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ORIGINAL RESEARCH PAPER

Enhanced tolerance of transgenic tall fescue plants overexpressing 2-Cys peroxiredoxin against methyl viologen and heat stresses

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Abstract Plant 2-Cys peroxiredoxins (2-Cys Prx) has both peroxidase and chaperon function. We overexpressed an *Arabidopsis* 2-Cys Prx in transgenic tall fescue (*Festuca arundinacea*) plants to confer tolerance against heat and methyl viologen (MV) stress. Transgenic plants were generated by *Agrobacterium*-mediated genetic transformation, and integration and expression of the transgene was confirmed by Southern, northern and western blot analyses. Compared to control plants, transgenic plants had significantly less electrolyte leakage and thiobarbituric acid-reactive substances (TBARS) when exposed to heat or MV. Under heat stress (42°C), transgenic plants maintained their chlorophyll fluorescence (Fv/Fm) for 24 h while control plants lost chlorophyll fluorescence very quickly. We conclude that the high levels of 2-Cys Prx proteins in transgenic plants protect leaves from oxidative damage probably due to chaperon activity.

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Introduction

Peroxiredoxins (Prx) are ubiquitous thiol-based peroxidases that detoxify various peroxide substrates through their catalytic cysteine residues by using thiol-containing proteins as reductants (Wood et al. 2003). The importance of Prx enzymes is underlined by their high abundance and involvement in multiple cellular processes such as antioxidant defense (Neumann et al. 2003), H_2O_2-mediated cellular signaling (Choi et al. 2005) and molecular chaperones (Jang et al. 2004). Based on the number of conserved Cys residues that participate in the catalytic cycle, Prxs are largely divided into two groups, 1-Cys Prxs and 2-Cys Prxs (Wood et al. 2003). Both the Prx contain a reactive peroxidatic cysteine (Cys_p-S^-) residue, which is oxidized to sulfenic acid (Cys_p-SOH) when exposed to peroxides. In the 1-Cys mechanism, Cys_p-SOH is directly reduced, whereas in the 2-Cys catalytic cycle, a second Prx cysteine residue, the resolving cysteine (Cys_r-SH), condenses with the sulfenic acid to form a disulfide. Finally, the 2-Cys disulfide is reduced by another biothiol, particularly thioredoxin- a low-molecular-weight protein with two vicinal cysteine residues (Wood et al. 2003).

In plant cells, there are five subfamilies of Prxs: 1-Cys Prx, 2-Cys Prx, type II Prx, Prx Q and

glutathione Prxs. Of these, the 2-Cys Prxs serve as a key protectant for the chloroplast against environmental stresses (Baier and Dietz 1999). Thus, antisense suppression of 2-Cys Prxs in transgenic *Arabidopsis* shows significant impairment of the photosynthetic apparatus and metabolic processes during the plant development (Baier and Dietz 1999). In addition, Kim et al. (2009) reported that dual functions of Chinese cabbage 2-Cys Prx acting as a peroxidase and as a molecular chaperone are alternatively switched by heat shock and oxidative stresses, accompanying by its structural changes. The stress-dependent structural and functional switching of 2-Cys Prx could play pivotal roles in plant cells to adjust antioxidative redox signaling cascades and to protect denaturation of intracellular macromolecules from a broad range of external stresses.

Tall fescue (*Festuca arundinacea*) is a cool-season perennial grass grown for forage and turf (Hoveland 2005). Although tall fescue grows best in cool, moist environments, it is not suited to extreme hot, cold or prolonged drought (Hannaway et al. 2005). Tall fescue especially suffers from extended periods of heat stress during summer months in the transitional zone (so called 'summer depression'). Heat stresses cause a decline in forage and turf quality that has been associated with reductions in root growth, leaf water potential, cell membrane stability, photosynthetic rate, photochemical efficiency, and carbohydrate accumulation (Jiang and Haung 2000). Heat injury induces oxidative stress, resulting from the production and accumulation of toxic oxygen species such as superoxide radicals $\left(O_2^-\right)$, hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$) (Foyer et al. 1994). Oxidative stress can lead to inhibition of the photosynthesis and respiration processes and, thus, plant growth. Plants have evolved enzymatic and nonenzymatic systems to scavenge active oxygen species. However, the function of the scavenging enzyme systems can be interrupted by drought or heat stress, which can result in increases in lipid peroxidation and consequent membrane damage (Dat et al. 1998).

