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Research article

Overexpression of 2-cysteine peroxiredoxin enhances tolerance to methyl viologen-mediated oxidative stress and high temperature in potato plants

Myoung Duck Kim [a], Yun-Hee Kim [a], Suk-Yoon Kwon [b], Bo-Young Jang [a], Sang Yeol Lee [c], Dae-Jin Yun [c], Ji-Hong Cho [d], Sang-Soo Kwak [a], Haeng-Soon Lee [a,*]

[a] Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yusong-gu, Daejeon 305-806, Republic of Korea
[b] Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yusong-gu, Daejeon 305-806, Republic of Korea
[c] Division of Applied Life Sciences, Gyeongsang National University, Jinju 660-701, Republic of Korea
[d] Highland Agriculture Research Center, National Institute of Crop Science, RDA, Ganswon 232-935, Republic of Korea

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ABSTRACT

Oxidative stress is one of the major causative factors for injury to plants exposed to environmental stresses. Plants have developed diverse defense mechanisms for scavenging oxidative stress-inducing molecules. The antioxidative enzyme 2-cysteine peroxiredoxin (2-Cys Prx) removes peroxides and protects the photosynthetic membrane from oxidative damage. In this study, transgenic potato (*Solanum tuberosum* L. cv. Atlantic) expressing *At2-Cys Prx* under control of the oxidative stress-inducible *SWPA2* promoter or enhanced CaMV 35S promoter (referred to as SP and EP plants, respectively) was generated using *Agrobacterium*-mediated transformation. The transgenic plants were tested for tolerance to stress. Following treatment with 3 μM methyl viologen (MV), leaf discs from SP and EP plants showed approximately 33 and 15% less damage than non-transformed (NT) plants. When 300 μM MV was sprayed onto whole plants, the photosynthetic activity of SP plants decreased by 25%, whereas that of NT plants decreased by 60%. In addition, SP plants showed enhanced tolerance to high temperature at 42 °C. After treatment at high temperature, the photosynthetic activity of SP plants decreased by about 7% compared to plants grown at 25 °C, whereas it declined by 31% in NT plants. These results indicate that transgenic potato can efficiently regulate oxidative stress from various environmental stresses via overexpression of *At2-Cys Prx* under control of the stress-inducible *SWPA2* promoter.

1. Introduction

Various environmental stresses, such as drought, salinity and extreme temperature, can induce reactive oxygen species (ROS) that generate oxidative stress at the cellular level. ROS, which include the superoxide anion radical ($O_2^{-}\bullet$), hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2), have been implicated in diverse environmental stresses in plants and appear to be common participants in most degenerative conditions [1,2]. Since antioxidant enzyme systems are effective mechanisms for scavenging ROS, modification of antioxidant gene expression is crucial for developing plants that can withstand various environmental stresses [3–5].

Peroxiredoxins (Prxs) are ubiquitous thiol-specific peroxidases that reduce toxic peroxides. They combat ROS generated by electron transport activities under different types of biotic and abiotic stresses. These peroxidases can be divided into three groups, typical 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx, each distinguished by their catalytic mechanisms and number of Cys residues [6]. In plants, the 2-Cys Prx protein is post-translationally targeted to chloroplasts, where it protects the photosynthetic membrane from oxidative damage [7,8]. Under strong oxidative or heat shock stress conditions, 2-Cys Prx rapidly switches from a low molecular weight structure to a high molecular weight complex. Accordingly, the protein also switches functionally from a peroxidase to a molecular chaperone that protects its substrate from stress [9]. In plants, studies have focused on the roles played by Prx as an antioxidant, a modulator of cell signaling pathways and a redox sensor [6,10–12]. In transgenic *Arabidopsis*, reduced 2-Cys Prx expression resulted in impaired photosynthesis and accelerated degradation of chloroplast proteins [7]. In addition, Chinese cabbage 2-Cys Prx performs two functions, acting as a peroxidase, or switching to

Abbreviations: 2-Cys Prx, 2-cysteine peroxiredoxin; MV, methyl viologen; ROS, reactive oxygen species; *SWPA2*, sweetpotato anionic peroxidase promoter 2.

* Corresponding author. Tel.: +82 42 860 4439; fax: +82 42 860 4608.
E-mail address: hslee@kribb.re.kr (H.-S. Lee).

