Transgenic Res (2008) 17:705–715
DOI 10.1007/s11248-007-9155-2

ORIGINAL PAPER

Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses

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Received: 4 July 2007 / Accepted: 18 October 2007 / Published online: 20 November 2007

Abstract In plants, nucleoside diphosphate kinase 2 (NDPK2) is known to regulate the expression of antioxidant genes. In this study, we developed transgenic potato plants (*Solanum tuberosum* L. cv. Atlantic) expressing *Arabidopsis* NDPK2 (AtNDPK2) gene in cytosols under the control of an oxidative stress-inducible *SWPA2* promoter (referred to as SN plants) or enhanced CaMV 35S promoter (EN plants) and evaluated their tolerance to various environmental stress, including methyl viologen (MV)-mediated oxidative stress, high temperature, and salt stress. When 250 μM MV was sprayed to whole plants, plants expressing NDPK2 showed significantly an enhanced tolerance compared to non-transgenic (NT) plants. SN ?tul?> plants and EN plants showed 51% and 32% less visible damage than NT plants, respectively. Transcript level of AtNDPK2 gene and NDPK2 activity in SN plants following MV treatment well reflected the plant phenotype. Ascorbate peroxidase (APX) activity was also increased in MV-treated SN plants. In addition, SN plants showed enhanced tolerance to high temperature at 42°C. The photosynthetic activity of SN plants after treatment of high temperature was decreased by about 10% compared to the plants grown at 25°C, whereas that of NT plants declined by 30%. When treated with 80 mM NaCl onto the plantlets, both SN plants and EN plants also showed a significant reduced damage in root growth. These results indicate that overexpression of NDPK2 under the stress-inducible *SWPA2* promoter might efficiently regulate the oxidative stress derived from various environmental stresses.

Keywords Ascorbate peroxidase · High temperature · Molecular breeding · Oxidative stress · Photosynthetic activity · Salt stress

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Introduction

Various environmental stresses, such as extreme temperature, chemical toxicity, and salinity can induce reactive oxygen species (ROS) in cellular level. ROS, which include the superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2), has been implicated in diverse environmental stresses in plants and appears to be a common participation in most degenerative conditions (Foyer et al. 1994; Asada 1999). Antioxidant enzyme systems are effective mechanisms for scavenging ROS caused by various environmental stresses. Direct modification of the expression of antioxidant genes has been assigned a key role in the strong protection of plants against various environmental stresses (Allen et al. 1997; Kwon et al. 2002; Tang et al. 2006; Lim et al. 2007).

Nucleoside diphosphate kinase (NDPK; EC 2.7.4.6) is believed to be a housekeeping enzyme that maintains the intracellular levels of all (d)NTPs used in biosynthesis except ATP. Recent studies suggested that NDPK also played a significant role in signal transduction pathways involved in oxidative stress (Otero 2000). In addition, NDPK function is associated with the phytochrome A response (Choi et al. 1999), UV-B signaling (Zimmermann et al. 1999), heat stress (Escobar Galvis et al. 2001) and growth (Yano et al. 1995). Moreover, constitutive over-expression of NDPK2 in *Arabidopsis* (AtNDPK2) conferred an enhanced tolerance to multiple environmental stresses (Moon et al. 2003). Constitutive over-expression of AtNDPK2 in plants induced numerous genes involved in cellular signal transduction and protection (Yang et al. 2003). Thus, AtNDPK2 regulates the expression of genes involved in antioxidant and protective functions and down-regulation of cellular redox state in AtNDPK2 transgenic plants is mediated by genes involved in antioxidant and protective processes (Yang et al. 2003). These results suggest that AtNDPK2 mediates multiple stress tolerance by signaling the transient expression of genes involved in antioxidant and protective, possibly through activation of the MAPK cascade (Yang et al. 2003).

Depending upon the stress, plants may keep the steady state level of ROS in cells by altering the balance between ROS production and ROS removal. In addition, due to the potential toxicity of ROS, a fine regulation is expected to occur between different ROS scavenging mechanisms.

Generally, a strong constitutive promoter such as CaMV 35S promoter is used for expression of foreign genes in plants. However, constitutive expression of a stress-inducible transcription factor caused retardation of the plant growth. The stress-responsive *rd29A* promoter could negate deleterious effects on plant growth (Kasuga et al. 1999). A more precise regulation of expression using an inducible promoter might be useful for development of stress-tolerant plants (Yoshida and Shinmyo 2000). Recently a novel oxidative stress-inducible peroxidase *SWPA2* promoter was isolated from cell cultures of sweetpotato and characterized its function in transgenic tobacco plants in terms of environmental stresses (Kwak et al. 1995, Kim et al. 1999, 2003). The *SWPA2* promoter contains several *cis*-element sequences implicated in oxidative stress, suggesting that the *SWPA2* promoter might be biotechnologically useful for the development of various transgenic plants with enhanced tolerance to multiple stresses (Wang et al. 2005; Tang et al. 2006; Lee et al. 2007; Lim et al. 2007).