In this study, we generated transgenic tall fescue plants overexpressing the *Arabidopsis* 2-Cys Prx gene under the control of CaMV 35S promoter. Approaches to engineer stress tolerant turfs and forages has been done in last decades. To the best of our knowledge, however, there have been no reports about overexpression of 2-Cys Prx in forage grasses. Previously, we established an *Agrobacterium*-mediated genetic transformation protocol for tall fescue (Lee et al. 2004, 2007a, b, c). Here we described stress tolerance of transgenic tall fescue against oxidative and heat stresses.

Materials and methods

Gene construct and *Agrobacterium*-mediated transformation

The *Arabidopsis* 2-Cys Prx cDNA fragment (accession no. AT3G11630) was ligated at the translation initiation codon within the 5′-untranslated sequence of the tobacco etch virus (TEV) that provided highly efficient translational initiation, and the chimeric gene cassette was then inserted into the *Eco*RI/*Pst*I site of pCAMBIA1300 binary vector and the final construct was named pCam2Prx (Fig. 1). Recombinant pCam2Prx was introduced into *Agrobacterium tumefaciens* strain EHA105, which was then used for genetic transformation. Callus induction and *Agrobacterium*-mediated genetic transformation were performed according to our previous protocol (Lee et al. 2004, 2007a, b, c).

Molecular analyses of transgenic plants

Hygromycin-resistant putative transgenic plants were selected by PCR and validated by Southern, northern and western blot analyses. Genomic PCR was performed using following primers: 2-Cys Prx gene: 5′-TCTAGAATGGCGTCTGTTGCT-3′ and 5′-TTC TCAGCTATTTAGGAGC-TC-3′; *hpt* gene, 5′-CCT GAACTCACCGCGACG-3′ and 5′-AAGA-CCAA TGCGGAGCATAT-3′. Southern and northern blot analyses were performed according to our previous protocol (Lee et al. 2006). Gene-specific DNA probes were amplified by PCR using 2-Cys Prx as the template and were used to generate a ^{32}P-labeled

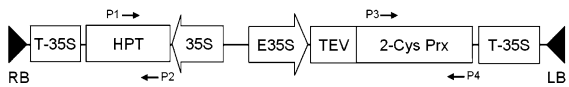

Fig. 1 Schematic representation of the T-DNA region of pCam2Prx, the expression vector used for transformation. *E35S* enhanced CaMV 35S promoter, *TEV* tobacco etch virus 5′-untranslated region, *2-Cys-Prx Arabidopsis* 2-Cys peridoxiredoxin, *HPT* hygromycin phosphotransferase

probe by random primer synthesis incorporating ^{32}P-dATP utilizing the Prime-it II kit (Stratagene, USA). Hybridization was performed as previously described (Lee et al. 2006).

Protein extraction and western blot analysis was conducted as described previously Lee et al. (2007b). Briefly, total proteins were subjected to SDS–PAGE, and were transferred to nitrocellulose membranes (Amersham Biosciences). The blotted membranes were blocked for 1 h in TTBs (50 mM Tris/HCl, pH 8.2, 0.1% v/v Tween 20, and 150 mM NaCl) containing 5% (w/v) nonfat dry milk and subsequently incubated with an anti-2-Cys Prx rabbit at 1:10,000 dilutions for 2 h followed by a secondary anti-rabbit IgG HRP (H + L) liquid (474-1506; KPL) antibody conjugated with peroxidase diluted 1:1,000 in TTBs. The immunoblot signals were detected using ECL (Perkin Elmer Life Sciences, USA) and visualized on X-ray films (Fuji Medical X-ray film, Japan).

Plant growth and imposition of stresses

Transgenic tall fescue plants were grown in soil pot in a growth chamber maintained at 25°C under a 16/8-h photoperiod. Six weeks after transplantation, the plants were exposed to stress and samples were collected for the physiological analyses as described earlier (Lee et al. 2007a, b, c). For methyl viologen (MV) treatment, leaf segments were floated on 20 ml 5 μM MV at 25°C for 12 h in darkness to allow diffusion of the MV into the leaf segments, according to Hajduch et al. (2001). Distilled water was used as control. For heat treatment, 6-week old plants were subjected to heat stress by increasing temperature of the growth chambers to 42°C with 60% humidity, while the control plants were maintained at 25°C. Leaf samples were harvested 24 h after treatment.

