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Identification of genes for cold hardiness in Japanese spurge

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ABSTRACT:

Pachysandra terminalis Sieb et Zucc is a cold hardy, but heat susceptible evergreen perennial plant. In this study transcript abundance and enzyme activity were measured for genes previously shown to be differentially expressed by temperature stress to identify those genes that may be useful for improving temperature stress tolerance. Rooted seedlings were treated at cold (4 ^{o}C), heat (38 ^{o}C) and control condition (22 ^{o}C) for four d, and young leaves were collected daily from each treatment. In the photorespiratory pathway, glycolate oxidase showed higher level of transcript and enzyme activity when plants were subjected to cold than heat stress; the enzyme activity of NADPH-dependent hydroxypyruvate reductase was suppressed by temperature treatments whereas the transcript level fluctuated during the treatment period. For the plastidal-targeted proteins, carbonic anhydrase was induced by cold, but suppressed by heat temperature. The treated plants contained higher transcript level of photosystem II 10 kD PsbR and Rubisco activase compared to the control. Results from this study have shown that gene expression and enzyme activity of glycolate oxidase and carbonic anhydrase were correlated with the cold hardiness and heat susceptibility of Japanese spurge, thus these two genes may be good candidate genes for cold tolerance in this species.

Keywords:
Pachysandra terminalis, carbonic anhydrase, glycolate oxidase, NADHP-dependent hydroxypyruvate reductase, PsbR, real-time PCR, enzyme activity.

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INTRODUCTION

Temperature is one of the most important environmental factors that affect plant growth and crop productivity (Crosatti et al., 1999). During the early spring season, chilling temperature (0 to 12 ^{o}C) and bright sunlight can cause significant damages (leaf necrosis and sunburn) to many chilling susceptible crops. Such damage can result in significant economic losses. Prolonged and excessively heat period in the summer season can also cause damages to many landscape plants. Broadening the temperature range that a plant can tolerate could make significant contributions to agriculture.

Normally, plant species are either cold tolerant and heat susceptible or heat tolerant and cold susceptible. For evergreen landscape plants in particular, it is important for their foliage to maintain tissue integrity despite rapid temperature fluctuation during their growing season. Genes that confer tolerance to extremely cold or heat temperatures can be used for developing tolerant varieties using marker-assisted breeding and genetic transformation approach.

Photosynthesis and carbon fixation are very sensitive to temperature conditions. For most of temperate C3 plants, the peak CO_2 assimilation often occurs at 25 to 30 °C. Increasing leaf temperature beyond this range inhibits photosynthesis. On the other hand, chilling-susceptible species would experience 30-60 % reduction in photosynthetic activity when air temperature is below 10 ^{o}C (Levitt, 1980; Powles, 1984). Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) is a bifunctional enzyme, it catalyzes either the carboxylation or the oxygenation of ribulose-1,5-bisphosphate with carbon dioxide or oxygen. In C3 plants, as temperature increases, the ratio of dissolved O_2/CO_2 and the specificity of ribulose-1, 5-bisphosphate carboxylase/oxygenase *(Rubisco)* for O_2 increases, thus favoring the oxygenase actvitiy (Monson et al, 1982).

The oxygenase reaction of Rubisco synthesizes very large amounts of 2-phosphoglycolate (2-PG) (Andrews et al., 1971; Bowes et al., 1971). The 2-PG molecule cannot be used by plants for biosynthetic reactions; it is a potent inhibitor of chloroplastic function (Anderson, 1971; Rizhsky et al., 2002). In plants, the toxic 2-PG molecules are converted into 3-phosphoglyceric acid (3-PGA) through photorespiratory pathway. On the other hand, cold stress under lighted condition induces photochemical damages (photoinhibition) to the photosynthetic machinery, the excessive photochemical energy is dissipated through the photorespiratory pathway for avoiding damages to photosystem I (PSI) and PSII (Kudoh and Sonoike, 2002; Takahashin et al., 2007). Thus when plants are growing under stress conditions, photorespiration play a key role in protecting the structural integrity of photosynthetic apparatus (Osmond et al., 1997; Park et al., 1996;), serving as an alternative electron sink and lowers the quantum yield of photosynthesis (Weis and Berry, 1988), and generating intermediates for synthesis of polyamines and other protection substances. Transgenic rice plants with higher photorespiratory activity showed enhanced tolerance to salt and chilling stress (Hoshida et al., 2000).