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a molecular chaperone upon heat shock or oxidative stress [13]. Although there have been few reports describing Prx over-expression in plants, transgenic tobacco plants overexpressing 1-Cys Prx and tall fescue plants overexpressing 2-Cys Prx have shown resistance to oxidative stress and increased antioxidant activity [14,15].

More precise regulation of expression may be achieved using an inducible promoter and such techniques could be useful for development of stress-tolerant plants [16]. Previously, we isolated the novel oxidative stress-inducible *SWPA2* promoter from sweet-potato and used transgenic tobacco plants to characterize its function with respect to environmental stresses [17]. Stress-tolerant transgenic plants (potato, sweetpotato, tall fescue) were then developed using the *SWPA2* promoter [4,5,18–20].

The 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 [21]. Although increased pathogen-resistance has been reported for potato plants, there has been only limited research into improving its tolerance to environmental stress via molecular breeding technology [4,18–20]. Potato tubers (cv. Atlantic) are grown widely for making chips and French fries. However, potato plants are relatively sensitive to high temperature and severe high temperature conditions may cause the tubers to develop internal brown spots or hollow hearts. In this study, we developed transgenic potato (cv. Atlantic) plants that express the *Arabidopsis 2-Cys Prx* (*At2-Cys Prx*) gene under control of the stress-inducible *SWPA2* promoter [17] or enhanced CaMV 35S promoter. These plants showed enhanced tolerance to methyl viologen (MV)-mediated oxidative stress and high temperatures.

2. Results

2.1. Transformation and regeneration

Transgenic potato plants expressing *At2-Cys Prx* under the control of the *SWPA2* promoter (referred to as SP) or E35S promoter (referred to as EP) were generated successfully by *Agrobacterium*-mediated transformation (*Fig. 1*). The kanamycin-resistant calli induced from leaf explants developed into numerous shoots. These shoots were converted to plantlets on MS medium supplemented with 100 mg l^{-1} kanamycin and grown under fluorescent light (70 μmol m^{-2} s^{-1} of light intensity). Regenerated plants were transferred to pots and grown in a greenhouse. No morphological differences were detected between non-transgenic (NT) and transgenic plants (SP and EP).

PCR analyses were carried out to confirm the presence of the introduced *At2-Cys Prx* and *nptII* genes in the genomic DNA of transgenic plants. DNA from 21 lines with the EP-K vector and 19 lines harboring the SP-K vector contained the predicted 800 and 750 bp internal fragments for *At2-Cys Prx* and *nptII*, respectively (data not shown). Genomic DNA from randomly-selected, PCR-positive plants was subjected to Southern blot analysis under high stringency. When the genomic DNA from seven transgenic plants were digested with *Eco*RI, hybridization with an *At2-Cys Prx*-specific probe showed one to four bands in SP and EP plants, no hybridization band was detected in genomic DNA from NT plants (Fig. 2). All transgenic lines were cultivated in a growth chamber for 8 weeks prior to use in experiments for enhanced tolerance against MV-mediated oxidative stress. Ion leakage was assessed over times, and the four transgenic lines (SP1, SP2 and EP1, EP2, i.e., 2 lines for each vector) demonstrating the greatest stress tolerance were selected for further characterization.

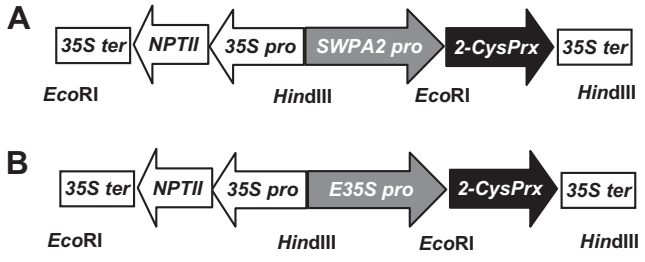

Fig. 1. Expression vector used for potato transformation. A, pSP-K; B, pEP-K. *SWPA2* pro, sweetpotato peroxidase (*SWPA2*) promoter; 2-Cys Prx, At2-Cys peroxiredoxin; 35S ter, CaMV 35S terminator; 35S pro, enhanced CaMV 35S promoter; NPTII, neomycin phosphotransferase.

