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Development of Selection Marker-free Transgenic Potato Plants with Enhanced Tolerance to Oxidative Stress

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A binary vector devoid of a plant selection-marker gene (designated as pSSA-F) was constructed to overcome bio-safety concerns about genetically modified plants. This vector carried chloroplast-targeted superoxide dismutase (SOD) and ascorbate peroxidase (APX) genes under the control of an oxidative stress-inducible (*SWPA2*) promoter, and was utilized to transform potato (*Solanum tuberosum* L.). Integration of these foreign genes into transgenic plants was primarily performed via PCR with genomic DNA. Twelve marker-free transgenic lines were obtained by inoculating stem explants. The maximum transformation efficiency was 6.25% and averaged 2.2%. Successful integration of the SOD and APX genes rendered transgenic plants tolerant to methyl viologen-mediated oxidative stress at the leaf-disc and whole-plant levels. Our findings suggest that this technique for developing selection marker-free transgenic plants is feasible and can be employed with other crop species.

Keywords: genetic transformation, marker-free transgenic plants, oxidative stress, potato

In the developing world, 840 million people are chronically undernourished and survive on only minimal calories each day (Christou and Twyman, 2004). This scarce food supply is due to many factors, including the effect of abiotic stresses on crops. Productivity could be increased substantially by decreasing those detrimental effects. Plant molecular breeding is advantageous to a large number of people because of its tremendous potential to develop crops with desired characteristics in a significantly shorter time span. However, the use of selection-marker genes has introduced environmental and consumer concerns over safety (Ramessar et al., 2007). Therefore, it is necessary to eliminate those genes if we are to harvest the maximum benefits from advancements made in the field of agricultural biotechnology.

Strategies used to eliminate marker genes have included 1) co-transformation of genes of interest with selectable marker genes, followed by segregation of the separate genes through sexual crosses (Ebinuma et al., 2001); 2) co-transformation with negative selection markers (Park et al., 2004); and 3) utilization of site-specific recombinases (Li et al., 2007). Unfortunately, marker-removal methods are often too immature and inefficient to allow their widespread adoption in product development (Koning, 2003), especially in asexually reproducing or vegetatively propagated crops. However, de Vetten et al. (2003) have used a T-DNA region free of a marker-gene cassette to create marker-free transgenic plants. This system provides a distinct advantage over other systems in that it does not require secondary screening and, subsequently, may be a less time-consuming bio-safety process.

Potato (*Solanum tuberosum* L.) is a major food crop in many regions, ranking fourth worldwide in production after wheat, maize, and rice (Newell et al., 1991). However, its plants are vulnerable to several abiotic factors, including oxidative stress (Kim et al., 2007). Mindful of the potential from genetic engineering, we must address the concerns of consumers toward GMO (genetically modified organisms) products. Here, we investigated selection marker-free plants that expressed pea Cu/Zn SOD and APX targeted to chloroplasts under the control of the stress-inducible *SWPA2* promoter. We monitored their expression of foreign genes for stability and examined their tolerance to oxidative stresses.

MATERIALS AND METHODS

Plant Material and Plasmid Construction

Sterile plants of potato (*Solanum tuberosum* L.; cv. Superior) were grown for our transformation experiments. They were propagated by sub-culturing shoot tips and stem nodal sections every 3 to 4 weeks on an MS medium containing 3% sucrose (Murashige and Skoog, 1962). The plant expression vector pSSA-F was constructed using pNL, a mini binary vector modified from pNC (Park et al., 2004; Xiang et al., 1999). This was accomplished by removing the *codA* and *nptII* genes and adding multiple cloning sites. The expression cassettes for pea Cu/Zn SOD (hereafter SOD; Sen Gupta et al., 1993) and APX (Allen et al., 1997) were transcriptionally fused to the oxidative stress-inducible *SWPA2* promoter of sweetpotato (Kim et al., 2003) and

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loaded into pNL using the *Hin*dIII and *Pst*I sites (Figure 1A). pSSA-F was then mobilized into the AGL0 strain of *Agrobacterium tumefaciens*.