Photorespiratory pathway involves three types of *organelles: chloroplasts, peroxisomes* and *mitochondria.* In leaf peroxisomes, glycolate oxidase is the first enzyme to receive glycolate formed from 2-PG in chloroplast, and convert the toxic molecule into glyoxylate. Hydroxypyruvate reductase is the last enzyme to catalyze the conversion of pyruvate into glycerate which enters the chloroplast to recycle the glycolate carbon back into the Calvin cycle. Within the chloroplast, carbonic anhydrase catalyzes the hydration of CO_2 to bicarbonate, thus increasing the local availability of CO_2 at the carboxylation reaction site of ribulose -1, 5-bisphosphate carboxylase oxygenase (Rubisco) (Graham and Reed, 1971). When cotton plants were subjected heat treatment, the low rate of photosynthesis coincided with reduced carbonate activity (Downton and Slatyer, 1972). Transcript expression of this gene was suppressed by cold stress in the warm season crop chickpea (*Cicer arietinum*) (Mantri et al., 2007). The 10-kDa PsbR protein is essential for stable assembly of the oxygen-evolving complex in PSII (Suorsa et al., 2006); it is also one of the primary targets of thermal damage in plants (Allahverdiyev et al., 2007). Rubisco activase functions as chaperone to promote and maintain the catalytic activity of Rubisco (Portis, 2003). Reduction of Rubisco activase activity limits the Rubisco activation and, therefore, the CO_2 assimilation rates at high temperatures (Yamori and von Caemmerer, 2009).

Japanese spurge (*Pachysandra terminalis*) is a cold hardy evergreen perennial in the boxwood family, Buxaceae. Plants can overwinter at below -33 °C in USDA zone 5 (A) (Sagebud, 2011). New growth

occurs when temperature is at 10 to 22 ^{o}C in spring, early summer and fall. Incubator experiment in our laboratory found that when plants were exposed to temperature below 0 ^{o}C, leaves were frozen, but they recovered without any sign of damages soon after being transferred to warmer temperature (unpublished data). The species is susceptible to heat stress. Plants normally cease putting out new growth when air temperature rises above 30 *°C, leaves become very chlorotic at above 35 °C under full sun condition.*

Five genes were isolated from cold-treated leaves of Japanese spurge by cDNA differential display and race-PCR (Zhou et al., 2005, 2006). These genes are glycolate oxidase (*PtGOX*, accession DQ442286) and NADPH-dependent hydroxypyruvate reductase (*PtHPR,* accession DQ442287) in the photorespiration pathway, photosystem II PsbR (*PtPsbR*, accession DQ781306), plastidal *β*-carbonic anhydrase (*PtCA,* accession DQ781308), and Rubisco activase (*PtRCA*, accession DQ486905). In this study, gene expression and/or enzyme activity in leaves of plants treated under cold (4 ^{o}C) or heat (38 ^{o}C) temperature conditions were measured to associate molecular and biochemical changes to physiological properties, and to identify candidate genes for cold tolerance in Japanese spurge.

MATERIALS AND METHODS

Plant material preparation and temperature treatments:

Rooted cuttings bearing four-six dark green mature leaves were obtained from Classic Ground Covers (Athens, GA). Upon arrival, these plants were maintained in the shade in a greenhouse at Tennessee State University (Nashville). Then, they were placed in incubators which were programmed at 22 ± 1 °C with a 12-h photoperiod provided by fluorescent lights (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiations) for four d. For temperature treatment, the incubator was reprogrammed at 4 ± 1 °C (for cold treatment) or 38 ± 1°C (for heat treatment). For the control treatment, plants were maintained at 22 ± 1 °C. The light-dark cycle was unchanged for the treatments.

Short durations of cold or heat often show little or no damage to plants, but exposure to unfavorable temperature conditions for a long length of time can be devastating to susceptible species. The experiment was designed to analyze daily changes over a four d period, in order to identify the mechanisms that result in tolerance for lengthy temperature stress conditions. Leaf samples were collected after 1 d, 2 d and 3 d and 4 d of incubation. Leaf samples collected from the day before the beginning of the experiment were reported as zero hour. To avoid effects of circadian rhythms, all tissues were harvested at 900 HR; five hour after the light cycle began. The temperature treatment experiment was repeated twice.