Potato (*Solanum tuberosum* L.) is a major food crop in many parts of the world and ranks fourth in world production after wheat, maize and rice (Ross 1986). To our knowledge, many efforts have been made to increase the pathogen-resistance of potato plants, but few researches were carried out to improve its tolerance to environmental stress by molecular breeding technology (Perl et al. 1993; Jeong et al. 2001). Potato cultivar Atlantic is widely grown for making chips and French fries. However, tubers may have internal brown spots or hollow hearts under severe high temperature conditions. Previously, we have generated transgenic potato plants expressing AtNDPK2 gene (Moon et al. 2003) under the control of *SWPA2* promoter (Kim et al. 2003) (referred to as SN plants) and enhanced CaMV 35S promoter (EN plants) and selected the plant lines (SN1, SN19, EN1, and EN2) with high tolerance to methyl viologen (MV) at the level of leaf discs for further study (Tang et al. 2004). In this study, we evaluated transgenic SN plants and EN plants for protection against oxidative stress induced by MV, high temperature, and salt (NaCl) on the level of whole plants in detail.

Materials and methods

Plant materials and vector constructions

Four lines for each transgenic potato (*Solanum tuberosum* cv. Atlantic) plant (SN1, SN19 and EN1, EN2) with high tolerance to methyl viologen (MV, 10 μM) at the level of leaf discs (Tang et al. 2004) were used in this study. Plants were propagated under sterile conditions in Petri dishes containing MS medium (Murashige and Skoog 1962) supplemented 100 mg l^{-1} kanamycin. Plants for MV and heat tolerance assays were transferred in pots and grown in a growth chamber under a 16-h photoperiod with light intensity (100 μmol m^{-2} s^{-1}), 60% (w/v) relative humidity at 25°C.

Two vectors were developed to express AtNDPK2 gene in the control of the stress-inducible *SWPA2* promoter (Kim et al. 2003) or enhanced CaMV 35S promoter. The full-length cDNA of AtNDPK2 (accession no. AF017640) was kindly provided by Prof. D.J. Yun (Choi et al. 1999; Moon et al. 2003). This cDNA was obtained by PCR using the forward primer with *Nco*I site, 5′-CACCATGGTGGGAGCG-ACT-3′ and reverse primer with *Xba*I site, 5′-TCTGT CTAGACAAGGATCA-3′. The cDNA was ligated into the corresponding site of pRTL2 vector (E35Sp::AtNDPK2/pRTL2). In addition, to generate SWPA2 promoter-AtNDPK2 vector, enhanced 35S promoter fragment was replaced with *SWPA2* promoter (SWPA2p::AtNDPK2/pRTL2). These completed chimeric gene cassettes were inserted into *Hin*dIII site of the binary vector pCAMBIA2300, respectively.

MV treatment to whole plants

MV (0, 150, 200, or 250 μM) dissolved in 0.1% (w/v) Tween 20 solution was sprayed on the leaves of plants grown in a greenhouse for 4 weeks using a spray booth (Model SB-6; DeVries Manufacturing, Hollandale, MN, USA). The MV solution (70 ml) was applied to five potato plants using an 8001 VS type nozzle, 0.5 inches s^{-1}, 0.22 MPa. The tests for visible damage analysis by MV applications were repeated in triplicate. The percentage of leaf damage that appeared on the leaves after MV spraying was evaluated at 5 days after treatment (0% indicated no damage to the leaves; 100% meant fully damaged leaves). The percentage of the dry weight of non-damaged leaf from the first to fourth leaves of NT, EN, and SN plants at 5 days after MV spraying was measured on the basis of the dry weight of untreated plants under the same conditions. The chlorophyll contents were determined from discs (1.3 cm in diameter) from central tissue of the fifth leaves of plants at 5 days after MV spraying.

High temperature treatment

For high temperature stress, four-week-old potato plants growing at 25°C growth chamber were transferred to 42°C for 20 h in the growth chamber. The treated plants were transferred to normal conditions (25°C, 100 μmol m^{-2} s^{-1}) for recovery from the stress. The tolerance of transgenic plants to high temperature stress was estimated as the photosynthesis activity (F_v/F_m) and the fresh weight of the plants after treatment. For the measurement of photosynthesis activity, the fifth leaves of plants were determined at 0, 10 and 20 h after treatment and 10 h after recovery from the stress. Photochemical efficiency was calculated by evaluating emission of chlorophyll fluorescence, which represented the maximal yield of the photochemical reaction on PSII, with a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England). Relative fresh weight was estimated by determining the fresh weight of the plants after 20 h heat stress at 42°C.

Salt stress treatment

For salt tolerance assays, plantlets (NT, EN, and SN plants) were propagated on MS medium in vitro. Salt stress was accomplished by transferring shoots to test tube containing MS medium (solid) supplemented with 80 mM NaCl. Plants were cultured in a growth chamber under a 16-h photoperiod with light intensity (100 μmol m^{-2} s^{-1}) at 25°C. Tolerance was estimated by determining the root length and the root dry weight after 20 days of treatment. The root dry weight was measured after drying the sample in the dry oven at 70°C for 48 h.

Reverse Transcriptase-PCR (RT-PCR)

For analysis of the transcriptional levels of AtNDPK2 gene, the fourth-fifth leaves from the top of potato plants (NT, EN and SN) sprayed with 150 μM MV were sampled over a time period. All plant materials were immediately frozen in liquid nitrogen and stored at −70°C until further experiments.