Plant transformation and Regeneration

Agrobacterium harboring the pSSA-F vector was cultured for 48 h at 28°C in 5 mL of a YEP medium containing 100 mg rifampicin L^{-1}. Transformations were carried out as described by Ahmad et al. (2008). The first shoot regenerates were acquired after one month and were transferred to a rooting medium (MS plus 3% sucrose and 400 mg cefotaxime L^{-1}).

PCR and Southern Blot Analyses of Putative Transgenic plants

To screen for selection marker-free transgenic plants, we harvested the leaf material from five independent shoots. Their pooled genomic DNA was extracted according to the protocol of Kim and Hamada (2005), and was tested for the presence of transformants via PCR with the following primers: APX, 5′-ATACAAAAAAACGAATCTC-3′ (forward) and 5′-CAGTCTACAACTCAGTGAAA-3′ (reverse); and SOD, 5′-ATACAAAACAAACGAATCTC-3′ (forward) and 5′-ACCACAC-CACAAGCTAATCT-3′ (reverse). PCR conditions included 94°C for 5 min (1 cycle); followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; then a final extension at 72°C for 7 min. Subsequent PCR analysis was conducted on the DNA isolated from individual plants of the PCR-positive pools to select for true transformants.

For Southern blot analysis, 20 μg of genomic DNA was digested by *Eco*RV, then subjected to electrophoresis on a 0.8% (w/v) agarose gel. Afterward, the gel was denatured in a bath of 0.4 M NaOH, then blotted to a Zeta-probe GT blotting membrane (Bio-Rad, CA, USA) by capillary transfer. The probe was labeled with [α-^{32}P] dCTP, using a Redi prime kit (Amersham, USA). This membrane was prehybridized at 65°C for 2 to 4 h in a buffer containing 0.25 M sodium phosphate buffer (pH 7.2) and 7% SDS. It was then hybridized at 65°C for 18 to 24 h in the presence of the denatured, labeled 0.4-kb chimeric CuZnSOD fragment and washed three times (10 min each) at 65°C in 0.02 M sodium phosphate buffer (pH 7.2) and 1% SDS. All subsequent experiments were conducted on the T_0 generation of transgenic plants.

MV-mediated Oxidative Stress Treatment

Discs were punched from the fifth to sixth leaf from the top of 5- to 7-week-old plants, and were floated on a solution containing 3 or 5 μM methyl viologen (MV) in 0.4% sorbitol solution. After incubation in the dark, these discs were placed under continuous light (150 mmol photon m^{-2} s^{-1}) at 25°C. Ion leakage of this solution was assessed with an ion conductivity meter (Model 455C; Isteck Co., Seoul, Korea) over a period of 0 to 60 h. Afterward, the samples were autoclaved for 15 min at 121°C to release all the solutes. Conductivity was measured again and values were used to calculate relative ion leakage.

For MV treatment at the whole-plant level, we followed the method described by Tang et al. (2008). Briefly, 4-week-old greenhouse-grown plants were treated with MV (0, 300, 350, or 400 mM) dissolved in 20% acetone and 0.1% Tween-20 solution. To test the effect of MV, applications were repeated in triplicate. Chlorophyll contents and the percentage of leaf damage were determined after 5 d of treatment.

RT-PCR Analysis

Leaf discs from 6-week-old soil-grown plants were treated with 5 μM MV. Samples were collected after 0, 24, 48, and 72 h of treatment and immediately frozen in liquid nitrogen. Total RNA was extracted via the cetyltrimethylammonium bromide (CTAB) method (Kim and Hamada, 2005), and treated with RNase-free DNase I to remove any contaminating genomic DNA. The RNA (2 μg) was used for reverse-transcription in accordance with the manufacturer's instructions (Promega, USA). PCR was conducted with 0.5 μL of first-strand cDNA. The primers for actin (as our internal control) were 5′-TGGACTACTGGTGATGGTGTC-3′ (forward) and 5′-CCTCCAATCCAAACACTGTA-3′ (reverse). For APX and SOD, the same primers were utilized as for the genomic DNA. PCR conditions for actin, APX, and SOD were as described in the previous section except in the extension time for actin, which was 30 s per cycle.

