Adaptation of *Pachysandra terminalis* Sieb. & Zucc. to Freezing Temperatures by the Accumulation of mRNA and Cold-induced Proteins

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Abstract. A cold acclimatization mechanism regulated by the accumulation of mRNAs and proteins has been tentatively identified in japanese spurge (*Pachysandra terminalis* Sieb. & Zucc.). Two polypeptides and several cDNA fragments were observed in leaf tissue after acclimation. When these proteins were probed with type III fish antifreeze antibodies, an immune-cross reaction occurred. Nonacclimatized young leaves and stems of japanese spurge survived 20-minute exposures at -5°C . Although newly emerged leaves and stems were damaged, plants resumed growth at higher temperatures. After acclimation by gradual cold treatments (4 to -5°C), new proteins began to accumulate in young leaves and plants were more tolerant to extended treatments at -5°C . Changes in accumulation of proteins and mRNA in leaf tissue of japanese spurge appear to be an adaptation mechanism to subfreezing conditions. This is the first report of the immune-cross reaction between antibodies of type III fish antifreeze proteins and plant proteins

Cold-tolerance is an important trait for ornamental plants. During production, minor phenotypic imperfections in ornamental plants resulting from cold or subfreezing conditions can result in loss of their market value. Each year, abiotic stress from cold temperatures causes yield losses wherever cold-sensitive crops are grown. Plant species vary in their responses to freezing temperatures, some species are able to survive cold stresses by using a super-cooling or a freeze-tolerance mechanism (Anston et al., 2001; Baardsnes and Davies, 2001; Meyer et al., 1999; Worrall et al., 1998). An understanding of antifreeze mechanisms in tolerant species is needed for developing strategies to produce cold-resistant cultivars. In this study, we investigated the effects of low temperature treatments on *Pachysandra terminalis* Sieb. & Zucc. (japanese spurge). Japanese spurge is an evergreen groundcover cold hardy in most of the 48 U.S. contiguous states. This species is widely planted in shade gardens in USDA zones 4 to 8.

This study was performed to determine the

cold-tolerance mechanism of japanese spurge by observing accumulations of protein and mRNA after low temperature treatments. Differential display (Xu et al., 1997) of leaf extracts of plants that were subjected to various cold treatments was used to isolate cDNA fragments of genes regulated by temperature changes and involved in freeze-tolerance (Leong et al., 2002; Zhou et al., 2002). To identify the accumulation of antifreeze proteins, total protein samples extracted from leaf tissues were probed with antibodies against Type III fish antifreeze proteins. Type III antifreeze proteins were first found in the Antarctic eel-pout (*Lycodichthys dearborni*) (De Vries et al., 1970). Type III antifreeze proteins have structural characteristics that make them good candidates for conferring freeze protection in frost-susceptible species (Anston et al., 2001; Miura et al., 2001). However, there is no report of their existence in plants.

The study is part of a larger program to discover genes in cold-tolerant plants that can be used to improve plant stamina to subfreezing conditions through genetic engineering. Improvement of cold-tolerance in tobacco plants through genetic engineering with antifreeze genes has recently been reported (Fan et al., 2002).

Materials and Methods

Plant materials and cold-temperature treatments. During December 2001, mature japanese spurge plants with multiple shoots grown in 11.5×15.2 -cm-deep square pots containing a soilless medium [shredded pine bark, Canadian peat and coarse builder's sand (1:1:1)] that had

been maintained in an unheated cold-frame in Nashville, Tenn., were relocated to a growth chamber at $24/20^{\circ}\text{C}$ (day/night) with a 16 h photoperiod at $30\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ flux for deacclimation. After new shoots began to grow (6 weeks at 24°C), the temperature was reduced to 10°C for 2 weeks before the initiation of cold-treatments. Treatments consisted of exposing 3 whole plants to the following exposures: $5^{\circ}\text{C}/3\text{ d}$, $4^{\circ}\text{C}/2\text{ d}$, $0^{\circ}\text{C}/24\text{ h}$, $-2^{\circ}\text{C}/2\text{ h}$, $-2^{\circ}\text{C}/20\text{ min}$, $-5^{\circ}\text{C}/2\text{ h}$, $-5^{\circ}\text{C}/20\text{ min}$, $-10^{\circ}\text{C}/30\text{ min}$, and $-10^{\circ}\text{C}/1\text{ h}$ in a freezer. Following treatments, plants were moved to an incubator at 7°C for 24 h for recovery and later returned to the growth chamber ($24/20^{\circ}\text{C}$ day/night). Cold-damage was assessed based on the degree of leaf and stem necrosis and shoot regeneration. Control plants were not acclimatized; they were taken from the greenhouse and immediately subjected to subzero temperatures. For each treatment, 3 plants with an average of 12 shoots each were used.

Newly emerged leaves (young) from each of the 3 plants in each treatment combination (18 treatment combinations) were harvested immediately after each temperature treatment, frozen in liquid N_2 and stored at -70°C until tissue analysis.

