Cold-induced Antioxidant Enzymes Changes in *Leucanthemum maximum* ‘Silver Princess’

Suping Zhou and Roger J. Sauvé¹

*Institute for Agricultural and Environmental Research, Tennessee State University,
Nashville, TN 37209-1531*

Margaret T. Mmbaga

Nursery Crop Research Station, 472 Cadillac Lane, McMinnville, TN 37110

Chaim Frenkel

*Department of Plant Biology and Pathology, Cook College, Rutgers–The State
University of New Jersey, New Brunswick, NJ 08901-8520*

Additional index words. catalase, cold stress, glutathione peroxidase, glutathione reductase, ROS, SOD, shasta daisy

Abstract. *Leucanthemum maximum* ‘Silver Princess’ plants, that were gradually acclimated for 7 days at 10 °C followed by 28 days at 7 °C, were subjected to the following cold treatments: 30 days at 4 °C; 4 or 5 days at 0 °C and for 3 hours at –1 °C to identify cold inducible proteins that may be responsible for cold tolerance in this cold tolerant species. Change in antioxidant enzymes activity in fully expanded leaves was assessed after each treatment. Catalase activity began to increase after 30 days at 4 °C and reached its peak after a 5-day exposure to 0 °C. The activity of cellular glutathione peroxidase and glutathione reductase significantly increased after a 4-day exposure to 0 °C. Changes in activity of four active superoxide dismutase isoforms, one basic guaiacol peroxidase and two *o*-dianisine peroxidase isoforms were also detected following the full series of cold treatments (30 days at 4 °C; 4 or 5 days at 0 °C and for 3 hours at –1 °C).

Low temperature is one of the environmental stresses that limits the distribution of plant species and causes significant agricultural damage each year. Cold can induce increases in the accumulation of reactive oxygen species (ROS) such as O₂, H₂O₂ and HO[•] (Purvis et al., 1995). ROS are highly reactive and toxic compounds. They can have nonspecific reactions with lipids, proteins and nucleic acids (Davies, 1995), and cause damage to plasma membranes (Livingston and Henson, 1998). Thus, ROS cause disruptions in the structural and functional integrity of cells and induce oxidative stress and death (Prasad et al., 1995; Kocsy et al., 2001).

All plants have evolved antioxidant defense mechanisms to combat the inevitable production and activity of ROS (Alscher et al., 1997). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase (GPX), and peroxidases, breakdown excess amount of H₂O₂ and other ROS in chloroplasts, mitochondria, peroxisomes, vacuoles and apoplasts (Wise and Naylor, 1987). Plants under oxidative stress have elevated SOD, ascorbate peroxidase (APX), GSH reductase (GR) and peroxidases activities (Lee and Lee, 2000).

Shasta daisies (*Leucanthemum maximum*) are cold and drought tolerant perennials; these plants are a mainstay in perennial borders or cutting flower gardens where they provide long-lasting summer and early fall blooms. Established plants have been observed to survive freezing temperatures ranging from –23 to –29 °C (USDA zones 5 to 11). In Middle Tennessee, they can remain evergreen during the month of January when temperatures can range from –10 to –27 °C. In this paper, we report on changes in antioxidant enzymes in fully expanded leaves of *L. maximum* ‘Silver Princess’ following progressive cold treatments (from 7 to –1 °C).

Materials and Methods

Plant material preparation. One hundred and forty two-month-old seedlings of *L. maximum* ‘Silver Princess’ obtained from Yoder’s Green Leaf Perennials™ (Lancaster, Pa.) were transplanted into 300-mL square containers containing a mixture of 1 shredded pine bark : 1 peat : 1 Perlite adjusted with lime to an initial pH of 6.5. Plants were watered as needed and fertilized weekly with 20N–10P–20K (20 mg·L^{–1}) soluble fertilizer (Grace Horticultural Products Co., Cambridge, Mass.) and maintained in a glasshouse for 30 d under natural daylight at 24 to 26 °C. To minimize the effect of previous handling on subsequent analysis, all fully expanded leaves, except the top two new leaflets, were removed and plants were returned to the greenhouse for another 30 d. For cold treatments, plants with four to six fully

expanded leaves were transferred to growth-chambers calibrated at the following durations and temperatures: 7 d at 10 °C, and 28 d at 7 °C under cool white fluorescent lights (16-h photoperiod at 40 µE·m^{–2}·s^{–1} light intensity at the pot level) for acclimation.

