transgenic plants by electrolyte leakage for 48 h. Data are the means of three replicates, with each replicate consisting of seven leaf discs. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

respectively. In addition, the SSAN transgenic lines exhibited greater MV tolerance as assessed by PSII photosynthetic efficiency and total chlorophyll content (Fig. 3C, D). The above results indicate that SSAN transgenic plants are more tolerant to MV-induced oxidative stress than NT, SN and SSA plants.

The enhanced tolerance of SSAN transgenic potato plants to oxidative stress correlates with an increase in activities of antioxidative enzymes

To understand the tolerance mechanism that is active in SSAN plants following MV-induced oxidative stress, we performed RT-PCR to determine temporal changes in the expression of the *CuZnSOD, APX* and *NDPK2* transgenes following treatment with 300 μ*M* MV. As shown in Fig. 4A, the expression of *CuZnSOD* and *APX* increased in SSA and SSAN plants, whereas that of *NDPK2* gene also increased in SN and SSAN plants

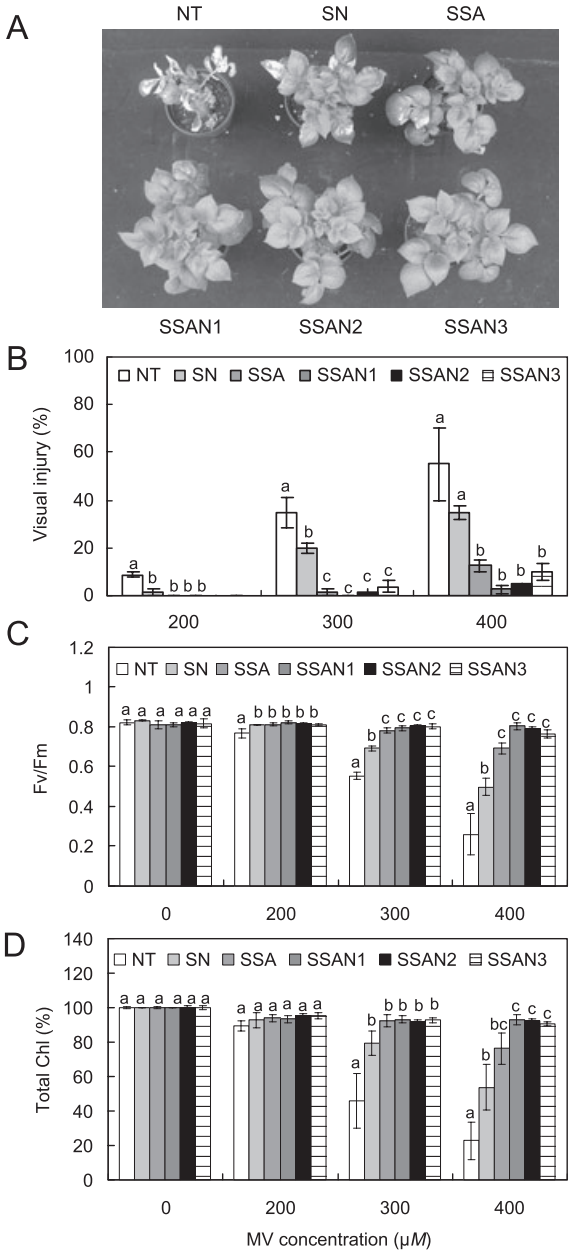

Fig. 3. Enhanced MV-mediated oxidative stress tolerance in the whole plant levels of SSAN transgenic potato plants. (A) Differential visible damages in the leaves of NT and transgenic (SN, SSA and SSAN1–3) plants at 5 days after treatment with 400 μ*M* MV. (B) Quantitative estimate of visible damage that appeared on leaves from NT and transgenic SN, SSA and SSAN plants by treatment with 0, 200, 300 and 400 μ*M* MV. (C) PSII photosynthetic efficiency in MV-treated NT and transgenic plants was analyzed with a chlorophyll fluorescence meter. Fv/Fm is a relative chlorophyll fluorescence showing PSII photosynthetic efficiency. Fv is the total amount of variable fluorescence and Fm is the maximum fluorescence yield. (D) Total chlorophyll contents in MV-treated transgenic plants were analyzed. Data represent the average of three replicates, each from five plants. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