2.2. Transgenic plants show enhanced tolerance to MV-mediated oxidative stress

To evaluate tolerance to MV-mediated oxidative stress, the four transgenic plant lines (EP1, EP2, SP1 and SP2) were cultivated in a growth chamber for 8 weeks. MV is a typical ROS-generating, redox-active compound and has been used as a non-selective herbicide [22]. Leaf discs were treated with 3 μM MV and, after 48 h, severe necrosis was observed in the discs from NT plants, while partial necrosis was observed at the edges of discs from SP plants. When leaf discs were subjected to MV for 72 h, SP and EP plants showed a reduction in membrane damage of approximately 35% and 15%, respectively, compared to NT plants (Fig. 3A). The tolerance of transgenic plants to radical stress was measured after treatment with 0.5 M and 1 M H_2O_2, and similar to the findings with MV treatment and after treatment with 1 M H_2O_2, membrane damage of SP and EP transgenic plants reduced for approximately 30% and 15%, respectively, compared to NT plants (Fig. 3B). These data strongly suggest that the transgenic potato plants exhibit enhanced tolerance to oxidative stress.

To investigate oxidative stress tolerance at the whole plant level, transgenic (SP and EP) and NT plants were sprayed with solutions containing 0, 100, 200, or 300 μM MV. NT plants showed symptoms of leaf damage, which became more severe as the concentration of MV increased. In contrast, SP and EP plants showed fewer symptoms of damage (Fig. 4A). At 200 μM MV, NT plants showed damage on 35% of their leaf area versus 20 and 23% of the area on SP and EP transgenic plants. Damage on NT plants became more severe (80%) upon exposure to 300 μM MV, compared with 28 and 33% damage

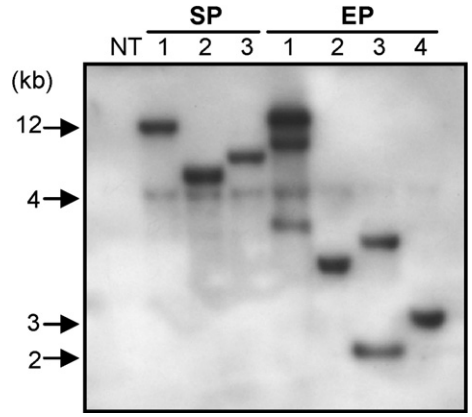

Fig. 2. Southern blot analysis of transgenic potato plants expressing *At2-Cys Prx* gene under the control of an oxidative stress-inducible *SWPA2* promoter (SP plants) or enhanced CaMV 35S promoter (EP plants). Genomic DNA prepared from transgenic and NT potato plants and hybridized with ^{32}P-labeled *At2-Cys Prx* gene. SP and EP, transgenic potato plants; NT, non-transgenic potato plant.