The top two fully-expanded light-green young leaves were harvested while plants were in the incubators. Leaves from four plants were pooled to form one biological replicate and three replicates were harvested at each collection period. Samples were frozen in liquid nitrogen and stored at -80 ^{o}C immediately after harvesting from plants. Enzyme and total RNA were extracted soon after completion of the temperature treatment experiment.

Enzyme extraction and activity assay:

Except as noted, all chemicals used were obtained from Sigma (St. Louis, MO). For enzyme extraction, leaf tissue samples were ground into a fine powder under liquid nitrogen. The powder (200 mg) was resuspended in 2 ml of prechilled 50 mM potassium phosphate buffer (pH 7.5) with 1 mM polyethyleneglycol (molecular weight: 8000), 8% polyvinylpyrolydone (molecular weight: 40,000), and 0.01% Triton X-100. Protein extraction was carried out by incubating the mixture for nine hours at 4 ^{o}C while being constantly mixed on a rotary mixer. After two centrifugations at 12,000 g_n and 4 ^{o}C for 10 min, the clear supernatant that contained enzymes was immediately used for analysis of enzyme activity described subsequently.

Measurement of carbonic anhydrase was done by following the method described by Iyer et al (2006) with minor modifications (Sigma, 1994). The reaction solution contained 20 mM Tris-sulfate buffer, pH 7.6, and 3 mM p-nitrophenyl acetate (MP Biomedicals, Solon, OH). The absorbance at 348 nm was detected every min for 10 mins. Catalytic activity of carbonate anhydrase was determined following the formation of p-nitrophenol (extinction coefficient of 5 $mM^{-1} \cdot cm^{-1}$) (Iyer et al,2006; Sigma,1994).

Glycolate oxidase was assayed essentially as described by Feierabend and Beevers (1972) by the increase of absorption at 324 nm every min for 10 min. The assay mixture contained 33 mM triethanoleamine buffer, pH, 7.8; 2.7 mM EDTA, pH 7.8; 0.0083% Triton X-100; 0.2 mM flavin

mononucleotide (FMN); 3.3 mM phenylhydrazine-HCl, pH 6.8 and 5 mM glycolic acid (neutralized to pH 7.0 with 1 M NaOH). Glycolate oxidase activity was determined by following the formation of glyoxylate phenylhydrazone (extinction coefficient of 17 $mM^{-1} \cdot cm^{-1}$).

For hydroxypyruvate reductase, the reaction mixture contained 25 mM potassium phosphate buffer, pH 5.8; 0.01% Triton X-100, 0.2 mM NADH; 1 mM dithiothreitol and 20 mM sodium glyoxylate. After the addition of NADH, the absorbance at 324 nm was measured every min for 20 min. Enzyme activity was determined by following the oxidation of NADH (extinction coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$) (Booker et al., 1997). The protein concentration was determined using the Biorad Protein Assay reagent (Biorad, Hercules, CA). A standard curve was constructed with bovine serum albumin (BSA). All absorbance measurements were performed on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA), using Costar 3635 UV transparent plates (Corning Incorporated, Corning, NY). The absorbance in each well was normalized to a pathlength of 1 cm using the factory-stored values in the SOFTMAX® PRO SOFTWARE (Version 5, Molecular Devices).

Quantitative-reverse-transcriptase-polymerase chain reaction (q-RT-PCR).

Total RNA was isolated using RNA Pure Reagent and genomic DNA was removed with DNase I in the Messenger Clean Kit (Genhunter, Nashville, TN). RNA samples were analyzed and quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

Q-RT PCR assay was done using a two-step procedure. In the first step, RNA was transcribed into cDNA using Oligo-dT primers for target genes and random primers for rRNA using the Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following the manufactory's manual. Real-time PCR primers were designed using the Primer Express version 2.0 software (Applied BioSystems). Initially three pairs of primers were tested for each gene including the housekeeping gene. After examining the PCR products on 2% agarose gel, the primer pair that produced one single band of the expected size was selected for q-PCR analysis.