Total RNA was extracted from leaves of potato plants (NT, EN and SN) with cetyltrimethylammonium bromide (CTAB) method (Kim and Hamada 2005). Total RNA samples were treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. First-strand cDNA was then synthesized using MMLV Reverse Transcriptase (Stratagene, USA) from 1 μg of total RNA in a 20 μl reaction volume, and 1 μl of the reaction mixture was subjected to subsequent PCR in a 50 μl reaction volume. AtNDPK2 (5′-GTTGGCCGCATTTCGTCCTCA-3′ and 5′-CCACTTGCATAGCTCGCCCTC-3′) and actin (5′-TGGACTCTGGTGATGGTGTC-3′ and 5′-CCTCCAATCCAAACACTGTA-3′) were amplified using the indicated primers for 30 cycles according to the following conditions for each cycle: 94°C for 0.5 min, 62°C for 0.5 min, and 72°C for 1 min. This was followed by a final cycle at 72°C for 5 min to allow completion of the polymerizations. Actin mRNA served as an internal control. Ten μl of reaction products were separated on 1% agarose gel, stained with ethidium bromide and visualized on UV.

NDPK and APX activity assays

For analysis of the activities of NDPK and APX, total soluble protein was extracted from the fifth-sixth leaves from the top of potato plants treated with 150 μM MV and determined protein concentration by Bio-Rad protein assay kit (Bradford 1976).

For assay of NDPK activity, crude protein was extracted in the extraction buffer [0.15 M Tris-HCl, pH 8.0; 25% glycerol; 0.8% mercaptoethanol; 100 μM phenylmethylsulfonyl fluoride; 10 μg ml^{-1} leupeptin; and 10 μg ml^{-1} pepstatin A]. NDPK enzyme activity was measured using the coupled reaction method with lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) at 25°C (Yano et al. 1995). NDPK activity was calculated based on the reduction of OD_{340} following a decrease in NADH. One unit of enzyme activity was defined as 1 μmol of ADP production per minute at 25°C. Measurements of enzyme activity were carried out in triplicate for each independent sample.

For the assay of APX activity, crude protein was extracted in 50 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and 1 mM ascorbate. APX activity was elicited out according to the methods of Nakano and Asada (1981) in 1 ml of a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.6 mM ascorbate, 1 mM H_2O_2, and 50 μl of enzyme extract. Oxidation of ascorbate was determined by monitoring the decrease in absorbance at 295 nm (extinction coefficient 0.36). One unit of APX was defined as the amount of enzyme oxidizing 1 μM of ascorbate per min.

Statistical analysis

All treatments of MV, heat, and salt stress were repeated three times. All measurements were subjected to Student's *t*-test using Microsoft Excell 2003. The significance refers to statistical significance at the $P \leq 0.05$ level.

Results

Enhanced tolerance to methyl viologen-mediated oxidative stress

To investigate the tolerance of transgenic plants to oxidative stress at the level of whole plant, NT and transgenic plants (SN, EN) were evaluated for visible damage 5 days after spraying with solutions containing 0, 150, 200 or 250 μM methyl viologen (MV) (Fig. 1). MV is a typical ROS generating redox-active compound, which has been used as non-selective herbicide. All plants showed a significant symptom of leaf damage in correlation with MV concentration. However, SN and EN plants showed reduced symptoms of damage compared to NT plants. Especially, young leaves from SN plants were almost unaffected even under high MV concentration (Fig. 1).

The percentage of visible damages that appeared on leaves from NT, EN, and SN plants were evaluated at 5 days after MV spraying by (Fig. 2a).

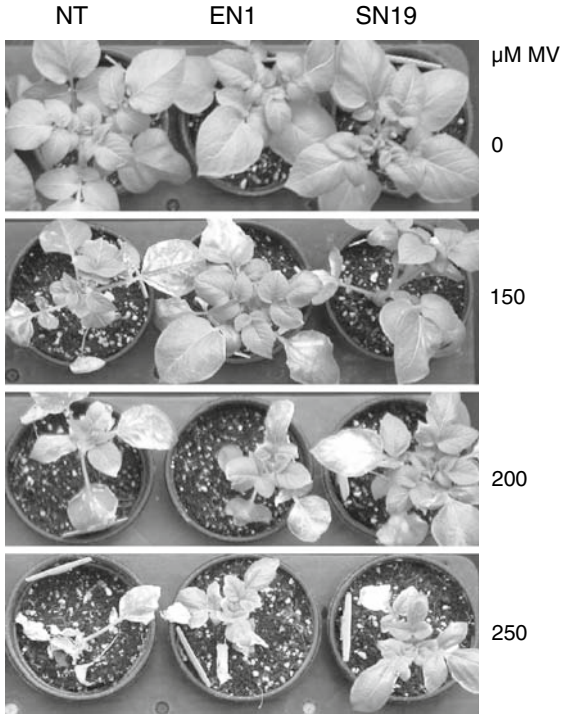

Fig. 1 Differential visible damages in the leaves of non-transgenic (NT) and transgenic (EN1 and SN19) potato (cv. Atlantic) plants at 5 days after treatment with 0, 150, 200, or 250 μM methyl viologen (MV). EN1 and SN19 indicate transgenic potato plants expressing AtNDPK2 under the enhanced 35S CaMV promoter and an oxidative stress-inducible *SWPA2* promoter, respectively