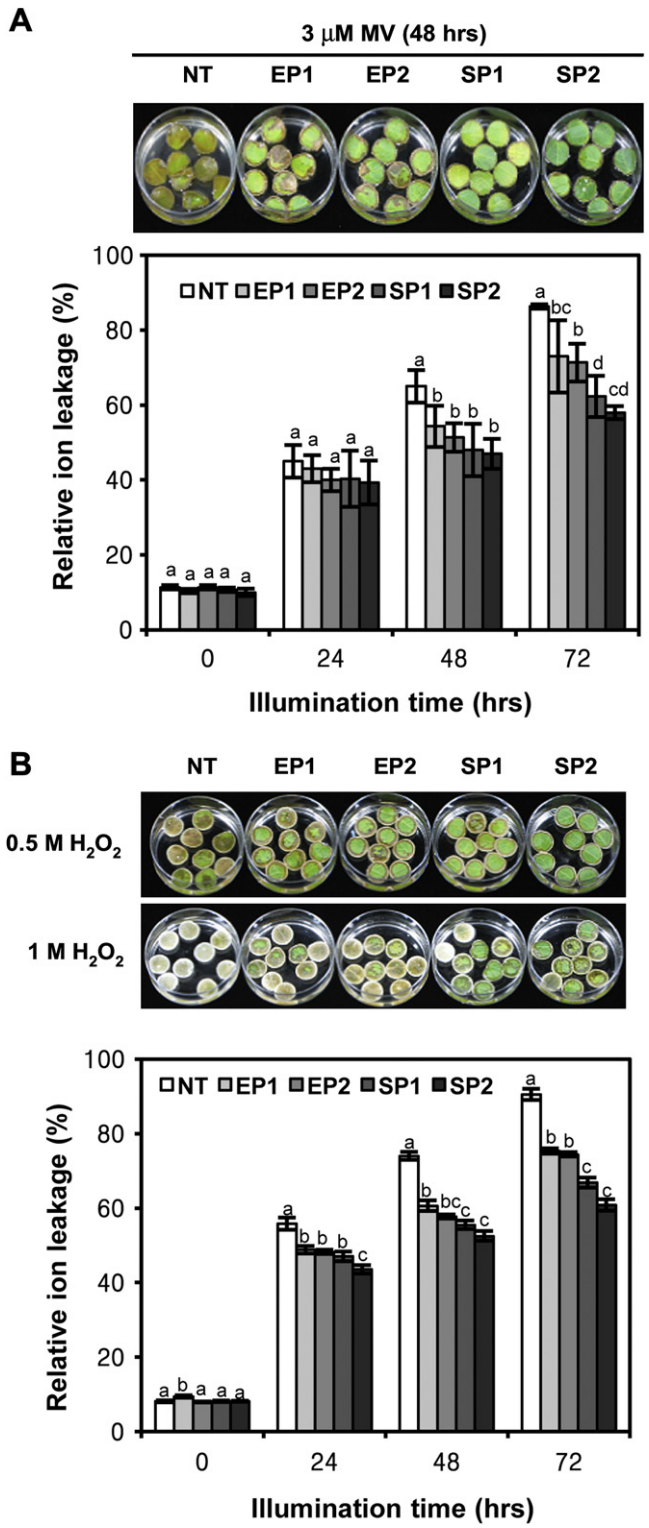

Fig. 3. Enhanced oxidative stress tolerance in transgenic potato plants. A, Photograph of leaf discs in the transgenic plants 48 h after 3 μM MV treatment and analysis of cellular damage in the MV-treated leaf discs of transgenic plants by electrolyte leakage for 72 h. B, Photograph of leaf discs in the transgenic plants 48 h after 1 M H_2O_2 treatment and analysis of cellular damage in the H_2O_2 treated leaf discs of transgenic plants for 72 h. Data are expressed as the mean ± SD of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

to SP and EP transgenic plants, respectively (Fig. 4B). The PSII photosynthetic efficiency in MV-treated potato leaves was estimated using a chlorophyll fluorescence determination of photochemical yield (F_v/F_m), which represents the maximal yield of the photochemical reaction in PS II. When transgenic and NT plants were treated with 300 μM MV, the PSII photosynthetic efficiency of NT plants was reduced significantly (approximately 60% of untreated plants), while the reduction in SP and EP plants decreased slowly by about 25% and 35%, respectively (Fig. 4C). Chlorophyll contents were determined using the central region of the fifth leaf from the top of plants. Under normal condition, Chlorophyll content did not differ significant between NT and transgenic plants (Fig. 4D). However, when treatment with 300 μM MV, NT plant chlorophyll contents were reduced by 60%, whereas in SP and EP plants, they were only reduced by 20% and 25%, respectively. These findings clearly suggest that transgenic potato plants expressing *At2-Cys Prx* exhibit enhanced tolerance to oxidative stress induced by MV.

The recombinant *At2-Cys Prx* expression pattern underlying tolerance to MV-mediated oxidative stress was examined in the transgenic potato plants (Fig. 5). Quantitative RT-PCR analysis of EP2 plants showed constitutively high levels of *At2-Cys Prx* transcription, whereas high levels of *At2-Cys Prx* expression were induced after 1 day of MV treatment in SP2 plants. The SP2 plant line exhibited the highest transcript level of *At2-Cys Prx* gene, consistent with the highest tolerance to MV stress. These results suggest that overexpression of *At2-Cys Prx* confers protection against MV-mediated oxidative stress in transgenic potato plants.

2.3. Enhanced tolerance to high temperature treatment

Tolerance to high temperature stress was assessed in the transgenic potato plants. When whole plants were exposed to high temperature at 42 °C for 10 h, the NT plants showed severe wilting from heat shock, whereas the SP plants appeared to remain healthy (Fig. 6A). Photosynthetic activity (F_v/F_m) was determined in the fifth leaf from each plant. Heat shock reduced the photosynthetic activity of NT plants by 31% after 10 h, while the activity of SP2 plants only decreased by 7% (Fig. 6B). Following 24 h of recovery at 25 °C, the F_v/F_m of SP plants had recovered to near normal levels, while that of NT plants remained low. EP plants showed intermediate levels between NT and SP plants. These results indicate clearly that SP plants exhibit enhanced tolerance to high temperature stress.