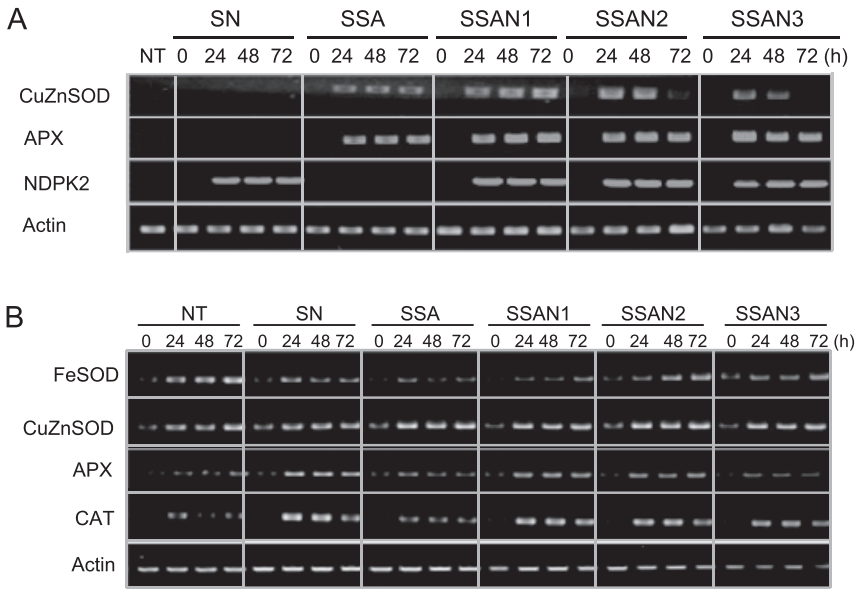

Fig. 4. RT-PCR analysis of NT and transgenic (SN, SSA and SSAN) potato plants subjected to 300 μ*M* MV treatments for 72 h. (A) The expressions patterns of *CuZnSOD*, *APX* and *NDPK2* transgenes in leaves from NT and transgenic (SN, SSA and SSAN1–3) potato plants subjected to 300 μ*M* MV treatments. (B) The expressions patterns of potato endogenous *FeSOD*, *CuZnSOD*, *APX* and *CAT* genes in leaves from NT and transgenic (SN, SSA and SSAN) potato plants subjected to 300 μ*M* MV treatments. Total RNA was extracted from leaves 0, 24, 48 and 72 h after treatment with MV. Actin was used to control for equal loading.

in response to MV exposure. However, the *CuZnSOD*, *APX* and *NDPK2* transgene transcripts were not detected in NT and untreated transgenic potato plants. We also investigated the expression of endogenous antioxidant genes in potato leaves subjected to 300 μ*M* MV (Fig. 4B). RT-PCR analyses clearly demonstrated that the expression of endogenous *APX* and *CAT* genes in the SN and SSAN plants showed higher levels than those in the NT and SSA plants under MV treatment. However, expression patterns of endogenous *FeSOD* and *CuZnSOD* showed similar levels in both the NT and transgenic lines. In addition, endogenous *SOD*, *APX* and *CAT* genes in potato were weakly expressed in untreated plants unlike transgenes.

For analysis of enzyme activity levels, the SOD, APX, NDPK and CAT activities in NT and transgenic plants (SSAN, SSA and SN) following treatment with 300 μ*M* MV with time course were also investigated (Fig. 5). The activity levels of SOD and APX in NT and transgenic plants increased following MV treatment; in particular, SOD and APX activities in SSAN plants underwent dramatic increases (Fig. 5A, B). SSA plants also showed high levels of SOD and APX activities after MV exposure. NDPK activity in SSAN plants increased markedly until 72 h after MV treatment, whereas that in NT and SSA plants slightly increased 48 h after MV spraying (Fig. 5C). The pattern of CAT activity in NT and transgenic plants was similar to that of NDPK activity (Fig. 5D). In parallel with NDPK activity, CAT activity was higher in SSAN and SN plants than in SSA and NT plants. We also investigated the antioxidant enzyme activity after 300 μ*M* MV treatment using native gel electrophoresis (Fig. 6). Native gel assay showed that enzyme activities of SOD, APX and CAT in transgenic plants increased after stress treatment, whereas the isoenzyme patterns were similar in NT and transgenic plants in untreated condition. SSA and SSAN plants showed six SOD and two APX isoenzymes including novel band of each transgene, a bold arrow-headed band, which were not found in NT and SN plants. Activity of CAT was high in SN and SSAN plants compared to NT and SSA, similar to the changes in enzyme activity shown in Fig. 5. Isoenzymes showed an increasing pattern after MV treatments in transgenic plants compared to the NT plants, indicating that the transgene somehow interacted with the endogenous potato isoenzymes under stress condition. These results suggest that protection against MV-induced oxidative stress can be ascribed to the elevated activities of the antioxidative enzymes SOD, APX and CAT in SSAN plants.