Physiological analyses of the transgenic plants

Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) in leaf homogenates prepared in 20% trichloroacetic acid (TCA) containing 0.5% 2-thiobarbituric acid and heated at 95°C for 30 min (Heath and Packer 1968). Malondialdehyde (MDA; $\varepsilon = 155\ mM^{-1}\ cm^{-1}$) content was determined from the A_{532} value and corrected for non-specific turbidity at 600 nm. Relative electrolyte leakage analysis was carried out following the procedure of Yan et al. (2006) as described previously (Lee et al. 2007a, b, c).

Statistical analyses

Results of the physiological parameters were statistically analyzed by using analysis of variance (ANOVA). Significant differences from control values were determined at $P < 0.05$ levels. All the results are represented as mean ± SE of at least three independent replications.

Results and discussion

Integration and expression of transgenes in transgenic plants

Eight independent primary transformants recovered from hygromycin-containing selection medium showed the presence of the gene by PCR screening using gene-specific primers for both the *hpt* and the *2-Cys Prx* gene (Fig. 2a, b). Southern analysis of four lines confirmed that genes of interest were successfully integrated into the genome of the transgenic tall fescue lines. Transgenic tall fescue lines contain at least one copy of the transgene and the transgenic events were truly independent (Fig. 2c). RNA gel blot suggest the high level expression of 2-Cys Prx mRNA as the genes are driven by a constitutively expressing CaMV 35S promoter. On the other hand no transcripts were observed in non-transgenic control plants (Fig. 3a). Further the expression of the transgene at protein level was confirmed by immunoblotting. As shown in the Fig. 3b, the antibody recognized a protein of ca. 29 kDa molecular mass in the samples obtained from the transgenic plants, whereas a very week signal was detected in the control plant. These results indicated that the transgene was successfully expressed transcriptionally and translationally in the transgenic plants.

Reduced electrolyte leakage and lipid peroxidation in transgenic plant

Stress-induced changes are frequently related to an increase in membrane permeability, affecting membrane integrity and cell compartmentation under stress conditions (Leshem 1992). Increased rates of solute

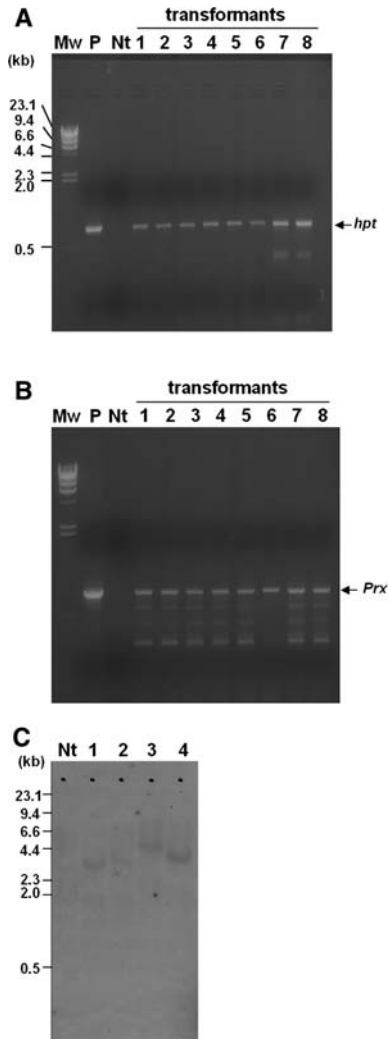

Fig. 2 Integration of transgene to the transgenic plant. PCR amplification of a 800 kb fragment of *hpt* (**a**) and *2-Cys Prx* gene (**b**) respectively. **c** Southern blot analysis of *2-Cys Prx* gene in transgenic tall fescue. *P* plasmid DNA of expression vector pCam2Prx, *Nt* non-transgenic plants