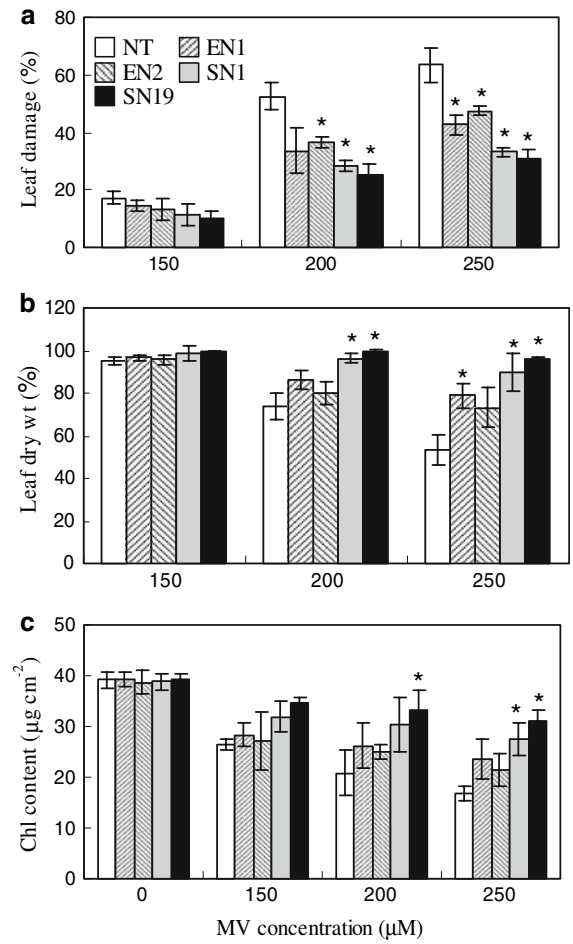

Fig. 2 Effect of methyl viologen (MV) on the leaves of non-transgenic (NT) and transgenic (EN and SN) potato (cv. Atlantic) plants at 5 days after treatment with 0, 150, 200, or 250 μM MV. (**a**) Quantitative estimate of visible damage that appeared on leaves from NT, EN, and SN plants. (**b**) Relative dry weight of leaves from non-damaged parts in NT, EN, and SN plants. The percentage of dry weight was calculated on the basis of the content of untreated plants (0 μM MV) under the same light conditions. (**c**) The chlorophyll contents (mg cm^{-2}) in the fifth leaves. Measurements were made on the central tissue (d = 1.3 cm) of the fifth leaves from NT, EN, and SN plants. EN and SN indicate transgenic potato plants expressing AtNDPK2 under the enhanced 35S CaMV promoter and an oxidative stress-inducible *SWPA2* promoter, respectively. Data are means ± SE of three independent measurements. Asterisk indicates significant differences between NT and transgenic plants (Student's *t*-test $P \leq 0.05$)

SN plants showed slightly higher tolerance to MV-mediated oxidative stress than that of NT and EN plants sprayed with 150 μM MV. When 200 and 250 μM MV were treated, NT plants increased visible leaf damages to 52.5% and 63.4%, respectively, whereas SN19 plants showed only 25% and 31% visible leaf damage, EN1 plants showed 34% and 43%, respectively. Although both SN and EN plants showed enhanced tolerance against MV treatment compared to NT plants, SN plants treated with 250 μM MV exhibited 28% more tolerance than EN plants. This result suggests that stress-inducible *SWPA2* promoter might be more effective than constitutive expressing CaMV 35S promoter for development of transgenic plants with an enhanced tolerance to oxidative stress.

In addition to visible damage, the effect of MV spray was also investigated at 5 days after MV treatment by determining the dry weight of non-damaged leaf tissues from the first to fourth leaves (Fig. 2b). Notably, no reduction in the dry weight of SN19 plants was shown at 150 μM MV treatment. At 200 or 250 μM MV treatment, the dry weight of non-damage leaves in NT plants was significantly

decreased to 27% and 48% compared to non-treated NT plants, respectively. However, SN19 plants showed about 1% and 4% reduction in dry weight at the same treatment.

The chlorophyll contents were also investigated in the central region of fifth leaves from the plants treated with MV at various concentrations (Fig. 2c). There were no significant differences in chlorophyll contents between NT and transgenic plants in untreated conditions. Chlorophyll content in the leaves of NT and transgenic plants after MV treatment well reflected the visible damages of each plant. When treated with 200 and 250 μM MV, the chlorophyll contents of NT plants were severely decreased to 49% and 59%, respectively. At the same treatment SN plants showed only 15% and 20% reduction of chlorophyll content compared to untreated plants, respectively. It is clear that transgenic potato plants expressing AtNDPK2 gene resulted in enhanced tolerance to oxidative stress induced by MV.