Enhanced tolerance of SSAN plants to high temperature stress

The Atlantic potato cultivar is sensitive to high temperature. Thus, it would be of interest to generate

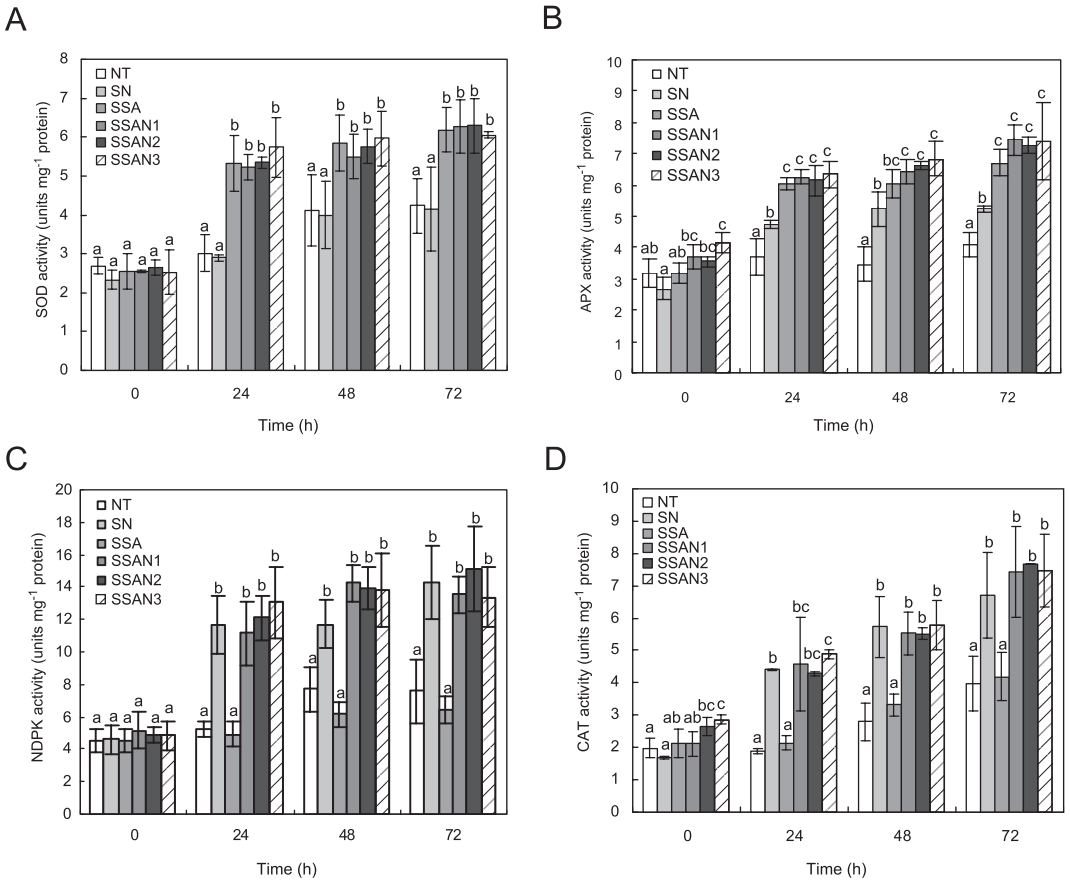

Fig. 5. Changes in activities of various antioxidant enzymes in leaves from NT and transgenic (SN, SSA and SSAN1–3) potato plants after 300 μ*M* MV treatment. Changes of specific activities of SOD (A), APX (B), NDPK (C) and CAT (D) in the NT and transgenic plants after MV treatments. Data represent the average of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

potato plants with a greater heat tolerance. Accordingly, we tested whether SSAN plants have an increased tolerance to heat stress, because SSA and SN plants exhibited this phenotype (Tang et al. 2006, 2008). Soil-grown SSAN plants were incubated for 24 h at 42°C and then returned to 25°C. After this treatment, the leaves of NT and SN plants were severely wilted and curled, whereas SSA plants were only slightly damaged and SSAN plants appeared to be healthy (Fig. 7A). We also monitored photosynthetic activity in potato plants (Fig. 7B). Twenty-four hours after exposure to high temperature, the photosynthetic efficiency (Fv/Fm) of SSAN plants decreased by 8%, whereas that of NT and SN plants decreased by 88 and 50%, respectively. Furthermore, the photosynthetic efficiency of SSAN plants that were allowed a 12-h recovery was almost that of initial levels, whereas NT plants exhibited a decrease in Fv/Fm to the lowest recorded level. These results suggest that SSAN potato plants are more resistant than NT, SN and SSA plants to high temperature stress as a result of the overexpression of *CuZnSOD*, *APX* and *NDPK2*.