The 18S ribosomal RNA (18S rRNA) was selected as the house keeping gene to normalize the real-time PCR data. Its primers were designed against an *Arabidopsis* 18S RNA sequence from the GenBank (NC-003071, region: 3706..5513). The forward/reverse primers were 5'-CATCAGCTCGCGTTGACTAC-3'/5'-CACTTCACCGGATCATTCAA -3', respectively, which produces an amplicon of 110 bp. The forward/reverse primers for target genes were designed against the open reading frame region. The primer sequences were *PtpsbR* - 5'-GAGCTTGAGGGATGGTCTTG-3'/5'-CACCACTTGGAGACCAATCA-3', amplicon size 137 bp; *PtCA* -5'-GACGATGGGACCACCTCTAC -3'/5'-CCGAACTCAGCTTTCACCTT-3', amplicon size 85 bp; *PtRCA* - 5'-GTTCAGCTCCCCGGTATGTA-3'/5'-CGGATAAGAGGGGCATACAA-3', amplicon size 97 bp; *PtGOX* - 5'-GTTTGTGGATGGTGGGATTC -3'/5'-CAGCACAGGTCGTCCAATAA-3', amplicon size 91 bp; and *PtHPR* - 5'-TGAGGACGAGCCTTACATGA-3'/5'-CAGCTAGAGTCGCCATTCCT -3', amplicon size 112 bp. All primers were synthesized by Invitrogen (Carlsbad, CA).

In a preliminary experiment, the copy number of 18S RNA in the samples was found to be 20- 25 fold higher than target genes. In the assay, the qPCR mixture (30µl) contained cDNA (equal to 100 ng total RNA for target genes, and 10 ng for 18S RNA), primer mix (250 nM each) and 2 x PCR master mix supplied in the SYBR-green PCR mix/ RT kit (Applied BioSystems). PCR amplification was performed using a program consisting of 40 cycles of 94 ^{o}C, 30 s, and 60 ^{o}C, 1 min on a 7000 Real Time PCR System (Applied Biosystems). At the end of the PCR cycles, all data were analyzed with the ABI Prism 7000 SDS software (Applied Biosystems) and presented as Ct value. To check the specificity of annealing of the oligonucleotides, dissociation kinetics was performed by the instrument at the end of each PCR run.

Data from control samples (22 ^{o}C) were used as the reference to determine the relative gene expression under treated conditions. The relative expression (fold change) compared to control was estimated using $2^{-\Delta\Delta CT}$, where $\Delta\Delta Ct = \Delta C_t$(4 ^{o}C or 38 ^{o}C) - ΔC_t(22 ^{o}C). Ranges of fold given for each treatment were determined as $2^{-\Delta\Delta CT + s}$ for upper limit, and $2^{-\Delta\Delta Ct - s}$ for lower limit, where s = the standard deviation of the ΔΔCt values among the six biological replicates (Applied Biosystems, 1997).

Statistical analysis was performed using the Student's t-test function (paired and one tail-

distribution) in Microsoft Excel 2007, and the significant difference was determined at $P<0.05$ level.

RESULTS

Effect of temperature treatments on stable expression of the internal reference gene:

The stability of internal reference gene expression is a prerequisite to standardize the target gene expression data. The Ct values of the 18S RNA gene were compared to determine the treatment conditions under which expression levels of the housekeeping gene remained constant. After four d treatment, Ct value of heat treated sample increased by nearly one cycle (which equals to two-fold change) compared to control (22 ^{o}C) and cold treatment (4 ^{o}C) (**Table 1**). This indicates that the 18S RNA gene transcription was affected by heat stress after 4 d of treatment, and q-PCR analysis should only be performed for treatments lasting three d.

Temperature effects on gene transcription

The two genes in the photorespiratory pathway showed different expression patterns under cold or heat treatments. For the glycolate oxidase, the transcript level increased 1.8-fold after one d of treatment, followed by gradual decrease to near the control level after 2-3 d of cold treatment. The transcript level in heat treated leaves was decreased significantly (-2-fold) after three d (**Fig.1A**). For the hydroxypyruvate reductase, after one d of treatment, transcript level was enhanced (2.5-fold) by cold, but suppressed (-1.3-fold) by heat treatment. During the remaining two d, gene expression in cold or heat treated tissues followed the same pattern, the transcript level declined (-2-fold) after two d but increased (>2.5-fold) after three d (**Fig. 1B**) of treatment.