Changes in activity of NDPK and APX after MV treatment

To understand the tolerance mechanism in transgenic potato plants to MV-mediated oxidative stress, we investigated changes in gene expression of introduced AtNDPK2 after 150 μM MV treatment with time course. As shown in Fig. 3, transcripts of AtNDPK2 gene were not detected in NT and non-treated SN plants as expected. The expression level of EN1 plants was high, but not significantly changed after MV treatment. Gene expression of AtNDPK2 in SN19 plants was induced at low level after 12 h of MV treatment and then increased gradually until 72 h, after which a slight decrease was observed. However, interestingly, transcript level of SN plants was higher than that of EN plants from 36 h after MV treatment. Transcript levels of AtNDPK2 in both SN and EN plants well reflected tolerance to MV. These results suggest that the protection from MV-mediated oxidative stress could be conferred by the over-expression of AtNDPK2 in transgenic potato plants.

In order to observe the response of NDPK enzyme in response to MV-induced oxidative stress, transgenic (SN, EN) and NT plants were sampled following 150 μM MV spraying for various periods of time (Fig. 4a). NDPK specific activity in NT plants showed increase at 12 h after MV spraying, and then decreased rapidly thereafter. NDPK activity in SN plants increased markedly until 72 h after MV treatment, and then slightly decreased. Interestingly, SN plants showed higher NDPK activity than EN plants with constitutive high level of activity after MV treatment. The NDPK activity in SN and EN plants was coordinated with transcript level of AtNDPK2 after MV treatment. The elevated NDPK activity in transgenic plants well reflected the tolerance to MV-mediated oxidative stress.

APX activity was also analyzed in NT and transgenic plants (EN, SN) following 150 μM MV treatment with time course (Fig. 4b). The pattern of APX activity in both NT and transgenic plants was similar to that of NDPK activity. Corresponding to NDPK activity, SN plants exhibited higher APX activity than EN plants, and its activity increased and reached a maximum level at 72 h after MV spraying, which was 2.7 fold than that before stress treatment. EN plants maintained with high level of APX activity

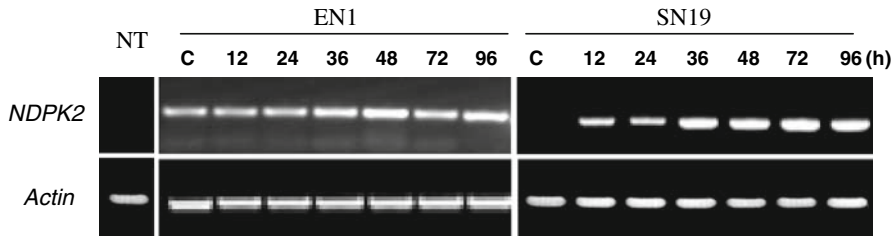

Fig. 3 RT-PCR analysis of the expression of NDPK2 gene in leaves from non-transgenic (NT) and transgenic (EN and SN) potato (cv. Atlantic) plants subjected to 150 μM methyl viologen (MV) spray. Total RNA was extracted from leaves 12, 24, 36, 48, 72, and 96 h after treatment with MV. First-strand cDNA synthesis and PCR were performed according to the instructions of the manufacturer. Actin was used to control for equal loading. Reaction products (10 μl) were analyzed by gel electrophoresis. C indicates non-treated transgenic plants. EN1 and SN19 indicate transgenic potato plants expressing AtNDPK2 under the enhanced 35S CaMV promoter and an oxidative stress-inducible *SWPA2* promoter, respectively

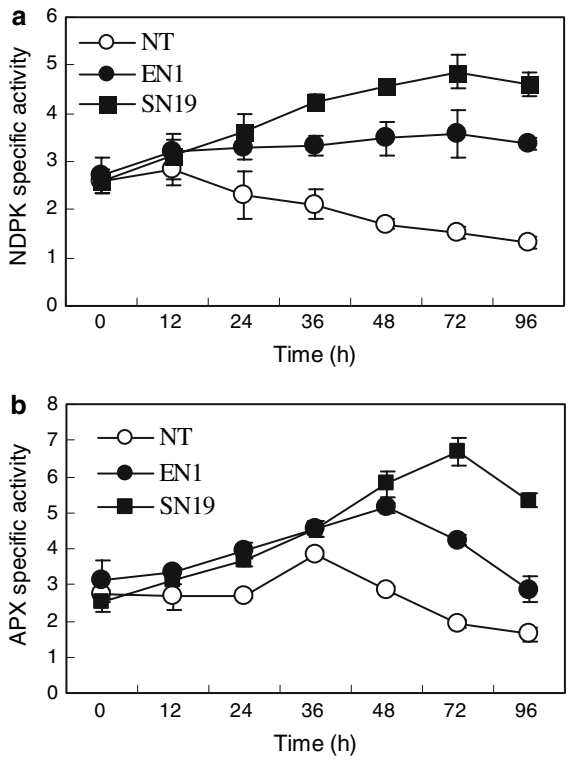

Fig. 4 NDPK (**a**) and APX (**b**) activities (units per mg protein) in leaves from non-transgenic (NT) and transgenic (EN and SN) potato (cv. Atlantic) plants subjected to 150 μM methyl viologen (MV) spray. Protein was extracted from leaves 12, 24, 36, 48, 72, and 96 h after treatment with MV. EN1 and SN19 indicate transgenic potato plants expressing AtNDPK2 under the enhanced 35S CaMV promoter and an oxidative stress-inducible *SWPA2* promoter, respectively

until 48 h after MV treatment, and then decreased thereafter. However, APX activity in NT plants increased at 24 h after MV spraying and then decreased rapidly. These results suggest that one of reasons for the protection against MV-mediated oxidative stress could be ascribed to the elevated NDPK activity in transgenic plants.