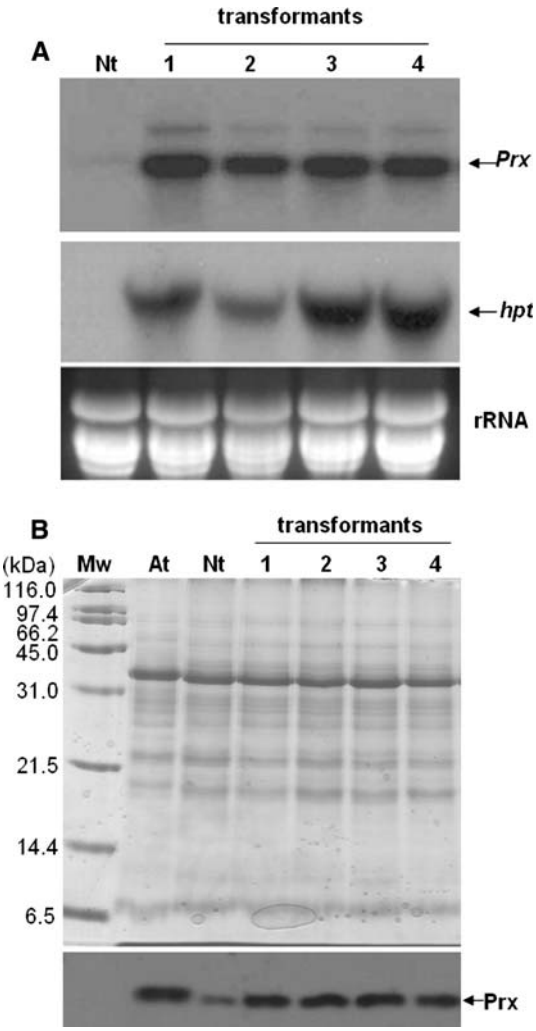

Fig. 3 Expression of 2-Cys Prx at RNA and protein level. **a** RNA gel blot analysis for the *2-Cys Prx* gene in control and transgenic tall fescue plants. **b** Western blot analysis of 2-Cys Prx in transgenic in tall fescue leaf protein (*lower panel*). Twenty-five microgram proteins were loaded in each lane, separated on 12.5% SDS–PAGE and visualized by CBB staining (*upper panel*). *M* marker, *At Arabidopsis* leaf proteins as positive control, *Nt* non-transgenic control plant, *1–4* transgenic lines

and electrolyte leakage occur in a variety of stress including heat and methyl viologen (Temsamani et al. 1995; Lee et al. 2007a, b, c). We quantified electrolyte leakage as a measure of cellular damage in response to heat and methyl viologen stress. Under control conditions, we did not detect any marked differences between control and transgenic plants. However, transgenic plants showed significantly lower electrolyte leakage in response to heat and MV treatments (Fig. 4a). This result suggests that the transgenic plants have greater capability of maintaining their membranes stability under stress condition.

Malondialdehyde (measured as TBARS content) is one of the final products of stress-induced lipid peroxidation of polyunsaturated fatty acids. TBARS formation in plants exposed to adverse environmental conditions is a reliable indicator of cellular free-radical generation. In order to determine the level of membrane damage caused by heat and MV treatments, we examined lipid peroxidation in transgenic and the control plants. As showing in Fig. 4b, transgenic plants accumulate significantly lower amount of TBARS

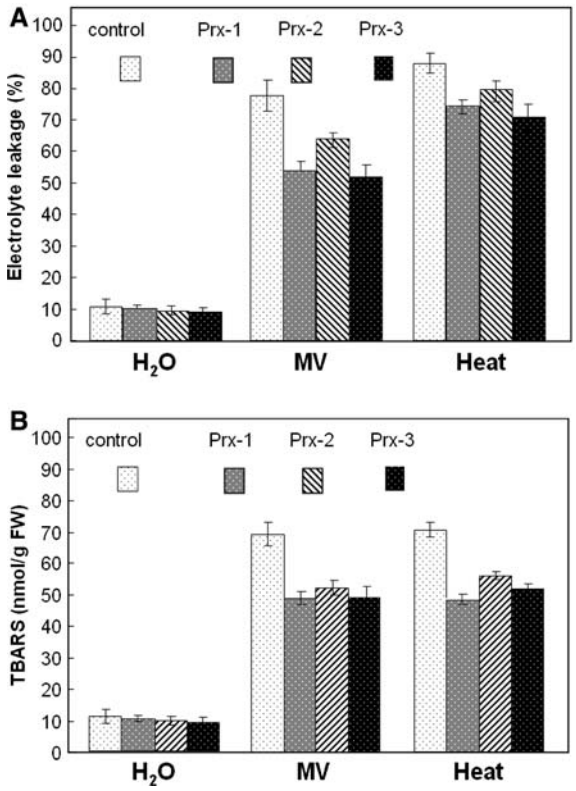

Fig. 4 Effects of MV and heat treatments on relative ion leakage (**a**) and TBARS concentration (**b**) in leaves of control and transgenic plants. Data represents the means and standard deviation (SD) of three independent measurements