Discussion

Overexpression of antioxidant enzymes in plants has been shown to enhance their tolerance to oxidative stress (Mittler 2002). Our previous studies reported that transgenic SSA plants (potato, tall fescue and sweet potato) overexpressing both *CuZnSOD* and *APX* showed a synergistic effect with regard to oxidative stress defense (Lee et al. 2007, Lim et al. 2007, Tang et al. 2006). In addition, transgenic SN plants (*Arabidopsis*, potato and sweetpotato) overexpressing *NDPK2* exhibited enhanced tolerance to environmental stresses (Kim et al. 2009, Moon et al. 2003, Tang et al. 2008). In this study, we successfully developed transgenic potato plants simultaneously expressing *CuZnSOD*, *APX* and

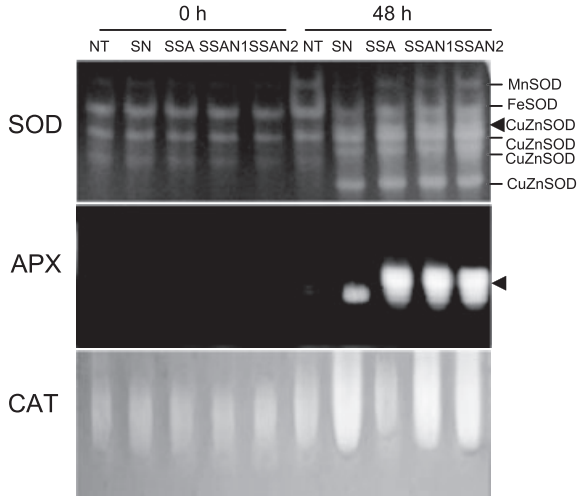

Fig. 6. Native gel analysis of SOD, APX and CAT isozymes in NT and transgenic (SN, SSA and SSAN) potato plants during 300 μ*M* MV treatments. SOD activity was detected with a negative staining solution using NBT after electrophoresis. APX activity was detected with a negative staining solution using NBT after electrophoresis. CAT activity was detected with a negative staining solution using DAB after electrophoresis. Sixty microgram of total protein concentrated from leaves were loaded in each line. Arrow-headed isoenzymes represent transgenic CuZnSOD and APX in SSA and SSAN plants.

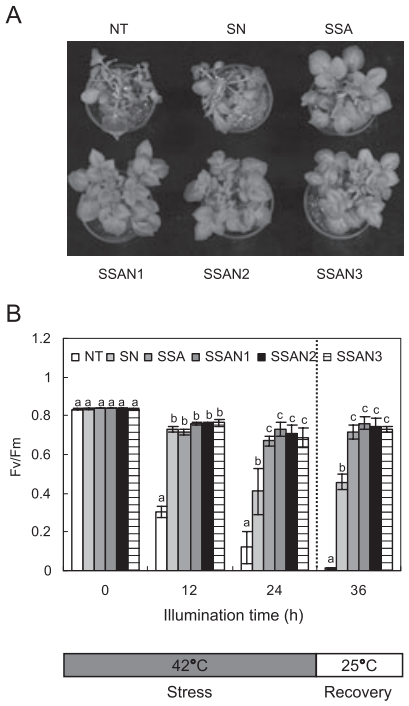

Fig. 7. Enhanced high temperature stress tolerance in the SSAN transgenic potato plants. (A) Visible differential damages in the leaves of NT and transgenic (SN, SSA and SSAN1–3) plants at 24 h after high temperature treatment. (B) Photosynthetic activity (Fv/Fm) in the leaves of NT and transgenic plants at 24 h post-high temperature treatment and at 12 h post-recovery at 25°C. Data are means of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

NDPK2 under the control of the oxidative stress-inducible *SWPA2* promoter (SSAN plants), which are more tolerant than SN or SSA plants to MV-induced oxidative stress and high temperature.