Analysis of SOD and APX Activities

Potato leaves treated with 0 or 5 μM MV were homogenized with a mortar on ice in a 0.1 M potassium phosphate buffer (pH 7.0). This homogenate was centrifuged at 12,000×g for 15 min at 4°C, and the supernatant was used immediately for enzyme assays. Protein concentration was determined according to the method of Bradford (1976). SOD activity was measured according to the protocol of McCord and Fridovich (1969), using xanthine, xanthine oxidase, and cytochrome c. One unit of SOD was defined as the amount of enzyme that inhibited the rate of ferricytochrome c reduction by 50%. APX activity was assayed according to the method described by Nakano and Asada (1981), using ascorbic acid as a substrate. The oxidation of ascorbate was initiated by H_2O_2; we monitored its decrease at 290 nm for 1 min 30 s. One unit of APX was defined as the amount of enzyme oxidizing 1 mM of ascorbate per min.

High-temperature Treatment

To determine the influence of high temperature at the whole-plant level, we reared transgenic and non-transgenic plants in a growth chamber. After 5 weeks, they were exposed to 42°C in the incubator. These stressed plants were then transferred to normal conditions (25°C, 100 μE m^{-2} s^{-1}) for recovery. To measure photosynthesis activity, their fifth leaves were evaluated at 0, 10, and 20 h after treatment. Fresh-weight percentages were calculated after 20 h of heat shock (42°C) as compared with untreated plants continuously grown at 25°C.

Statistical Analysis

All MV treatments and analyses of enzyme activity were

conducted three times. Measurement data were subjected to Student's t-tests, using Microsoft Excel 2003, and statistical significances were defined at $P \leq 0.05$.

RESULTS

Generation of Selection Marker-free Plants

The 'Superior' potato, an important cultivar for table and industrial usage, was transformed to obtain selection marker-free plants. In seven independent experiments, around 500 explants were inoculated with the *Agrobacterium*-harboring pSSA-F vector (Figure 1A). Over two months of inoculation, one or two regenerated shoots were harvested and transferred to a rooting medium. Using gene-specific SOD or APX primers, we obtained 12 PCR-positive lines that contained the transgene (Figure 1B). Selection marker-free (SSAF) plants exhibited successful transcription of the transgenes, as evidenced via RT-PCR after treatment with methyl viologen (data not shown). We also performed Southern blot analysis of two selected lines to confirm the integration of T-DNA into the potato genome. Genomic DNA from the PCR-positive Lines SSAF4 and SSAF6 was blot/hybridized against a CuZn SOD gene-specific probe to verify further this proper integration. Both lines showed positive signals depicting multiple copies of T-DNA. These data demonstrated successful transformation and integration of transgenes into the potato genome.