Leaf protein extraction and Western blot analysis. Frozen leaf samples were homogenized in liquid N_2 and mixed with a protein extraction buffer (2:1, v/w) and incubated on ice at 0°C for 6 h (12). The extraction buffer consisted of a 1:1 dilution of Tricine sample buffer (Biorad, Hercules, Calif.). Cell debris was removed by centrifugation at 20,000 rpm for 15 min at 4°C . Protein samples ($15\text{ }\mu\text{g}$) were denatured at 100°C for 3 min, separated on 10% SDS-PAGE, subjected to electrophoresis and electroblotted onto an Immobilon-P transfer membrane (Millipore Corporation, Bedford, Mass.). Equivalent loading was verified by staining blots with Ponceau S (Sigma). After blocking for 2 h at room temperature and 16 h at 4°C in TBST (Tris-buffered saline supplemented with Tween) containing 3% (w/v) low fat milk powder, blots were probed with the primary antibody, antibodies against SP and QAE fragments of type III antifreeze proteins, and with the secondary antibody IgG-ab7007 (Abcam Ltd, Cambridge, U.K.). Primary and secondary antibodies were diluted 1:100 and 1:500 (v/v), respectively. Chemiluminescence produced by a secondary antibody obtained from Enhanced Chemiluminescence (Pierce, Rockford, Ill.) was detected on radiographic film (12).

RNA fingerprinting using cDNA differential display. For cDNA differential display analysis, leaf samples were removed from plants that received a cold treatment of either 5, 0 (6 h), and -5°C (2 h). Plants growing at 24°C were used as control. Total RNA was isolated from each leaf sample with the B10101 FastRNA kit-Green (Q-BIOgene, Rutherford, Calif.), and tested for DNA contamination in formaldehyde agarose gels. Only pure RNA was used for cDNA synthesis and differential display. The Delta Differential Display PT1173-1 kit (Clontech, Palo Alto, Calif.) and Perkin Elmer radioactive ^{32}p -dATP label (Albany, Mass.) were used. After amplification, PCR products were separated

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Fig. 1. Western blot (a), with AFPIII QAE, (b) with AFPIII SP, (c) with rabbit antiserum.

in 5.5% denature acryl amide gels and signals were recorded on X-ray film by overnight exposure at -70°C . Unique bands present in specific treatments or bands that exhibited differences in intensity were considered to contain DNA fragments of putative low temperature responsive genes.

Results and Discussions

Fully expanded (mature) green leaves have higher freeze tolerance than newly formed leaves and cold-acclimation improves cold tolerance in newly formed leaves. At temperatures warmer than -2°C , young stems and leaves of Japanese spurge plants were not damaged. When the temperature was decreased to -5°C for 20 min, young stems and leaves of nonacclimated plants were damaged or killed while acclimated ones were not. The survival rates of acclimated plants were 100% and 90% for young shoots (stems) and leaves, respectively while the rate for nonacclimated plants was 70% for both young shoots and leaves. Assessments were tabulated by counting the leaves and stems on each plant that recovered after plants were returned to room temperature. However, when the cold-treatment was extended to 2 h, the survival rate of acclimated plants was 90% and 50%, young shoots and leaves, respectively while it was only 15% for nonacclimated plants (young shoots

and leaves). Fully expanded leaves of acclimated as well as nonacclimated plants were not damaged after 2 h at -5°C . Both young and old leaves (older than 30 d) of acclimated and nonacclimated plants were killed when the temperature was reduced to -10°C for 30 min or longer. Growth did not resume on these plants after 1 week in the growth-chamber.

New proteins accumulated after plants were subjected to low temperature treatments. To detect for possible accumulation of new proteins in leaves of Japanese spurge, Western blot analysis was performed with leaf proteins probed with antibodies produced against different fragments of fish AFPIII proteins. After treatments at 4, 0, -2 , and -5°C , new proteins began to accumulate in young leaves. Two new peptide bands (about 50 kDa against SP and 5 kDa against QAE (Fig. 1a and b) were detected in all leaves probed with antibodies of type III antifreeze proteins after being subjected to temperatures $<4^{\circ}\text{C}$. These bands were not detected in the Western blot assay against rabbit pre-serum (Fig. 1c).

This is the first report of the immune-cross reaction between antibodies of type III fish antifreeze proteins and plant proteins. Results suggest that similarities in the epitope structure of proteins identified in Japanese spurge and in fish exist. Presently, we are purifying these proteins to determine conclusively their homology to AFP III proteins by using peptide mapping and amino acid analysis.

Regulation of gene transcription is involved in cold tolerance of Japanese spurge. To test whether gene transcription is being regulated during the acclimation process, we carried out RNA fingerprinting using cDNA differential display (Leong et al., 2002; Zhou et al., 2002). Some genes maintained a constant transcription level (Fig. 2, P1), while others were down-regulated as indicated by lower intensities of cDNA bands following cold treatments (Fig. 2, P2). The intensities of some cDNA fragments were higher after low temperature treatments (Fig. 2, P3) and new cDNA bands appeared after cold treatment (Fig. 2, P4). In total, 290 cDNA bands were identified, of which, 152 had higher intensities after cold treatments and the rest had decreased intensities.

Most banding profiles were similar after 5, 0, and -5°C treatments. These results suggest that low temperature treatments can initiate up- and down-regulation of genes that may be participating in the cold acclimation process.

In conclusion, fully expanded leaves and stems of Japanese spurge can survive -5°C with no sign of tissue damage. Newly formed leaves from acclimated plants are tolerant to a cold-shock at -5°C for 20 min. The accumulation of new proteins and mRNA following exposures to 4 and 5°C suggests that alterations in gene expression occur during the process of cold acclimation. Such alterations in gene expression have been reported (Miura et al., 2001).

Sequence analysis and functional characterization of these proteins and cDNA fragments may lead to the identification and isolation of antifreeze genes in Japanese spurge for use in genetic engineering of ornamentals to improve their tolerance to freezing temperatures during the growing season.

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Fig. 2. cDNA profiles of cDNA differential display. Arrows indicate bands that differed between treatments: 1) 24°C ; 2) 5°C for 15 d, 3) 0°C for 24 h, 4) 5°C for 2 h.