To avoid irreversible leaf damage, –1 °C for 3 h was used as the coldest treatment. In previous experiments, we found that leaves of shasta daisies can freeze after more than 3 h at –1 °C. However, if they were returned to the greenhouse after up to 3 h at –1 °C, the partially frozen leaves resumed a normal appearance. Extending the cold treatment beyond 3 h or decreasing the temperature further resulted in irreversible leaf tissue damage.

Enzyme protein extraction. For all treated plants, the inner-most four fully expanded leaves were collected for analysis. Leaf samples from each treatment were combined and homogenized in liquid nitrogen to a fine powder. For enzyme extraction, 1 g of leaf powder was combined with 3 mL of extraction buffer (0.125 M Tris-HCl, pH 6.8), mixed for 2 h at 0 °C on a rotary shaker, and centrifuged twice (15,000 rpm) for 20 min at 4 °C. Pellets were discarded and the supernatant that contained the total proteins, were analyzed for enzyme activity.

Measurement of antioxidant status and enzyme activities. The following Calbiochem kits (EMD Biosciences) were used: the total antioxidant status assay kit, the glutathione reductase (GR) assay kit, the cellular glutathione peroxidase (GPX) assay kit, and the catalase assay kit. All assays were replicated four times. GR and GPX were determined spectrophotometrically at 340 nm, catalase activity was measured at 520 nm and the peroxidase activity was estimated using Bergmeyer’s (1974) guaiacol peroxidation protocol.

Detection of active isoforms of antioxidant enzyme on native PAGE gel. Protein extracts were diluted 1:1 by volume in a native sample buffer (Biorad, Hercules, Calif.) and 10 µg total protein aliquots were loaded onto 10% PAGE gel or on 1% agarose gel. PAGE electrophoresis was performed at 4 °C following Zilinskas and Mittler’s procedure (1993) and electrophoresis of the agarose gel was carried out according to Zhou et al. (2002). After electrophoresis, SOD activity was located using Beauchamp and Fridovich’s procedure (1971). Gels were soaked in 30 mL solution of 1.23 mM NBT in pH 7.0 potassium phosphate buffer for 15 min, rinsed twice in double-distilled H₂O, and soaked in 30 mL of 28 mM tetra methyl ethylene diamine (TEMED) and 2.8 × 10^{–2} mM riboflavin in the dark for another 15 min. Gels were rinsed twice in double-distilled H₂O and illuminated on a light box for 10 min to initiate the photochemical reaction for negative staining of active enzyme isoforms. To terminate the staining reaction, gels were rinsed in double-distilled H₂O.

The effect of temperature on the formation of catalase isoforms was determined by separating the total proteins from leaf extractions on 10% native acrylamide gels (pH 8.0). The location of catalase activity was determined following the procedure of Clare et al. (1984). Gels were soaked in a solution containing horseradish peroxidase (50 µg·mL^{–1}) in pH 7.0 potassium

Received for publication 1 Oct. 2004. Accepted for publication 11 Dec. 2004. The authors thank Chris Catanzaro and Sarabjit Bhatti for critical review of the manuscript, Fur-Chi Chen for biochemistry analysis and Safdar Muhammad for performing the statistical analysis. The project was financially supported through an 1890-Capacity Building Grants Program 2002-38814-12598 and Evans-Allen funds.

¹To whom all correspondence should be directed; e-mail rsauve@tstate.edu.

phosphate buffer (PPB) for 45 min. Then, 5 mM H_2O_2 was added to the PPB and gels were soaked for 10 more min, rinsed twice in double-distilled H_2O and submerged in a solution containing 0.5 mg·mL⁻¹ of diaminobenzidine in PPB until the staining reactions were completed.

The molecular weight of charged catalase isomers was determined by the separation of leaf proteins on a 10% native acrylamide gel. The protein standards used were HMW Native (Amersham Pharmacia Biotech, Piscataway, N.J.), which contained thyroglobulin 669,000, ferritin 440,000, catalase 232,000, lactate dehydrogenase 140,000, bovine serum albumin 67,000. These standards were loaded on two separate lanes of the same gel as the leaf proteins. One lane loaded with the standard protein was used for activity staining and the other lane was stained with Coomassie blue R-250 (Biorad). To determine the apparent molecular weight of catalase, an equation was generated based on the migration distance of the standards.