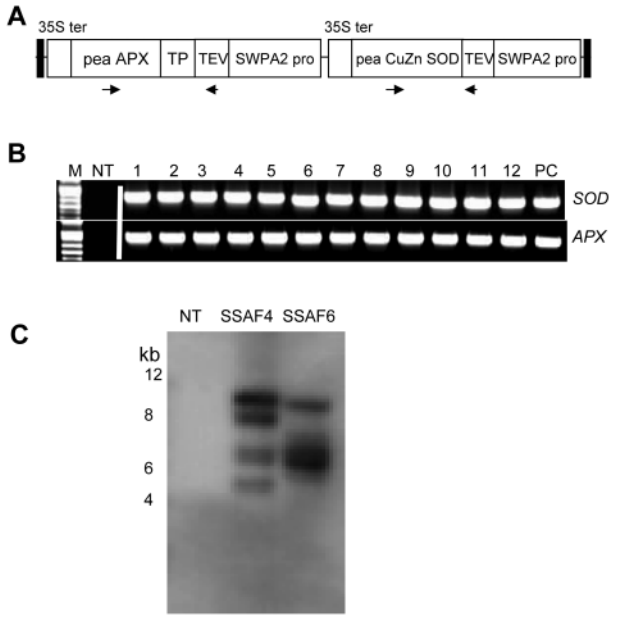

Figure 1. Generation of selection marker-free transgenic potato. **A**, Plant transformation vector pSSA-F. Horizontal arrows indicate PCR primer sites. *SWPA2* pro, sweetpotato peroxidase anionic 2 promoter; SOD, pea Cu/Zn superoxide dismutase; APX, pea ascorbate peroxidase; TEV, tobacco etch virus 5-UTR; 35S ter, CaMV 35S terminator; TP, chloroplast-targeted transit peptide. **B**, Genomic DNA PCR analysis of SOD and APX genes from transgenic plants. Numbers represent independent transgenic lines. SM, size marker; NT, non-transgenic plant; PC, positive control. **C**, Southern blot analysis of selection marker-free (SSAF4, SSAF6) transgenics. Genomic DNA from transgenic and non-transgenic plants was hybridized with CuZn SOD gene-specific probe.

Table 1. *Agrobacterium*-mediated transformation efficiency of potato plants.

Experimental numbers	Regenerated plants	PCR positive plants	Efficiency (%)
1	89	1	1.12
2	130	1	0.77
3	113	1	0.88
4	80	3	3.75
5	32	2	6.25
6	51	2	3.92
7	61	2	3.27
Total	556	12	2.2

In all, 556 regenerated shoots were screened via PCR to isolate true transgenics; 12 plants were obtained, for an average transformation efficiency of 2.2% (Table 1) and a maximum efficiency of 6.25%.

Line Selection for Further Characterization

Treating plants with MV led to their production of ROS, with this over-accumulation inducing oxidative stress that disrupted membrane integrity. All 12 of the SSAF lines were maintained in a growth chamber for six weeks, and leaf discs were evaluated for enhanced tolerance against MV-mediated oxidative stress. Discs were treated with 3 mM MV and ion leakage was measured over a time course. Among the 12 lines, 8 showed greater tolerance as manifested by lower levels of ion leakage (data not shown). The two most tolerant lines (SSAF4 and SSAF6) were selected for further characterization.

Oxidative Stress Tolerance of Transgenic SSAF Potato plants

Leaf discs were prepared from non-transgenic (NT) and SSAF plants of the same age, and were incubated in 5 μM MV. The protective effect of SOD and APX was proven after 24 h, and SSAF plants from both lines (SSAF4, 29%; SSAF6, 31%) had less ion leakage after the 60-h treatment period compared with NT plants (60%) (Figure 2).

To investigate oxidative-stress tolerance at the whole-plant level, both SSAF and NT stock were evaluated for visible damage 5 d after being sprayed with solutions containing 0, 300, 350, or 400 μM MV (Figure 3A). At 300 μM, NT plants showed damage on 28% of their leaf area versus 10 to 12% of the area on SSAF plants. Damage on NT plants became more severe (80%) upon exposure to 400 μM MV, compared with 25% and 30% damage to Lines SSAF4 and SSAF6, respectively (Figure 3B). Chlorophyll contents also were measured in the central portion of the fifth leaf from MV-treated plants (Figure 3C). Under normal conditions, chl values did not differ significantly between NT and SSAF samples. However, when treated with 400 μM MV, NT plants exhibited a 51% decrease in their level of chlorophyll compared with the untreated NT plants. Moreover, chlorophyll contents in the SSAF4 and SSAF6 plants were attenuated by 18% and 23%, respectively, after exposure to the