3. Discussion

Environmental stresses cause oxidative stress in plant cells via the rapid and excessive accumulation of ROS [1,2]. Many studies have focused on enhancing plant tolerance to stress, often by overexpressing genes associated with stress tolerance. 2-Cys Prx is an antioxidant that may act as signal to induce protection mechanisms [23]. In plant cells, 2-Cys Prx proteins function as both peroxidases and molecular chaperones [9,24]. In this report, we showed that overexpression of *2-Cys Prx* enhances tolerance to MV-mediated oxidative stress and high temperature stress in transgenic potato plants (Figs. 4 and 6). These results are consistent with a few reports showing that *2-Cys Prx* expression can provide increased tolerance to diverse stresses including MV and heat [15]. Transgenic *Arabidopsis* plants with partially suppressed *2-Cys Prx* expression exhibit impaired photosynthesis and increased oxidative damage to chloroplast proteins during early plant development [7]. Inactivated *2-Cys Prx* causes pale-green leaves, growth inhibition, reduced chlorophyll pigment content, decreased photosynthetic efficiency, and defective chloroplast development in *Ostrxm*

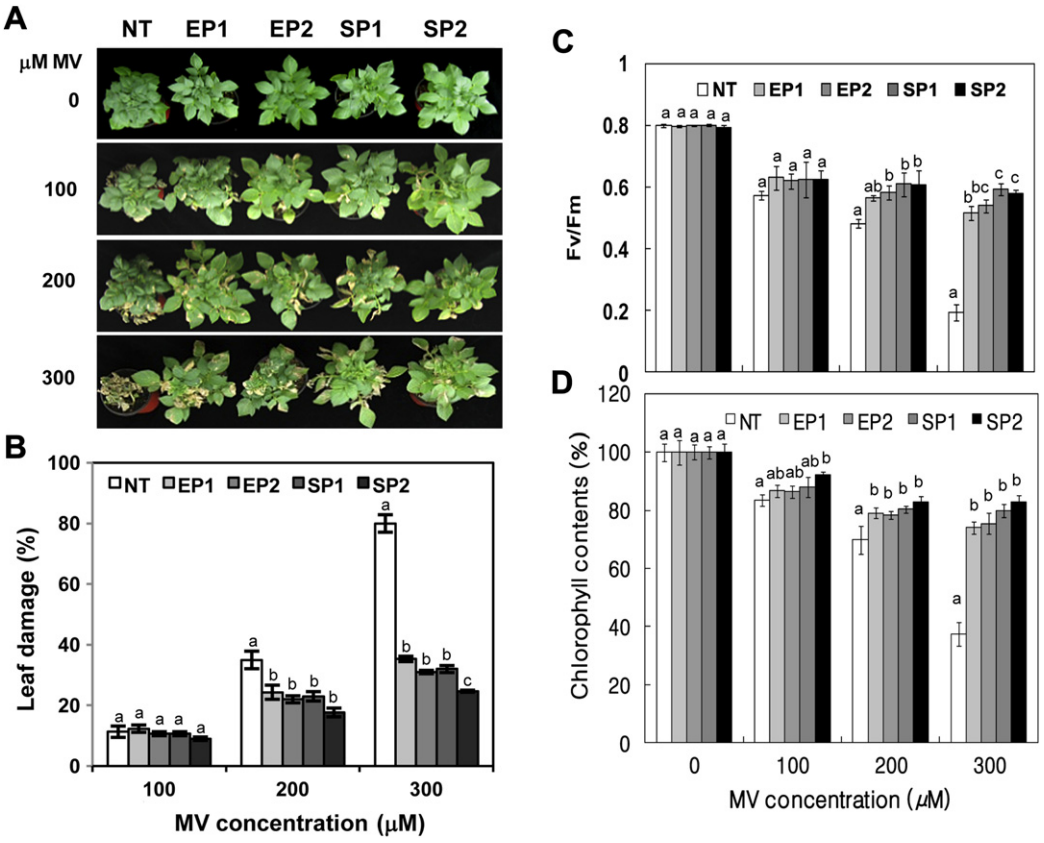

Fig. 4. Effect of MV-mediated oxidative stress on non-transgenic (NT) and transgenic (EP and SP) plants. A, Differential visible damages on leaves of transgenic potato plants. Photos were taken 5 days after treatment with 0, 100, 200 and 300 μM MV. B, Quantitative estimate of visible damage on leaves from NT, SP and EP plants. C, The photosynthetic activity (F_v/F_m). D, Total chlorophyll contents after MV treatment. Data are means ± SE of three independent measurements. Data are expressed as the mean ± SD of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

RNAi rice plants [25]. The findings described above and the results of this study suggest that *2-Cys Prx* overexpression protects transgenic potato plants from oxidative damage.