To determine the molecular weight of catalase subunits, the total leaf protein was first separated on a 10% native acrylamide gel. After locating the position of catalase by activity assay, the catalase was collected and electro-eluted for 20 h at 25 °C in a buffer solution with an Electro-Eluter Concentrator (C.B.S. Scientific) at 67 volts. The buffer solution consisted of 50 mM Tris-acetate and 1 mM ethylenediamine-tetraacetic acid (EDTA) adjusted to pH 8.0. Eluted proteins were precipitated overnight in acetone (1:3, v/v) at -20 °C and centrifuged at 15,000 rpm for 10 min. Pellets were air dried, dissolved in Tricine Sample Buffer (Biorad) and denatured for 5 min at 100 °C. For Western-blot analysis, proteins were separated using the Laemmli system. Sodium dodecylsulphate (SDS)-acrylamide gels (12.5%) were used. Gels were electro-blotted onto polyvinylidene difluoride (PVDF) membranes. For Western blot assays, membranes were incubated for 18 h at 4 °C in a 1:500 dilution of the catalase antibody conjugated with HRP (Abcam, Novus Biologicals). Signals were detected using chemiluminescence (Supersignal, Pierce, Ill.).

For the detection of guaiacol and *o*-dianisidine peroxidase isoforms, the loaded agarose gels were incubated in potassium phosphate buffer (pH 7.0) at 25 °C for 15 min. The buffer consisted of 2% (v/v) guaiacol or *o*-dianisidine (Sigma) and 0.5% H_2O_2 (Fisher Scientific).

Statistical analysis. Significant differences between means were calculated using Duncan's multiple posthoc *t* test. Total protein quantification was assayed using the Bradford method (BioRad). Bovine serum albumin fraction V was used as the standard.

Results

Changes in enzyme activity at different cold-stress regimes. The activity of ROS detoxifying enzymes such as catalase, glutathione peroxidase and glutathione reductase increased with decreasing temperatures in the leaf tissues of shasta daisies. Elevation in catalase activity was detected after 30 d at 4 °C and reached its peak when the temperature was lowered to 0 °C for 4 and 5 d (Table 1). Activities of cel-

lular glutathione peroxidase and glutathione reductase showed only minor increases after 30 d at 4 °C. However, their activity increased significantly after plants were held at 0 °C for 4 d; then, began to decrease when the treatment duration was extended to 5 d (Table 1).

The total antioxidant status (TAS) was not significantly affected by the 4 °C treatment. However, TAS increased to a maximum level after 4 d at 0 °C, and began to decrease when the treatment was extended to 5 d. When plants were held at -1 °C for 3 h, leaves were partially frozen and slightly damaged, causing the activity of the antioxidant enzyme and of TAS to be significantly reduced (Table 1). The activity of guaiacol peroxidase did not change with decrease in temperature (Table 1).

Active catalase isoforms and subunit composition. When the total leaf proteins were stained to detect catalase activity, only one band was observed on native acrylamide gels. No change in isoforms was detected following treatments at 4, 0, or -1 °C (Fig. 1).

The following equation was used to determine the migration of standard proteins: $y = 4577.7x^2 - 3358.1x + 646.6$, $R^2 = 0.9719$ where *x* is the migration distance and *y* is the

molecular weight. The relative migration distance for catalase was calculated to be 0.1508 and the molecular weight estimated to be about 244.36kD (Fig. 2).

To determine molecular size of the catalase subunits, the enzyme was eluted from native gels and separated on SDS-PAGE. Western blot of the catalase-antibody yielded one band. This band had an apparent molecular weight of 62kD indicating that this enzyme is a homotetramer made-up of 62kD basic subunits (Fig. 2).

Isoforms of SOD and POX. Four SOD isoforms were detected in shasta daisy leaves and no new isoform was detected after cold treatments. However, an increase in the band intensity of some SOD isoforms was observed after the 0 °C treatment (Fig. 3).

No new guaiacol peroxidase isoform was detected after the cold treatments. But, the band intensity was stronger subsequent to the 5-d treatment at 0 °C (Fig. 4). *O*-dianisidine peroxidase isoforms differed among treatments. Two isoforms (P1 and P2) appeared in the control treatment. The P2 band vanished during the 4 °C treatment and both P1 and P2 bands were very weak after 4 d at 0 °C. However, the P2 band was slightly stronger after 5 d at 0 °C (Fig. 4).

Table 1. Enzyme activity and antioxidant status in leaf tissues of *Leucanthemum maximum* 'Silver Princess' following cold temperature treatments. Results were analyzed using Duncan posthoc *t* test.

Treatment	Catalase (U/mg.protein)	Guaiacol peroxidase (U/mg protein)	Cellular glutathione peroxidase (mU/mg protein)	Glutathione reductase (mU/mg protein)	Total antioxidant status (mM)
20 to 22 °C, 30 d	119.66 a ²	0.70 b	46.63 a	111.95 a	0.27 ab
4 °C, 30 d	228.33 b	0.44 ab	52.54 ab	114.96 a	0.13 a
0 °C, 4 d	229.77 b	0.35 a	73.11 c	261.78 b	1.12 c
0 °C, 5 d (wilt)	432.41 c	0.75 b	63.46 bc	212.79 b	0.76 bc
-1 °C, 3 h	171.90 ab	0.76 b	64.80 bc	256.31 b	0.43 ab

²Means within a column not sharing the same letter are significantly different at the 0.05 level. Control plants were maintained in a greenhouse for 30 d at temperatures ranging from 24 to 26 °C.