Enhanced tolerance to high temperature treatment

Next, we assessed the tolerance to high temperature stress in transgenic potato plants. When whole plants of SN, EN, and NT were exposed to high temperature at 42°C for 20 h, NT plants were severely wilted from heat shock after 10 h, whereas SN and EN plants appeared to remain healthy (Fig. 5a). Photosynthetic activity, as assesses by the fluorescence

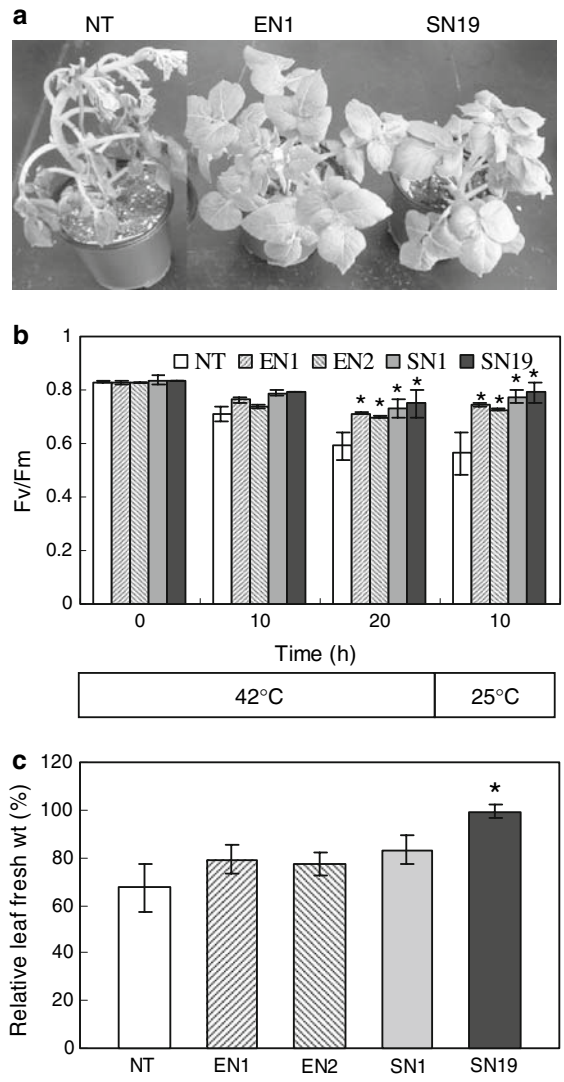

Fig. 5 Effects of high temperature (42°C) on non-transgenic (NT) and transgenic (EN and SN) potato (cv. Atlantic) plants. (**a**) Visible differential damages in the leaves of NT, EN1, and SN19 plants at 20 h after treatment; (**b**) Photosynthetic activity (F_v/F_m) in the leaves of NT, EN, and SN plants at 20 h after treatment; (**c**) Fresh weight of plants at 20 h after treatment. The percentage of fresh weight was calculated on the basis of untreated plants grown at 25°C. EN1 and SN19 indicate transgenic potato plants expressing AtNDPK2 under the enhanced 35S CaMV promoter and an oxidative stress-inducible *SWPA2* promoter, respectively. Data are means ± SE of three independent measurements. Asterisk indicates significant differences between NT and transgenic plants (Student's *t*-test $P \leq 0.05$)

parameter (F_v/F_m), was determined in the fifth leaf from each plant (Fig. 5b). Photosynthetic activity of SN plants was lightly declined during treatment at

42°C, whereas in NT plants the reduction was higher and irreversible damage occurred. The photosynthetic activity of NT plants reduced 29% at 20 h after heat shock relative to the values before treatment, while that of SN19 plant decreased only 10%. Moreover, at 10 h of recovery (25°C) following heat treatment, F_v/F_m in SN plants almost recovered to the initial level, while that in NT plants remained low. EN plants showed intermediate results between NT and SN plants. The tolerance in transgenic (SN, EN) plants treated with high temperature stress for 20 h was estimated as the percentage of initial fresh weight that remains after 42°C treatment (Fig. 5c). NT plants showed a 33% decrease in FW. However, SN plants remained at similar fresh weight compared with the SN plants grown at 25°C, showing only a decrease of 0.6% (SN19). The fresh weight of EN plants was a lower than that of SN plants. These results clearly indicated that SN plants had a tolerance to high temperature stress.