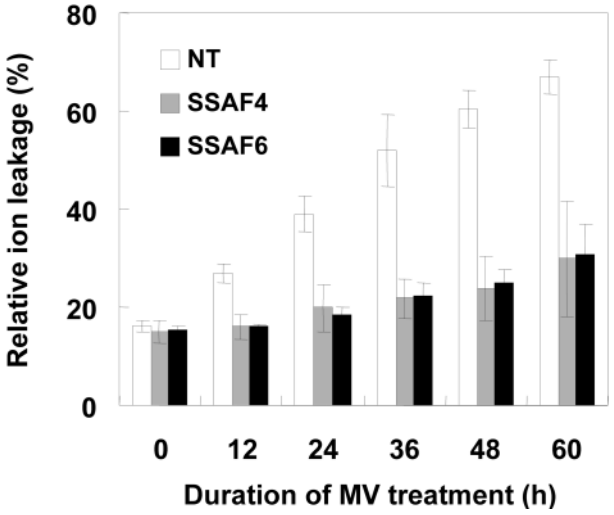

Figure 2. Effect of MV-mediated oxidative-stress treatment on ion leakage by SSAF4 and SSAF6 independent transgenic plants. Leaf discs were treated with 5 μM MV, then incubated at 25°C under light intensity of 150 μmol photons $m^{-2} s^{-1}$. Ion leakage was measured after 0, 12, 24, 36, 48, and 60 h of treatment. Percentages of ion leakage were calculated using 100% to represent values obtained after autoclaving. NT, non-transgenic. Data are expressed as means ± standard deviation (SD) of 3 replicates. Bars labeled with *asterisk* show significant differences between NT and SSAF plants by t-test at $P = 0.05$.

same MV concentration (Figure 3C).

RT-PCR and Analysis of SOD and APX Activities in Transgenic Plants

To further evaluate the successful introduction and transcription of foreign genes into SSAF plants, we analyzed their transcript levels by RT-PCR. Leaf discs were treated with 5 μM MV to induce the expression of SOD and APX. The foreign genes were profoundly expressed after 24 h of stress whereas, under non-stressed conditions, i.e., at 0 h, transcripts were not detected (Figure 4A). Expression levels of SOD and APX were slightly higher in Line SSAF4 than in SSAF6. We also evaluated changes in enzyme activities in treated leaf discs. Transgenic plants maintained higher SOD activity than the NT at 24 h of MV treatment compared with no significant differences in activities from either NT or SSAF plants that were not stressed (Figure 4B). Similarly, APX activity was higher in SSAF plants (Figure 4C). Our native page analysis also showed markedly induced SOD- and APX-specific activities in SSAF and NT plants (data not shown). These results demonstrate not only the successful transcription and post-transcriptional processes of foreign genes in SSAF plants but also the mechanism for enhanced protection against MV-mediated oxidative stress.

Tolerance to High-temperature Stress by Transgenic Potato Plants

Five-week-old greenhouse-grown NT and SSAF plants were exposed to high temperature (42°C) for 20 h. The former showed pronounced wilting afterward, whereas leaves on SSAF plants were clearly less sensitive (Figure 5A).