The present work describes development of transgenic potato plants expressing *At2-Cys Prx* under the control of the *SWPA2* promoter (SP plants) or the enhanced CaMV 35S promoter (EP plants), and evaluation of tolerance to oxidative stress induced by MV and high temperature. Five days after exposure to a high concentration of MV (300 μM), SP plants showed very little visible damage compared to NT plants. In addition, MV spray dramatically decreased the photosynthetic efficiency (F_v/F_m) and chlorophyll contents of NT plants, whereas these were only reduced slightly in SP plants (Fig. 4). The results for EP plants were intermediate between SP and NT plants. For development of stress-tolerant transgenic plants, the stress-inducible *SWPA2* promoter appears to be more efficient than the CaMV 35S promoter at providing appropriate overexpression of stress-tolerance genes. This study shows clearly that SP plants expressing *At2-Cys Prx* under control of the *SWPA2* promoter exhibit a significant increase in stress tolerance.

Temperature fluctuations can induce the formation of ROS [26]. Since the potato cultivar Atlantic is sensitive to high temperature conditions, the transgenic plants were tested for tolerance to stress caused by high temperature. After 10 h of heat shock at 42 °C, NT

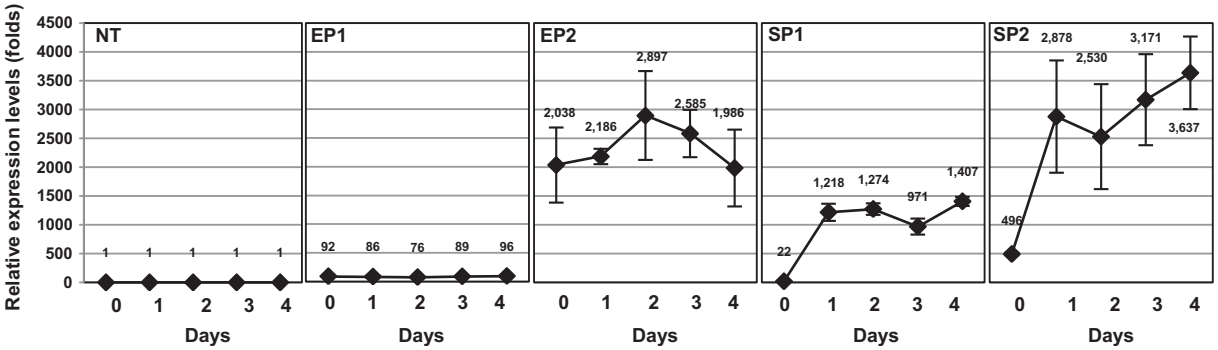

Fig. 5. Quantitative RT-PCR analysis of the expression of *At2-Cys Prx* gene in leaves from non-transgenic (NT) and transgenic (SP and EP) potato plants subjected to 100 μM MV spray. Data represent the means of three replicates.