For the carbonic activatase, transcript level increased after one d followed by gradual decline to the control level in cold treatment. Under heat treatment, the transcript level remained stable after one d, followed by rapid decline during the next two d (**Fig. 1C**). For Rubisco activase, transcript level had a drastic increase (6-fold) after one d, a fallback to 2.6-fold at two d, and a more drastic increase (8-fold) after three d under heat treatment condition. The same gene was also induced by cold temperature, but significant increase (fold) of transcript abundance was delayed to after 3 three d of incubation (**Fig. 1D**). Gene expression of PtpsbR gene was induced by temperature treatments, the transcript level in treated tissues was 1.5 to 2.5 fold higher than the control (**Fig.1E**).

Temperature effects on enzyme activity

Cold treatment activated enzyme activity of glycolate oxidase, enzyme activity increased by 2.5 - 4 units compared to the untreated control after two d and three d incubation, respectively. As cold treatment extended to three d, the enzyme activity started to drop to the control level and remained at the same level after four d (Fig.2A). Glycolate oxidase activity was suppressed but not significantly by heat treatment. For hydroxypyruvate reducatase, enzyme activity fluctuated daily during the experiment, but it was always lower in the treated tissues than those in the control (Fig.2B). For the carbonic activate, the enzyme activity was induced after one d of cold treatment, which was followed by gradual declining to the control level. In the contrary heat suppressed carbonic activase. The enzyme activity was reduced by 1, 1.5, and 2.0 units after 1, 2 and 3 d of heat treatment (**Fig. 2C**).

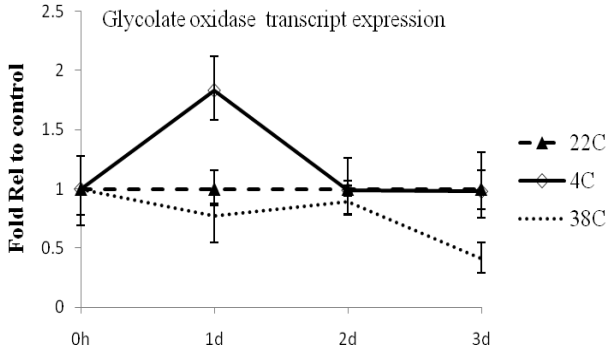
Table 1 The Ct[z] value of 18S rRNA in leaves of *Pachysandra terminalis* under cold and heat temperature treatments

Treatment	Time period[y]				
	0 h[x]	1 d	2 d	3 d	4 d
22 ^{o}C	18.98 ± 0.29a[w]	18.83 ± 0.15a	18.95± 0.09a	18.90 ± 0.15a	18.85± 0.05a
4 ^{o}C	18.98 ± 0.29a	18.79± 0.12a	18.71± 0.11a	18.95 ±0.24a	18.89± 0.12a
38 ^{o}C	18.98 ± 0.29a	18.97 ± 0.06a	18.82± 0.04a	18.98± 0.09b	19.95± 0.15

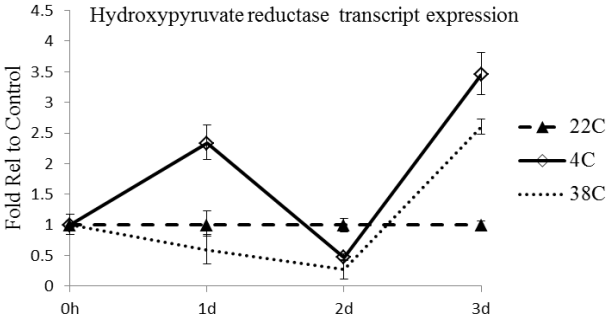
[z] The cycle threshold (Ct) which is defined as the number of cycles required for the fluorescent signal to cross the threshold in a quantitative polymerase chain reaction assay.
[y] Number of days under temperature treatment.
[x] Samples collected before the treatment.
[w] Ct value: mean ± SD (standard deviation among three biological replicate samples).
Any two means within a row not followed by the same letter are significantly different at $P<0.05$ by the Student's t-test function (paired and one tail-distribution).