The quantum yield of PSII, defined by the fluorescence

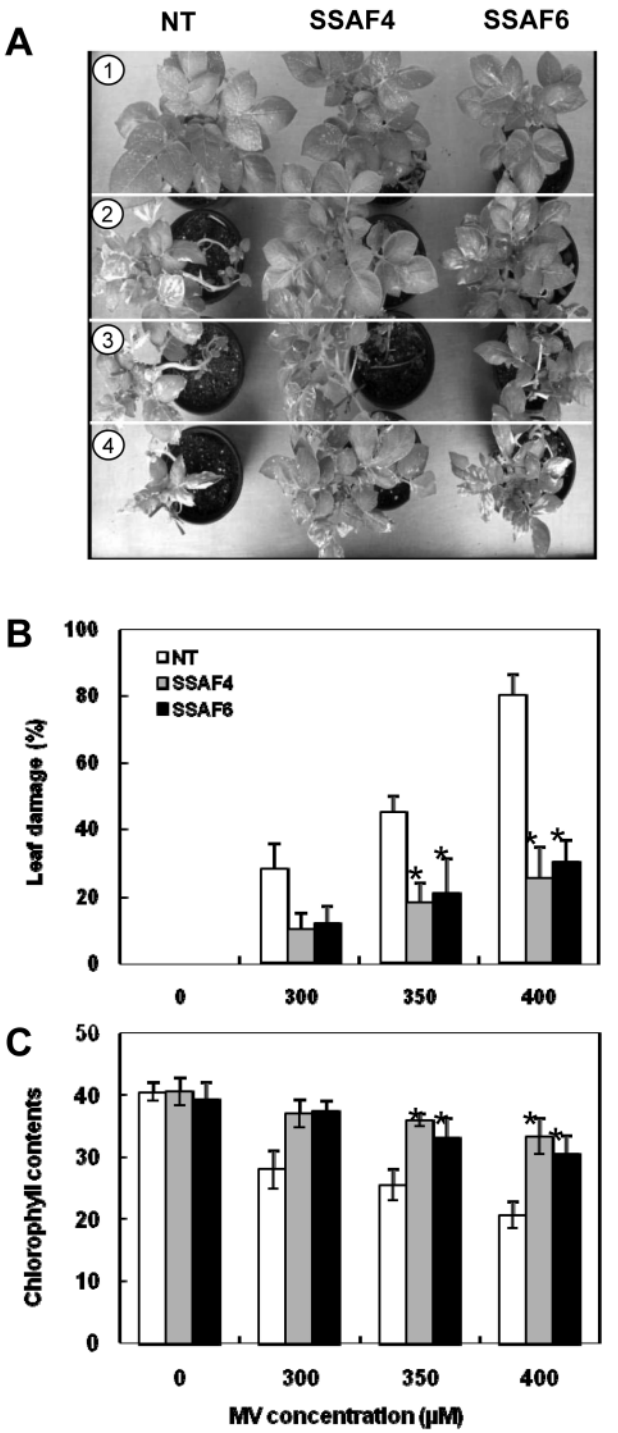

Figure 3. Effect of MV-mediated oxidative stress on non-transgenic (NT) and SSAF transgenic plants. Differential visible damage on leaves. **A**, Photos taken 5 d after treatment with 0 (1), 300 (2), 350 (3), or 400 mM MV (4). **B**, Quantitative estimate of visible damage on leaves from NT and SSAF plants 5 d after MV treatment. **C**, Chlorophyll contents in fifth leaf from NT and SSAF plants. Data are expressed as means ± SD of 3 replicates. Bars labeled with *asterisk* show significant differences between NT and SSAF plants by t-test at $P = 0.05$.

parameter Fv/Fm, was determined in the fifth leaf from the top of each plant. Measurements showed that photosynthesis by SSAF plants was slightly diminished during high-tem-

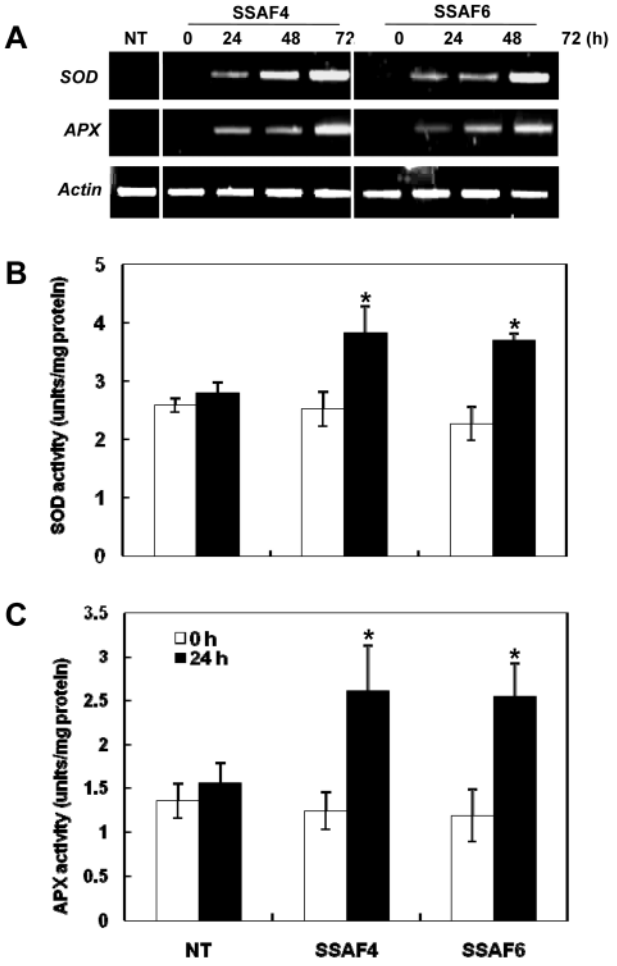

Figure 4. RT-PCR of SOD and APX genes and analysis of enzyme activity in leaves from NT and SSAF plants subjected to 5 mM MV. **A**, Total RNA was extracted from leaves 0, 24, 48, and 72 h after treatment. Actin served as internal control. **B**, SOD activity in leaves of NT and SSAF plants subjected to 5 mM MV. Total cellular activity measured spectrophotometrically after 0 and 24 h of treatment. **C**, APX activity in leaves of NT and SSAF plants subjected to 5 mM MV. Total cellular activity was measured at 0 and 24 h after treatment. Data are expressed as means ± SD of 3 replicates. Bars labeled with *asterisk* show significant differences between NT and SSAF plants by t-test at $P = 0.05$.