Fig. 1. Catalase isoform detected in leaves of *Leucanthemum maximum* 'Silver Princess' following cold temperature treatments. The total protein was separated on 10% PAGE (pH 8.0) with 5% stacking gel (pH 6.0). The active catalase isoform was stained with diaminobenzidine. Catalase from HMW native protein standard (Amersham Pharmacia Biotech) was stained as the control. The right lane contains the native protein molecular standard stained with Coomassie Blue R-250.

Fig. 2. Estimation of the molecular size of catalase isolated from leaf tissue of *Leucanthemum maximum* 'Silver Princess' and of the molecular weight of its subunits. (A) Catalase from HMW native protein standard (Amersham Pharmacia Biotech) stained diaminobenzidine. (B) Catalase active isoform stained with diaminobenzidine. The molecular weight 244.36kD was estimated by using the relative migration of HMW native protein with thyroglobulin 669,000, ferritin 440,000, catalase 232,000, lactate dehydrogenase 140,000 and bovine serum albumin 67,000 (Amersham Pharmacia). (C) Stained with Coomassie Blue, the molecular weight of 232.00kD is shown. (D) The catalase protein from leaf tissues were collected from native gels. The protein was denatured and separated on a 10% SDS-PAGE gel. The subunits were detected using catalase antibody (Abcam). The molecular weight was estimated using the Precision Plus Protein Standards (Biorad).

Fig. 3. SDS isoforms detected in leaves of *Leucanthemum maximum* 'Silver Princess' after cold treatments. Ten micrograms of total proteins were separated on a 10% PAGE gel. The activity of SOD was determined by using Beauchamp and Fridovich's procedure (1971).

Fig. 4. Peroxidase isoforms in leaves of *Leucanthemum maximum* 'Silver Princess' following cold treatments. The total protein was separated on 1% agarose gel (pH 8.0). For the detection of guaiacol and *o*-dianisidine peroxidase isoforms, the loaded agarose gels were incubated in potassium phosphate buffer (pH 7.0) at 25 °C for 15 min. The buffer consisted of 2% (v/v) guaiacol or *o*-dianisidine (Sigma) and 0.5% H₂O₂ (Fisher Scientific).

Discussion

Antioxidant system was up-regulated by cold treatment depending on degree of tissue damage. This study demonstrates that the antioxidant status and activity of antioxidant enzymes increase in response to decrease in temperature. The total antioxidant enzyme activity was higher at 0 °C than at 4 °C, indicating that plants were experiencing high oxidative stress at 0 °C. Enzymes were being mobilized to metabolize ROS to maintain a stable oxidative level. However, leaf injuries caused by below freezing temperatures (−1 °C) or by dehydration (5-d exposure at 0 °C) damaged the subcellular structures such as the cell membranes and the collapse of the antioxidant system.

At temperatures above 0 °C, the antioxidant status and the activity of antioxidant enzymes increased in response to decrease in temperature. Antioxidant enzymes and its components are regulated by the cell oxidative status. Increased catalase activity at 4 °C may be a defense response to the increased production of H₂O₂ and other peroxides. Increases in mitochondrial catalase (CAT3) and peroxidase (POX) activities during cold stress of sensitive plants have been reported to be an indicator of oxidative stress (Prasad et al., 1994).

Different antioxidant enzymes are activated at different stages of cold stress. In vivo ROS levels depend on the balance between production and on the capacity of cold-treated tissues to remove them. Low temperature sensitivity, stability and the effective response of individual antioxidant enzymes during low temperature stress have critical roles in ameliorating ROS-induced damage and increasing cold tolerance in plants. In this study, catalase was the most stable and reactive enzyme at low-temperatures. Its activity began to increase after the 4 °C treatment and kept on increasing until plants were injured. When plants were exposed

to low temperatures, catalase was the main antioxidant mechanism that operated above 0 °C. When the temperature was lowered to 0 °C, the oxidative stress in mitochondria and in the cytosol began to increase, inducing the activation of antioxidant enzymes such as glutathione peroxidase. GPX catalyzes the reduction of various organic hydroperoxides, as well as hydrogen peroxide, with GSH as the hydrogen donor. It has been suggested that this enzyme protects the cell membrane system against peroxidative damage (Nakagawa and Imai, 2000). The increase in GPX activity at 0 °C shows that an increase in lipid hydroperoxide caused by lipid peroxidation in the membrane is occurring at 0 °C treatment and that the oxidative stress in the cell membrane system may be one of the main challenges for survival at this temperature.