DISCUSSION

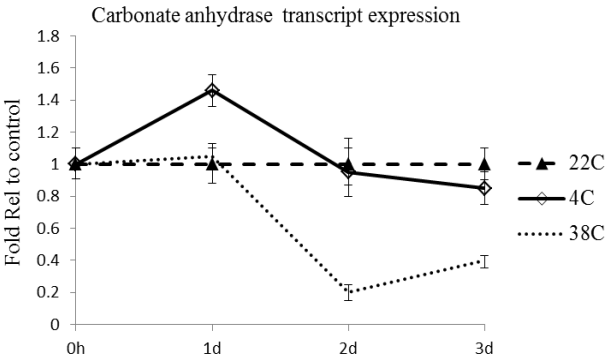
Glycolate oxidase is the first enzyme in photorespiratory pathway, it is localized in peroxisomes in green leaves of higher plants (Zelitch et al., 2009). Sufficient activities of glycolate oxidase are required to maintain normal activities of photosynthetic system (Fock and Krotkov, 1969; Yamaguchi and Nishimura, 2000). In this study, we have found that glycolate oxidase was induced by cold but suppressed by heat treatment. This result suggests that plants growing under cold or heat stress conditions are using



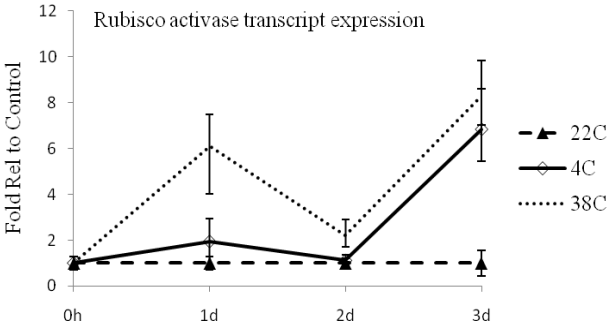
A: glycolate oxidase



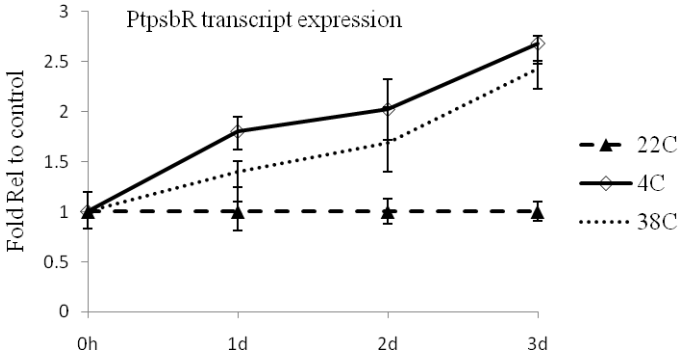
B: NADPH-dependent hydroxypyruvate reductase



C: carbonic activase



D: Rubisco activase

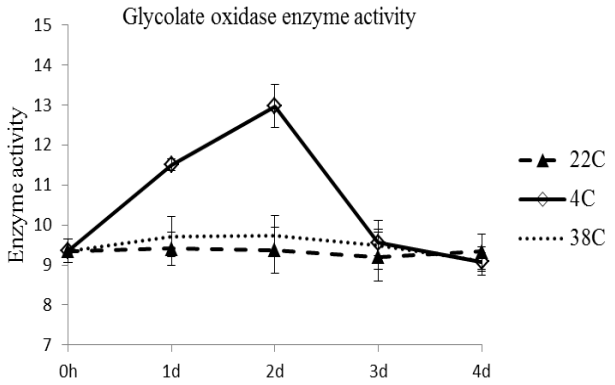


E: photosystem II PsbR.

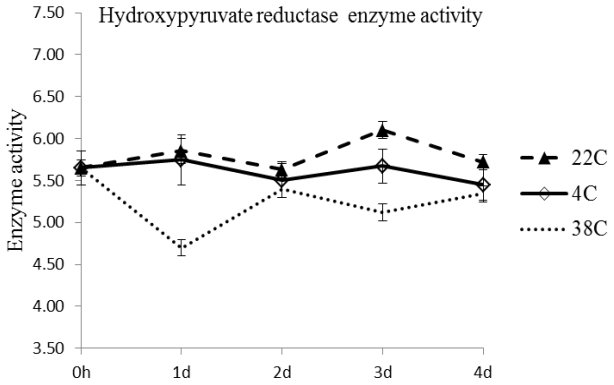
Fig.1. Effect of temperature stress on transcript abundance in Japanese spurge.
Rooted cuttings of *Pachysandra terminalis* were treated at cold (4 ^{o}C) or heat (38 ^{o}C) conditions for 1, 2, 3 and 4 d. The relative abundance of gene transcripts was compared between cold (4 ^{o}C) and heat (38 ^{o}C) to that of control (22 ^{o}C) in young leaves after 1 d, 2 d, and 3 d of treatments. Values are given in fold change from treated tissues to the control. Bars indicate the upper and lower ranges of the fold values. Values of folds and ranges were determined by evaluating the expression: $2^{-\Delta\Delta CT}$ with ΔΔCt + s and ΔΔCt – s, where s = the standard deviation of the ΔΔCt values among the six biological replicates for each treatment.