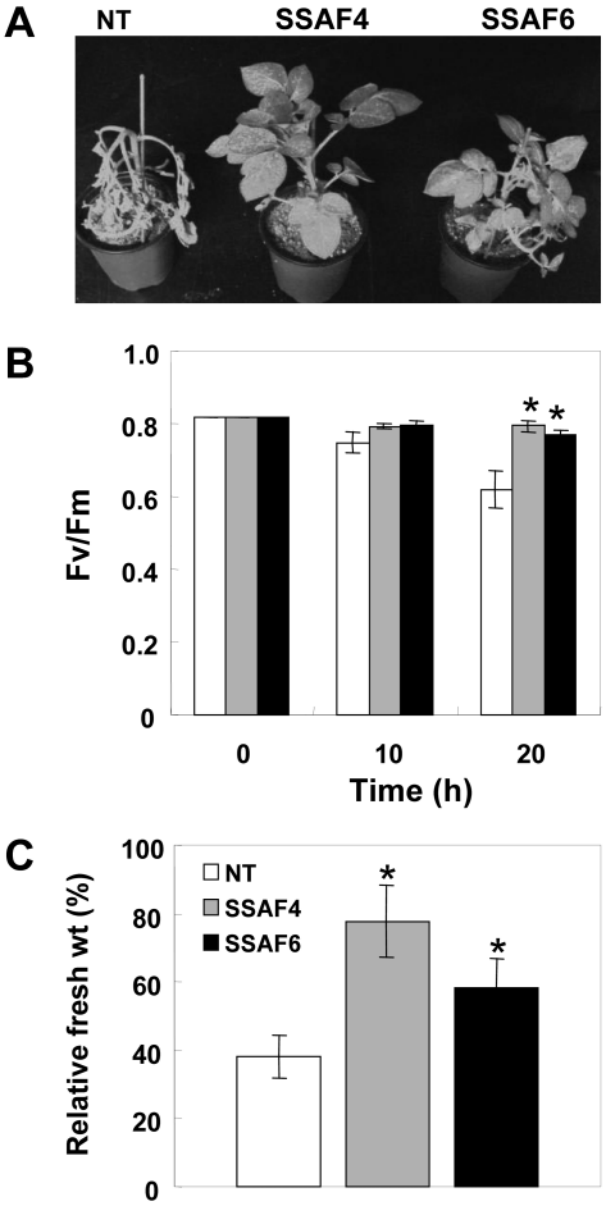

Figure 5. Effects of high temperature (42°C) on non-transgenic (NT) and transgenic SSAF plants. **A**, Visible differential damage in leaves 20 h after treatment. **B**, Photosynthetic activity (Fv/Fm) in leaves of NT and SSAF plants for 20 h after treatment. **C**, Plant fresh weights at 20 h after treatment. Percentages of fresh weight were calculated on basis of untreated plants grown at 25°C. Data are means ± SD of 3 independent measurements. Bars labeled with *asterisk* show significant differences between NT and SSAF plants by t-test at $P = 0.05$.

perature treatment compared with a greater reduction in stressed NT plants (Figure 5B). PSII activity in NT plants declined 25% at 20 h after heat shock from pre-stress values, while activity in SSAF4 and SSAF6 plants decreased by only 3% and 6%, respectively (Figure 5B), relative to the NT.

Fresh weights for heat-stressed plants (20 h at 42°C) were also compared with those of controls grown at 25°C (Figure 5C). In NT plants, high-temperature treatment caused a significant decrease in fresh weight (62%) from that of the control plants. In contrast, fresh weights for SSAF4 and SSAF6 plants remained higher than for NT plants, dropping 23% and 42%, respectively (Figure 5C).