GSH has been reported to have a role in responses to stress and during storage and transport of reduced sulfur. GSH is also involved in several detoxification reactions. The regulation of the size and redox state of the GSH pool is necessary to assure the redox-buffering capacity to process active oxygen species (Noctor et al., 1998). The oxidized form, GSH disulfide (GSSG), must be reduced to the active form by GR. At temperatures near 0 °C, GSH is a substrate for the removal of ROS and for increasing the GSSG level. GR converts GSSG to GSH and maintains the GSH pool. Cold sensitive plants, such as maize, have a limited capacity for regenerating GSH; the limited capacity for regenerating GSH may be related to its cold sensitivity (Pastori et al., 2000). Pinhero, et al., (1997) observed an increase in GR activity in chill tolerant maize during cold temperatures. A similar increase in GR activity at low temperatures was also observed in shasta daisies; this increased activity may be partially responsible for its tolerance to cold temperatures.

Literature Cited

- Alscher, R.G., J.L. Donahue, and C.L. Cramer. 1997. Reactive oxygen species and antioxidants: Relationships in green cells. *Physiol. Plant.* 100:224–233.
- Beauchamp, C. and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44:276–287.
- Bergmeyer, H.U. 1974. *Methods of enzymatic analysis* 1. 2nd ed. Academic Press, New York.
- Clare, D.A., N.D. Minh, D. Darr, F. Archibald, and I. Fridovich. 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stain for catalase. *Anal. Biochem.* 140:532–537.
- Davies, K.J. 1995. Oxidative stress: the paradox of aerobic life. In: C. Rice-Evans, G. Kocsy G. Gabor, and C. Brunold. 2001. Role of glutathione in adaptation and signaling during chilling and cold acclimation in plants. *Plant Physiol.* 113:158–164.
- Lee, D.H. and C.B. Lee. 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. *Plant Sci.* 159:75–85.
- Livingston, D.P. and C.A. Henson. 1998. Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat: Response to second-phase cold hardening. *Plant Physiol.* 116:403–408.
- Nakagawa, Y. and H. Ima. 2000. Novel functions of mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPX) as an anti-apoptotic factor. *J. Health Sci.* 46:414–417.
- Noctor, G., A. Arisi, L. Jouanin, and C.H. Foyer. 1998. Manipulation of glutathione and amino acid biosynthesis in the chloroplast, *Plant Physiol.* 118:471–482.
- Pastori, G., P. Mullineaux, and C.H. Foyer. 2000. Post-transcriptional regulation prevents accumulation of glutathione reductase protein and activity in the bundle sheath cells of maize. Implications on the sensitivity of maize leaves to low temperatures. *Plant Physiol.* 122:667–675.
- Pastori, G., P. Mullineaux, and C.H. Foyer. 2000. Low temperature-induced changes in the distribution of H₂O₂ and antioxidants between the bundle sheath and mesophyll cells of maize leaves. *J. Expt. Bot.* 51:107–114.
- Prasad, T.K., M.D. Anderson, B.A. Martin, and C.R. Stewart. 1994. Evidence for chilling-induced oxidative stress in maize seedlings and regulatory role for hydrogen peroxide. *Plant Cell* 6:65–74.
- Prasad, T.K., M.D. Anderson, and C.R. Stewart. 1995. Localization and characterization of peroxidases in the mitochondria of chilling acclimated maize seedlings. *Plant Physiol.* 108:1597–1605.
- Pinhero, R.G., M.V. Rao, G. Paliyath, D.P. Murr, and R.A. Fletcher. 1997. Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. *Plant Physiol.* 114:695–704.
- Purvis, A.C., R.L. Shewfelt, and J.W. Gegogine. 1995. Superoxide production by mitochondria isolated from green bell pepper fruit. *Physiol. Plant* 94:743–749.
- Thomashow, M.F. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50:571–599.
- Wise, R.R. and A.W. Naylor. 1987. Chilling enhanced peroxidation: the peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. *Plant Physiol.* 83:272–277.
- Zhou, S.P., R.J. Sauvé, and E.F. Howard. 2002. Identification of a cell wall peroxidase in red calli of *Prunus incisa* Thunb. *Plant Cell Rpt.* 21:380–384.
- Zilinskas, B. and R. Mittler. 1993. Ascorbate peroxidase native gel assay. *Anal. Biochem.* 212:540–546.