The tolerance of SSAN plants to MV-induced oxidative stress appeared to correlate with the transcriptional levels of *CuZnSOD*, *APX* and *NDPK2* (Fig. 4A). In addition, MV treatment of SSAN potato plants increased the activities of SOD, APX and NDPK under the control of the *SWPA2* promoter (Fig. 5). We previously showed that SOD and APX activities in SSA sweetpotato chloroplasts were strongly increased after MV treatment (Lim et al. 2007). It is likely that the CuZnSOD and APX proteins are targeted to chloroplasts in these plants (Lim et al. 2007). We can assume that CuZnSOD and APX were successfully targeted to the chloroplasts in SSAN potato plants, because we used the same expression vector as that reported by Lim et al. (2007) and Ahmad et al. (2010). In this study, the scavenging of O_2^- and generation of H_2O_2 by CuZnSOD and the detoxification of H_2O_2 by APX were observed in SSAN plants under oxidative stress conditions. Thus, chloroplast ROS can be deactivated by SOD and APX, which is important for plant productivity under challenging environmental conditions. These results have been confirmed in other reports (Lee et al. 2007, Tang et al. 2006). Yang et al. (2003) also demonstrated that *Arabidopsis NDPK2* regulated the expression of antioxidant genes including APX. SN potato plants also showed tolerance to MV-induced oxidative stress, with high NDPK and APX activities after MV treatment (Tang et al. 2008). Therefore, our results suggest that the higher levels of APX activity in SSAN plants might be a synergistic effect of the simultaneous expression of *CuZnSOD*, *APX* and *NDPK2* under stress conditions.

NDPK2 is involved in the H_2O_2-activated mitogen-activated protein kinase (MAPK) signaling and its overexpression in *Arabidopsis* induces the high expression of various antioxidant genes, such as POD, APX, CAT, thioredoxin and peroxiredoxin (Moon et al. 2003, Yang et al. 2003). In addition, overexpression of *NDPK2* in SN potato and sweetpotato plants increased NDPK, APX and CAT activities (Kim et al. 2009, Tang et al. 2008). These transgenic plants exhibited the enhanced tolerance to multiple stresses (Kim et al. 2009, Moon et al. 2003, Tang et al. 2008). Consistent with these results, SSAN plants in this study showed increased levels of APX and CAT activities under stress condition (Figs 5 and 6). This study also found that the activities

of APX were high in SSA and SSAN plants, whereas CAT activity in SN and SSAN plants compared with NT and SSA plants (Figs 5 and 6). APX and CAT are the major enzymes responsible for H_2O_2 scavenging during oxidative stress in plants. APX is a component of the ascorbate–glutathione pathway, which plays a key role in H_2O_2 scavenging (Foyer et al. 1994, Mittler 2002). CAT is also essential for removing H_2O_2 produced in peroxisomes by photorespiration (Dat 2000, Noctor et al. 2002). It is known that NDPK2 positively regulates the expression of antioxidant genes under the stress conditions and induced the enhanced tolerance to stress. Therefore, we suggest that the higher APX and CAT activities of SSAN plants are likely as a result of the increased expression of the *NDPK2*-regulated antioxidant genes and are correlated with environmental stress defense mechanisms involving an H_2O_2-regulated stress signaling.

Cellular ROS levels operate as critical signals for plant adaptation to environmental stresses. Thus, it is necessary to tightly control the efficient and accurate signaling during stress conditions. Therefore, we expect that the oxidative stress-inducible *SWPA2* promoter would be very useful for developing stress-tolerant plants (Ahmad et al. 2010, Kim et al. 2009, Lee et al. 2007, Lim et al. 2007, Tang et al. 2006, 2008). Accordingly, the expression of the *CuZnSOD*, *APX* and *NDPK2* genes controlled by the *SWPA2* promoter in transgenic potato plants was induced by MV-dependent oxidative stress (Fig. 4). In contrast, these transgenes were not expressed in plants unexposed to MV, suggesting their tight regulation by the *SWPA2* promoter. These results indicate that the *SWPA2* promoter strictly controls *CuZnSOD*, *APX* and *NDPK2* transgene expression in response to oxidative stress in SSAN plants.

In conclusion, we successfully generated and characterized SSAN transgenic potato plants expressing three stress-related genes under the control of the stress-inducible *SWPA2* promoter. These plants exhibited enhanced tolerance to multiple environmental stresses including MV-induced oxidative stress and high temperature by increasing the *NDPK2*-regulated activities of SOD, APX and CAT. Further characterization of the SSAN potato in natural field conditions is under investigation. We anticipate that the transgenic potato plants in this study might be useful for sustainable agriculture. Also, the strategy for the simultaneous expression of diverse genes in a gene-stacking manner may be employed for engineering crops for growth in marginal soils, which may help to overcome the existing food security problems of the world.

Acknowledgements – This work was supported by grants from the Biogreen21 (20070301034015), Rural Development Administration, Korea; from the MEST/NRF to World Class University Program (R32-10148), from the MEST/NRF to Korea-China Joint Research Program, and KRIBB initiative program.

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Edited by K.-J. Dietz