DISCUSSION

To obtain selection marker-free transgenic potato plants, we constructed a pSSA-F vector. Potato is a highly heterozygous crop, so vegetative propagation is preferable for maintaining elite genotypes. Compared with other methods for developing marker-free transgenic plants, the technique employed here has various advantages. We based our idea on the regeneration of transgenic plants without the use of a selection-marker gene so that genetic segregation was not necessary. This strategy is suitable for breeding vegetatively propagated crops such as potato (Gleave et al., 1999; Zuo et al., 2001). Transposon and recombinase methods also have been applied, but both require another cycle of regen-

eration to remove that marker gene, a step that makes those transgenic plants more vulnerable to somaclonal variation while also lengthening the production process. In contrast, our system entails a significantly shorter time span to develop and characterize selection marker-free transgenic plants.

From the 556 regenerated shoots screened via PCR, we isolated 12 transgenic plants, with average and maximum transformation efficiencies of 2.2% and 6.25%, respectively. Because only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without such a selection marker are usually low. Therefore, we might attribute the differences in transformation efficiency presented here to other factors during the transformation and regeneration steps (Miki and McHugh, 2004).

Plants are highly susceptible to abiotic stress, which can then reduce crop yields up to 80% (Bary et al., 2000). Oxidative stress is always implicated, and may cause severe cell damage and, ultimately, plant mortality (Allen et al., 1997). In this context, it is important to develop transgenic plants with enhanced stress tolerance, adopting approaches that can be accepted easily for commercial uses and that will ensure better performance in harsh environments. The SOD or APX isoenzymes in chloroplasts have key roles in protecting plants against such stress (van Camp et al., 1997; Badawi et al., 2004). In addition to selecting effective genes for imparting tolerance in transgenic plants, the use of a suitable promoter is very critical because inducible promoters can negate the unexpected adverse effects on a transgene phenotype (Kasuga et al., 1999). While considering the overwhelming acceptance of stress-inducible promoters, we have now generated selection marker-free transgenic plants that express chloroplast-targeted SOD and APX under control of the *SWPA2* promoter. Likewise, transgenic potato plants expressing NDPK2 (nucleoside diphosphate kinase 2) driven by the *SWPA2* promoter perform better than plants with constitutive expression (Tang et al., 2008).

Our selection marker-free SSAF plants showed enhanced tolerance to MV-mediated oxidative stress (Figures. 2, 3). To validate and understand this tolerance mechanism, we investigated post-treatment changes in transcripts and enzymatic activities of the SOD and APX genes. The degree of stress tolerance by SSAF4 and SSAF6 plants appeared to be correlated with transcript level and enzymatic activity (Figure 4). SOD and APX are important components of the antioxidative mechanism, and are effective in protecting plants against oxidative stresses.

We also evaluated the enhanced tolerance of SSAF transgenics to high-temperature stress at the whole-plant level. When temperatures are higher than 29°C, tuber formation is adversely affected (Khedher and Ewing, 1985). Here, NT plants were severely wilted after 20 h of exposure to 42°C, while SSAF plants had only minor wilting symptoms (Figure 5). When Fv/Fm values were calculated, photosynthesis activity in the transgenics was slightly reduced, while that process was significantly decreased in the NT plants. Other evidence has been reported previously about the relationship between high temperature-induced oxidative stress and the induction of antioxidant enzymes in plants (Gong et al., 1998; Storozhenko et al., 1998). Here, our results confirm the role of such enzymes in conferring tolerance to high temperatures.

In conclusion, we have demonstrated the feasibility of a method for developing transgenic potato plants that are tolerant to heat and oxidative stresses. Our system, which introduces a T-DNA region devoid of a selection-marker gene, holds advantages over contemporary protocols, such as being able to characterize transgenic plants over a similar time span but without the need for secondary screening. This faster approach can lead to commercialization that requires less time and fewer resources. We anticipate that these findings will be equally applicable to other important crops, and may be helpful for providing environment- and consumer-friendly GMO products.

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