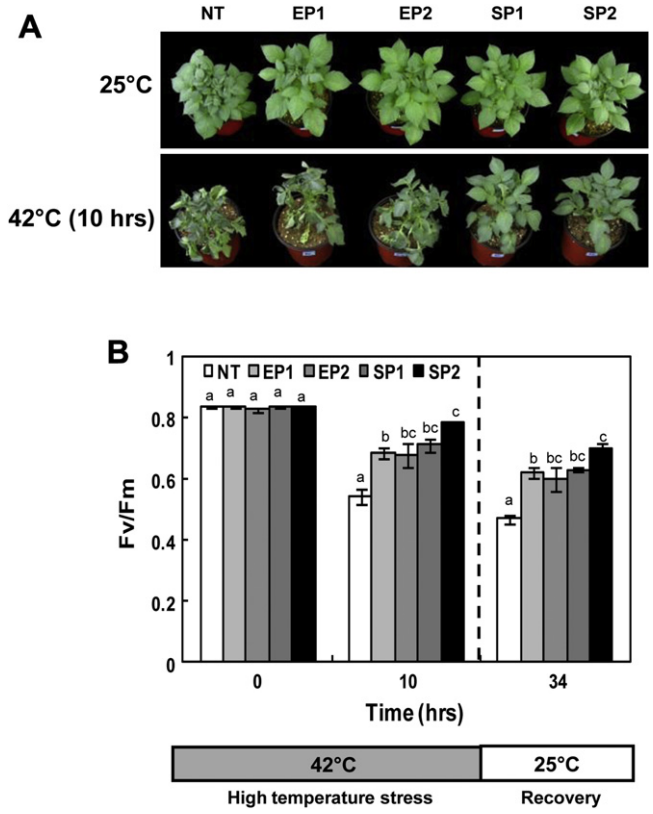

Fig. 6. Effect of high temperature (42 °C) on non-transgenic (NT) and transgenic (SP and EP) potato (cv. Atlantic) plants. A, Visible differential damages in the leaves of NT, SP and EP plants at 10 h after treatment and before treatment. B, Photosynthetic activity (F_v/F_m) in the leaves high temperature treatment and recovery. NT, non-transgenic potato plants; SP1-2 & EP1-2, transgenic potato plants. Data are expressed as the mean ± SD of three replicates. Bars carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

plants were severely damaged, whereas SP plants appeared to remain healthy (Fig. 6). In addition, the photosynthetic activity of SP plants recovered to near pre-stress levels after 24 h at 25 °C, but NT plants did not recover. Similar results have been reported in other studies that expressed antioxidative enzymes under stress conditions [4,5,18,27]. The results of this study suggest that transgenic potato plants expressing *At2-Cys Prx* exhibit enhanced tolerance to high temperature stress.

In conclusion, transgenic potato plants expressing *At2-Cys Prx* under the control of the stress-inducible *SWPA2* promoter or constitutive CaMV 35S promoter were developed successfully. These plants exhibit enhanced tolerance to environmental stress including MV-induced oxidative stress and high temperature, with the best tolerance shown by SP plants expressing *At2-Cys Prx* under control of the *SWPA2* promoter. Although these transgenic potato plants require further characterization in terms of yield and growth under field conditions, it is anticipated that they might be useful for sustainable agriculture in marginal lands.

4. Materials and methods

4.1. Plant materials and vector construction

Potato plants (*S. tuberosum* L. cv. Atlantic) were propagated via sub-culturing of shoot tips and stem nodal sections every 3–4 weeks on MS [28] basal medium containing 3% sucrose. Two transformation vectors were constructed, which expressed *At2-Cys Prx* under the control of the stress-inducible *SWPA2* promoter [17] or enhanced CaMV 35S promoter. Full-length *At2-Cys Prx* (accession no. Y10478/At3g11630) cDNA was amplified by PCR using a forward primer (5′-ATGGCGTCTGTTGCTTCTTCAAC-3′) containing an *Xba*I site and a reverse primer (5′-CTAAATAGCTGAGAAGTACTCTTT GCTGAG-3′) with a *Sac*I site. The cDNA was ligated into the corresponding sites in pRTL2 (E35Sp::At2-Cys Prx/pRTL2). In order to generate a vector containing the *SWPA2* promoter, the enhanced 35S promoter (E35Sp) fragment was replaced by the *SWPA2* promoter (SWPA2p::At2-Cys Prx/pRTL2). These completed chimeric gene cassettes were inserted into the *Hin*dIII site of the binary vector pCAMBIA2300 and the constructs were named pEP-K and pSP-K, i.e., E35Sp::At2-Cys Prx/pCAMBIA2300 and SWPA2p::At2-Cys Prx/pCAMBIA2300, respectively (Fig. 1). The recombinant vectors were then transformed into *Agrobacterium tumefaciens* EHA105.

4.2. Plant transformation and selection

For potato transformation, leaf explants from 3- to 4-week-old plants were pre-cultured for 2 days on MS basal medium containing 0.2 mg l^{-1} 2,4-D (pre-culture medium). These explants were inoculated for 10 min with *Agrobacterium* harboring the expression vectors pEP-K or pSP-K, and then co-cultured on pre-culture medium for two days in darkness. After co-culture, the explants were grown on regeneration medium (MS medium containing 0.01 mg l^{-1} NAA, 0.1 mg l^{-1} GA_3, 2 mg l^{-1} zeatin, 400 mg l^{-1} cefotaxime, and 100 mg l^{-1} kanamycin), and then transferred to fresh medium at 2 week intervals. When the regenerated shoots were 15 mm long, they were cut and transferred to rooting medium (MS medium containing 3% sucrose, 400 mg l^{-1} cefotaxime and 100 mg l^{-1} kanamycin). Plantlets capable of developing good root systems on selection medium were selected for further study.