separate regulatory mechanism for glycolate oxidase. NADH-dependent hydroxypyruvate reductase is another enzyme in the same pathway. While the gene transcript level had a significant increase after 3 d incubation under cold or heat conditions, the enzyme activity declined in the treated tissues. There is a disparity between transcript and enzyme activity for this gene. NADH-dependent hydroxypyruvate reductase activity is not totally confined to the peroxisomal compartment in plant cells. The enzyme is localized in the cytosol (70% in *Pisum sativum* L.), chloroplast and peroxisomes (Kleczkowski et al., 1988). In this study, the protein was extracted from whole leaf tissues, thus the measurement value is the total cellular enzyme activity. Reduction in enzyme activity of NADH-dependent hydroxypyruvate reductase may not necessarily affect the photorespiratory pathway.

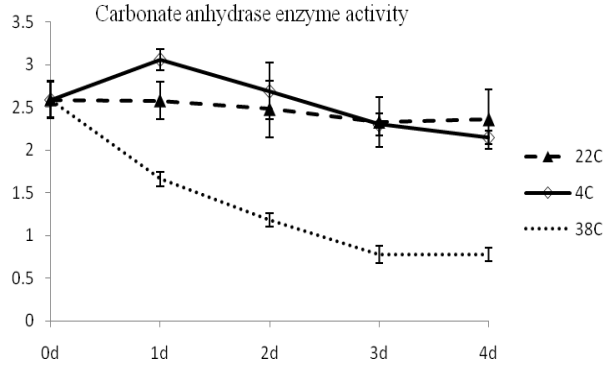
Carbonic anhydrases catalyzes the reversible hydration of carbon dioxide, facilitating the diffusion of inorganic carbon into the cell and allowing the accumulated inorganic carbon within the cell to generate higher levels of CO_2 around



A: Glycolate oxidase. Absorbance at A324 was recoreded every min for 20 min, and enzyme acitivty was determined following formation of glyoxylate phenylhydrazone (extinction coefficient of 17 $mM^{-1}\cdot cm^{-1}$). Enzyme activity is shown as units/mg protein.



B: NADPH-dependent hydroxypyruvate reductase. Absorbance at A324 was recoreded every min for 20 min, enzyme activity was determined by following the oxidation of NADH (extinction coefficient of 6.2 $mM^{-1}\cdot cm^{-1}$) (Booker et al., 1997). Enzyme activity is shown as units/mg protein.



C: Carbonic activase. Absorbance at A348 was recoreded every min for 10 min, and enzyme activity was determined following the formation of p-nitrophenol (extinction coefficient of 5 $mM^{-1}\cdot cm^{-1}$). Enzyme activity is shown as units/mg protein.

Fig. 2. Effects of temperature on enzyme activity in Japanese spurge.
Rooted cuttings of *Pachysandra terminalis* were treated at cold (4 ^{o}C) and heat (38 ^{o}C) conditions for 1, 2, 3 and 4 d. Enzyme activity in young leaves after 1 d, 2 d, and 3 d of treatments were measured. Means (values on the Y-axis) ± standard deviation (standard error bars) from six biological replicates are shown.

Rubisco (Villard et al., 1997). Similar to glycolate oxidase, carbonic anhydrase was induced by cold, and suppressed by heat stress in Japanese spurge. The response of carbonic anhydrase gene correlates to the cold hardiness and heat susceptibility physiological properties of the species.

Expression of the 10 kDa polypeptide PsbR and Rubisco activase was induced under cold or heat temperature treatment conditions. The induction by the two temperature extremes indicates that these genes are not directly involved in cold tolerance; instead they are more likely components of the general response mechanisms to various stress conditions in Japanese spurge.

Tolerance of plants to environmental stress is regulated by a complex molecular network consisting of multiple genes. In this study, a distinctive response to cold or heat temperature was identified for glycolate oxidase and carbonic anhydrase, which correlates with the cold hardiness and heat susceptibility of Japanese spurge. These two genes may be good candidate genes for selection and breeding for cold tolerance of nursery crops.

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