Enhanced tolerance to salt stress

The tolerance to salt stress was estimated by determining the root growth (elongation and dry weight) in NT and transgenic (EN, SN) plants after growing for 20 days in a medium containing 80 mM NaCl. NT and transgenic plants showed no differences in root development under normal conditions (Fig. 6). However, significant differences between NT and SN plants were clearly apparent at the phenotypic level from 20 days after salt treatment. The root elongation and root dry weight in SN19 plants subjected to salt stress displayed 67% and 64% compared to untreated plants. NT plants only showed 11% and 18% of the root elongation and root dry weight compared to control conditions (Fig. 6). EN plants showed intermediate results between NT and SN plants. These results suggest that transgenic potato plants (SN and EN) expressing AtNDPK2 had a considerable tolerance to salt stress.

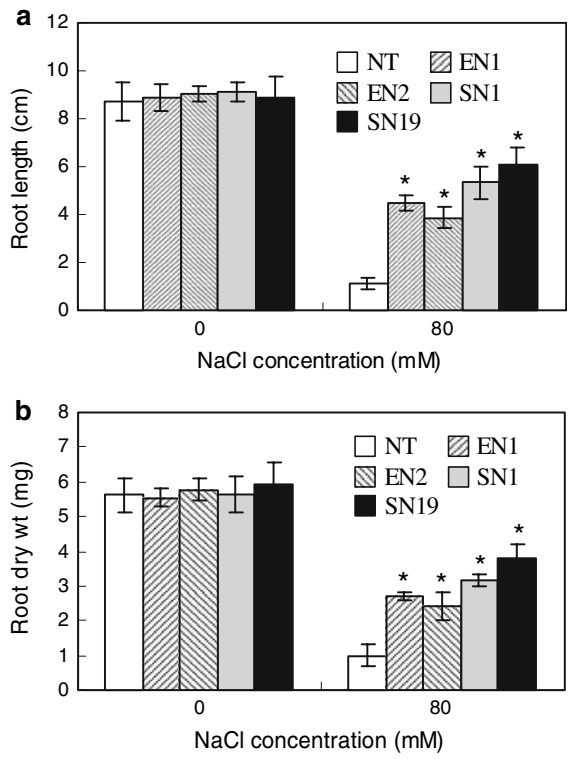

Fig. 6 Effects of salt stress (80 mM NaCl) on non-transgenic (NT) and transgenic (EN and SN) potato (cv. Atlantic) plants. (**a**) Root length of NT, EN (EN1 and EN2), and SN (SN1 and SN19) plants for 20 days after treatment; (**b**) Root dry weight of NT, EN, and SN plants for 20 days after treatment. EN and SN indicate transgenic potato plants expressing AtNDPK2 under the enhanced 35S CaMV promoter and an oxidative stress-inducible *SWPA2* promoter, respectively. Data are means ± SE of three independent measurements. Asterisk indicates significant differences between NT and transgenic plants (Student's *t*-test $P \leq 0.05$)

Discussion

Oxidative stress by excessive production of ROS under environmental stress conditions results in reduced yields for crop plants. Antioxidative mechanisms are an effective system for removal of ROS. Another efficient approach is to manipulate the genes that regulate the expression of these antioxidative enzymes. Transgenic *Arabidopsis* plants expressing AtNDPK2 with enhanced tolerance to multiple stresses have been reported (Moon et al. 2003). Yang et al. (2003) demonstrated that *Arabidopsis* NDPK2 (AtNDPK2) also regulates the expression of genes involved in antioxidants including APX, suggesting that it will be is attractive enzyme to regulate the oxidative stress.

Since the transgene products affect a critical pathway associated with stress tolerance, maintenance of redox potential and cell signaling, a high level of activity of an introduced transgene may cause

too much disruption and lead to a negative effect. In addition, under stress conditions, ROS may act either as signals to induce protection mechanisms or to accelerate injury (Dat et al. 2000). To allow for these different roles, cellular levels of ROS should be tightly controlled. Thus we expect that an oxidative stress-inducible *SWPA2* promoter might be very useful for development of stress-tolerant plants (Kim et al. 2003; Wang et al. 2005; Tang et al. 2006; Lim et al. 2007). More precisely controlled expression of foreign genes might avert unintended adverse effects on phenotypes.

The present study describes transgenic potato plants expressing AtNDPK2 gene under the control of *SWPA2* promoter (referred to SN plants) or CaMV 35S promoter (referred to EN plants), respectively, and their enhanced tolerance to oxidative stress induced by MV, high temperature, and salt stress. SN plants showed much less leaf damage than NT plants at 5 days after 250 μM MV spraying (Figs. 1 and 2). EN plants showed an intermediate result between SN and NT plants. In addition, chlorophyll contents in NT plants following MV spray were dramatically decreased, but SN plants retained with high chlorophyll levels. Therefore, we predicted that SN plants expressing AtNDPK2 gene could show effects that would lead to significantly increased stress tolerance. These data indicate that a stress-inducible *SWPA2* promoter is more efficient than CaMV 35S promoter for the development of stress-tolerant transgenic plants.