To select transgenic plants, genomic PCR was performed using following primers: for the *2-Cys Prx* gene, 5′-TCTAGAATGGCGT CTGTTGCT-3′ and 5′-GAGCTCCTAAATAGCTGAGAA-3′; and the *nptII* gene, 5′-GAGGCTATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGC-GATACCGTA-3′. For Southern blot analysis, genomic DNA was isolated from the leaves of non-transgenic (NT) and transgenic (SP, EP) plants. Twenty μg of genomic DNA were digested with *Eco*RI, electrophoresed on a 0.8% (W/V) agarose gel and blotted onto Zeta-probe GT membranes (Bio-Rad, CA, USA). The blots were hybridized with α ^{32}P-labeled PCR probes amplified from *At2-Cys Prx*. Hybridization was carried out in 0.5 M sodium phosphate buffer (pH 7.2), 7% SDS and 1 mM EDTA at 65 °C.

4.3. Ion leakage analysis using leaf discs

The leaf disc assay for oxidative stress tolerance was conducted as described by Kwon et al. [3]. Leaf discs (8 mm in diameter) were obtained using the fifth and sixth leaves from the top of 8-week-old plants cultivated in a growth chamber. Discs were floated on a 0.4% sorbitol solution containing 3 μM MV and H_2O_2 (0.5 and 1 M) solution. The samples were incubated in darkness for 12 h to allow for MV diffusion and then illuminated under continuous light (150 μmol photon m^{-2} s^{-1}) at 25 °C. Ion leakage of solution was assessed using an ion conductivity meter (model 455C, Isteck Co., Seoul, Korea) over a time period ranging from 0 to 72 h. At the end of the specified time period, the samples were autoclaved for 15 min at 121 °C in order to release all of the solutes. For calculations of the relative ion leakage at different time points, the conductivity was re-measured, and this value was considered to represent 100% of ion leakage.

4.4. Stress treatment of whole plants

MV (0, 100, 200, or 300 μM) dissolved in 0.1% (w/v) Tween 20 solution was sprayed on the leaves of whole plants cultivated in a growth chamber for 8 weeks. The MV solution (70 ml) was applied to five potato plants using a spray booth (Model SB-6: Devries Manufacturing, Hollandale, MN, USA) with an 8001 VS-type nozzle, 0.5 inches s^{-1} and 0.22 MPa. Photosynthesis activity (F_v/F_m), chlorophyll contents and the percentage of leaf damage were determined after 5 days of treatment.

For high temperature stress, 8-week-old potato plants cultivated at 25 °C in a growth chamber were transferred to a 42 °C growth chamber for 10 h. Treated plants were then returned 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 by photosynthesis activity (F_v/F_m).

4.5. Determination of PSII photosynthetic efficiency and total chlorophyll content

In leaves treated with MV or high temperature, PSII photosynthetic efficiency was estimated using chlorophyll fluorescence determination of photochemical yield (F_v/F_m). Measurements were performed with a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England). Chlorophyll content was measured in 0.1 g fresh weight leaf material, which was quickly frozen in liquid nitrogen and then extracted with 2 ml methanol. Samples were centrifuged at 12,000 *g* for 15 min at 4 °C, and supernatant chlorophyll content was analyzed using a spectrophotometer, as described by Porra et al. [29].

4.6. Gene expression analysis

Total RNA was isolated from leaves of potato using TRIzol reagent (Invitrogen, Carlsbad, CA). Samples were treated extensively with RNase-free DNase I, in order to remove any contaminating genomic DNA. RT-PCR amplification was conducted using an RT-PCR kit (Promega, Madison, WI), in accordance with the manufacturer's instructions. MMLV reverse transcriptase was used to generate first-strand cDNA from total RNA (1 μg). The *At2-Cys Prx* primer set (5′-TCGACAAGGAAGGAGTGATCCA-3′, 5′-TCTTTGCTGAG TTTTGGGTCGG-3′) was used to amplify a 184 bp product from cDNA encoding *At2-Cys Prx*. As an internal standard, *actin* gene-specific primers (5′- CCATGTTCCCTGGTATTGCTGA-3′, 5′-TCGTCA-TACTCTGCCTTTGCAATC-3′) were used to amplify a 181 bp product from total cDNA. For quantitative RT-PCR, fragments of interest were amplified by real-time PCR using gene-specific primers and EverGreen fluorescent dye.

4.7. Statistical analysis

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

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