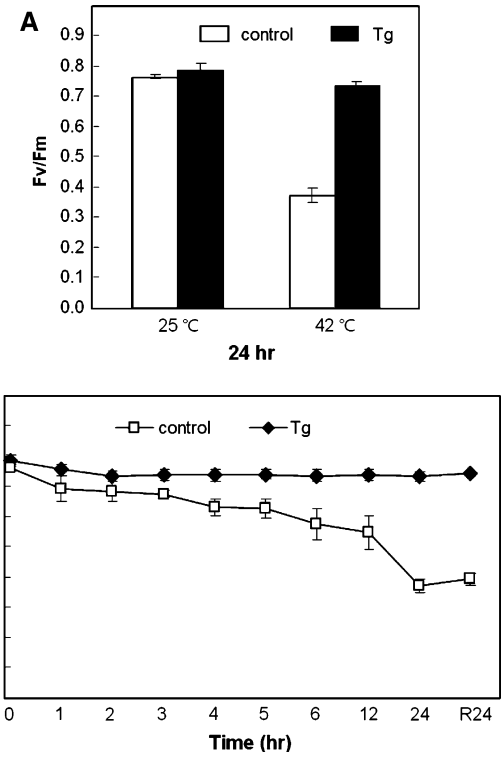

Fig. 5 Effects of heat treatment on chlorophyll florescence (Fv/Fm) of leaves of control and transgenic plants. Data represents the means and standard deviation (SD) of three independent measurements

compared to control when exposed to MV and heat stresses. However, the accumulation of TBARS in transgenic plants was similar as in control plants under control condition. This result is correlated with the relative electrolyte leakage data. Our result is consistent with several studies showing increased scavenging of ROS by transgene expression and maintaining increased membrane stability and tolerance against a wide range of stresses including heat, MV and heavy metals (Zhao et al. 2005; Lee et al. 2007b).

Maintenance of photosynthesis under stress condition in transgenic plant

Chlorophyll fluorescence (Fv/Fm) is a reliable indicator of the overall rate of photosynthesis in plant. It gives us the potential to estimate photosynthetic performance under conditions in which other methods would fail and in a manner that is almost instantaneous. In particular, Fv/Fm can give insights into the ability of a plant to tolerate environmental stresses and into the extent to which those stresses have damaged the photosynthetic apparatus (Maxwell and Johnson 2000). As shown in Fig. 5, photosynthetic activity of control plants gradually declined during treatment at 42°C. Photosynthetic activity of control plants reduced 50% at 24 h after heat treatment relative to the values before treatment, while that of transgenic plants was almost not changed. Moreover, at 24 h recovery of control plants following heat treatment, remained low. ROS modify the chlorophyll protein by facilitating its subsequent degradation by proteases (Desimone et al. 1996). Like the yeast and mammalian 2-Cys Prxs, plant 2-Cys Prx also behaves as a molecular chaperone under oxidative stress conditions. Using its chaperone function, the protein efficiently prevented the denaturation of citrate synthase and insulin from heat shock and dithiothreitol (DTT)-induced chemical stresses in Chinese cabbage (Kim et al. 2009). Thus our results suggests that overexpression of 2-Cys Prx in transgenic tall fescue plants may enhance scavenging of ROS and/

or switching to chaperon function which provide better stress tolerance under heat and MV stress.

In conclusion, we successfully introduced a dicot 2-Cys Prx to monocot forage crop tall fescue by *Agrobacterium*-mediated transformation for the first time. The transgene was fully functional and the transgenic plants showed enhanced tolerance to MV-induced oxidative stress and heat stress. Further characterization of transgenic tall fescue is under investigation in term of abiotic stresses including salt, cold and drought stresses. In addition, our results demonstrate the known dual functions, peroxidase and molecular chaperone, of 2-Cys Prx. Future studies will focus on inheritance of transgene in descendents and their field evaluation.

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