Next, we checked the effects of the SN and EN plants on the tolerance to high temperature stress. It was known that temperature fluctuations could induce the formation of ROS (Payton et al. 1997; McKersie et al. 2000). Potato plants, specially, cv. Atlantic is sensitive to high temperature. NT plants were wilted after 10 h of heat shock at 42°C, whereas SN and EN plants appeared to remain healthy (Fig. 5). Fluorescence measurements (F_v/F_m) showed that photosynthesis of SN was transiently reduced during heat stress, while that of NT plants was highly reduced. These results suggested that transgenic potato plants expressing AtNDPK2 had enhanced tolerance to high temperature. There was increasing evidence for considerable correlation between the responses to heat stress and oxidative stress. Phosphorylation of NDPK was affected by heat stress in sugarcane (Moisyadi et al. 1994). In pea seedling, NDPK was involved in heat stress response through its interaction with heat shock protein (Escobar Galvis et al. 2001).

The root elongation and dry weight of transgenic plants after NaCl treatment provided to be an estimate of their salt tolerance. Similar to high temperature stress, both SN and EN plantlets subjected to NaCl showed enhanced tolerance to salt stress compared to NT plants (Fig. 6). Notably, the SN plants showed the high tolerance to salt stress. Here, our results demonstrated that transgenic potato plants expressing AtNDPK2 could lead to increased tolerance to multiple stresses including salt.

Transgenic potato plants expressing AtNDPK2 gene showed enhanced tolerance to diverse environmental stresses (Figs. 2, 5, 6). In order to understand the tolerance mechanism in transgenic plants to MV-mediated oxidative stress, changes in transcript and enzymes of transgene after MV treatment were investigated. The tolerance levels of SN and EN plants against oxidative stress appeared to correlate with the transcriptional level and protein activity of NDPK (Figs. 3, 4b). SN plants with highest level of NDPK activity showed the strongest tolerance to MV-induced oxidative stress. In addition, SN plants showed higher APX activity correlated with NDPK activity after MV treatment (Fig. 4b). These data indicate that the increased tolerance to MV-mediated oxidative stress exhibited by SN and EN plants is likely a consequence of their high levels of AtNDPK2-regulated APX activity. APX is one of the major antioxidants that regulate the reduction/oxidation (redox) states. In *Arabidopsis* plants overexpressing AtNDPK2 with tolerance to multiple stresses, several antioxidant genes including APX are expressed (Yang et al. 2003). APX-suppressed tobacco cells showed tolerance to heat and salt stress through the up-regulation of stress-related genes via the activation of NDPK2 gene (Ishikawa et al. 2005). It is likely that AtNDPK2 localized in nucleus and cytoplasm may mediate multiple stress tolerance by gene expression associated with antioxidant enzymes (Moon et al. 2003).

SN plants with more tolerance to diverse stresses showed higher activities of NDPK and APX than that of EN plants (Fig. 4). This pattern of changes in NDPK activity in SN plants, particularly, may be attributed to an oxidative stress-inducible *SWPA2* promoter. Consistent with this result, expression of

transgene under the control of *SWPA2* promoter showed an inducible increase in transgenic tobacco cell cultures (Kim et al. 2003). The results highlighted the potential utility of the oxidative stress-inducible *SWPA2* promoter for the development of stress-tolerant transgenic plants. Increase of NDPK activity endows the SN plants with a considerable tolerance to oxidative stress, heat, and salt stress. Although the exact mechanism by which NDPK2 specifically regulates expression of APX gene is still unclear, it is clear that NDPK2 overexpression resulted in enhanced tolerance to oxidative stress by increased activity of various antioxidants including APX.

It was noted that a higher protection was observed in SN plants under the control of *SWPA2* promoter compared with the EN plants under the control of CaMV E35S promoter (Figs. 2, 5, 6). These results suggested that an oxidative stress-inducible *SWPA2* promoter might provide more precise regulation of foreign gene, indicating that this promoter might be useful for the development of efficient plants with enhanced tolerance to multiple stresses. In our previous study using *SWPA2* promoter, transgenic potato and sweetpotato plants expressing both genes of CuZnSOD and APX in chloroplasts showed an inducible activity of introduced genes (Tang et al. 2006; Lim et al. 2007). In addition, simultaneous overexpression of both CuZnSOD and APX in transgenic tall fescue, a monocotyledonous forage grass, under the control of *SWPA2* promoter also confers increased tolerance to a wide range of abiotic stress (Lee et al. 2007). These results suggest that *SWPA2* promoter might be useful for all kinds of plant species.

In conclusion, we successfully generated transgenic potato plants expressing AtNDPK2 in cytosols under the control of stress-inducible *SWPA2* promoter or constitutive CaMV 35S promoter. Transgenic potato plants, especially SN plants under *SWPA2* promoter exhibited enhanced tolerance to environmental stress including MV-induced oxidative stress, high temperature and salt stress. Further characterization of transgenic potato plants is under investigation in terms of multiple stresses including drought and cold stress at the level of whole plants. We anticipate that transgenic potato plants in this study might be useful in marginal soils for sustainable agriculture. In addition, we are trying to further introduce NDPK2 gene into cytosols of transgenic potato plants expressing both genes of CuZnSOD and APX in chloroplasts (Tang et al. 2007) to manipulate the oxidative stress in both chloroplasts and cytosols.

Acknowledgements This research was supported by grants from BioGreen21 Program (code #20070301034015), Rural Development Administration, Korea, from the Environmental Biotechnology National Core Research Center, KOSEF/MOST, Korea, and from the Korea Foundation for International Cooperation of Science and Technology (KICOS).

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