Cloning and Characterization of Glycolate Oxidase and NADH-Dependent Hydroxypyruvate Reductase Genes in *Pachysandra terminalis*

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Abstract. Photorespiration provides a protection mechanism in plants by diverting excessive energy accumulated from photochemical reaction, metabolizing toxic products and producing some protective molecules. The authors report cloning and characterization of a glycolate oxidase gene (GOX; NCBI accession DQ442286) and a NADH-dependent hydroxypyruvate reductase gene (HPR; NCBI DQ442287) from *Pachysandra terminalis*. The DQ442286 had the predicted GOX-like-Riboflavin-5'-phosphate (FMN) conserved domain and the DQ442287 had the predicted adenosine 5'-(alpha-thio)diphospho-5'-ribofuranosylnicotinamide adenine dinucleotide (NAD) binding domain (2-Hacid_DH_C). C-terminal peroxisome targeting signal was predicted to be -ARL for DQ442286 and -SKL for DQ442287. Both genes encoded enzyme proteins that are located in peroxisome and are involved in the photorespiration process. Real-time quantitative reverse-transcriptase polymerase chain reaction was performed to compare transcript level of the cloned genes after cold treatment. The 18s Ribosomal RNA (rRNA) was included to calibrate the data. The relative cycle threshold values (gene/18s rRNA) were 1.4, 1.5, and 1.5 for GOX and 1.2, 1.3, and 1.3 for HPR in the treatments of 4 °C 4 h, 4 °C 12 h, and control. The data revealed that gene expression was enhanced by only short-term (4-h) cold treatment. A ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) activase gene (DQ 486905) was also cloned and analyzed following the same procedure.

Chilling (5–10 °C) is one of the major abiotic stresses periodically experienced by different species of crops and native plants growing in temperate regions (Tsonev et al., 2003). Low temperature can enhance O₂ uptake processes (Flexas et al., 1999) as oxygen solubility increases from 4 to 5 mg/L at 50 °C to 10 to 11 mg/L at 10 °C and 13 to 14 mg/L at 0 °C (www.omega.com/techref/ph-1.html). A high concentration of O₂ in the cellular spaces will activate or enhance photorespirations (Byrd and Brown, 1989). Although photorespiration results in the loss of as much as 25% of the carbon that is fixed during photosynthetic carbon assimilation (Ludwig and Calvin, 1971), it is considered to be an essential metabolic pathway (Somerville and Ogren, 1982; Wu et al., 1991). Inhibitors of photorespiration en-

zymes are known to be phytotoxic. Photorespiration is necessary for healthy plant growth, especially under environmental stresses when high oxidative stress can damage membrane systems and disturb normal metabolism (Hendrickson et al., 2004; Kozaki and Takeba, 1996). It can also produce the intermediates for other metabolic processes, such as glycine for the synthesis of glutathione, which is also involved in stress protection (Wingler et al., 1999, 2000).

Photorespiration results from the oxygenase reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Fig. 1). This reaction produces 3-phosphoglycerate and 2-phosphoglycolate. The 2-phosphoglycolate is toxic and it is immediately dephosphorylated into glycolate. The glycolate enters peroxisomes and is oxidized to glyoxylate and then transaminated to glycine (Ellen et al., 1973; Kisaki and Tolbert, 1969; Ogren, 1984; Tolbert, 1971). Glycine leaves the peroxisomes and is converted to serine and CO₂ in mitochondria (Andrews, et al., 1971). Serine returns to the peroxisomes and is transaminated to hydroxypyruvate, which is reduced by NADH to glycerate. Glycerate leaves the peroxisomes and is converted to sugars in the chloroplasts (Kisaki and Tolbert, 1969).

Glycolate oxidase (GOX) and NADH-dependent hydroxypyruvate reductase (HPR) are two major enzymes located in peroxisomes involved in the photorespiration process (Fig. 1). These proteins are encoded in the nucleus and are synthesized in the cytoplasm on free ribosomes (de Hoop and Ab, 1992; Lazarow and Fujiki, 1985; Subramani, 1993). They are targeted to peroxisomes by a noncleaved C-terminal tripeptide of the prototype SKL, the peroxisome targeting signal type 1 (PTS1), or conservative variations thereof (Gould et al., 1987, 1989; Olsen et al., 1993; Reumann, 2004). The PTS as well as the pathway is conserved to a large extent throughout the eukaryotic kingdom (Frederich et al., 1973; Gould et al., 1990).

Different GOX genes have been isolated from different plant species that are cold tolerant, such as *Arabidopsis* (*Arabidopsis thaliana*) (Sato et al., 2000) and spinach (*Spinacea oleracea*) (Cederlund et al., 1988), and those that are cold sensitive, such as tomato (*Lycopersicon esculentum*) (Speirs et al., 1995) and pumpkins (*Cucurbita* sp.) (Tsugeki et al., 1993). The activity of GOX is affected by low-temperature stress. Its activity was estimated to be about one-third at 4 °C compared with that at 25 °C in the high-mountain plant species *Ranunculus glacialis* (Streb et al., 2005). Maintaining stable level of GOX activity is essential for protection from photoinhibition during irradiation in transgenic tobacco (Yamaguchi and Nishimura, 2000). The NADH-dependent HPR catalyzes the reaction for serine to be transaminated to hydroxypyruvate in the peroxisomes (Fig. 1). HPR gene expression is regulated by light, hormone, and CO₂ concentrations as well as water status (Wingler et al., 1998). Rubisco activase is an adenosine triphosphate (ATP) hydrolyzing (ATPase) enzyme. It causes a conformational change in the ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) from closed to open form, from inactive to active state. The activity of Rubisco activase is temperature dependent (Michael and Crafts-Brandner, 2004). Reducing the activity of Rubisco activase can cause reduction in photosynthesis (Salvucci et al., 2001).

Pachysandra terminalis is an evergreen plant species. It grows best at 20 to 24 °C and can survive growing zone 5 in the United States, where the low temperature can drop to –10 to –20 °C. Chilling (0–10 °C) is one of the major environmental stresses facing the nursery industry. It is also a process when over-winter plants acclimatize and prepare to survive subzero freezing temperatures. Understanding the molecular mechanism involved in this process is very important for breeding and selection of cold-tolerant genotypes. We report cloning and characterization of GOX and NADH-dependent HPR as well as the Rubisco activase genes from the cold-hardy *P. terminalis* plants. Regulation of gene expression under cold treatments was compared through real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Results from this research may

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Fig. 1. Diagram of photorespiration process.

contribute to the understanding of the mechanism involved in the adaptation of ever-green plants to cold stress.

Materials and Methods

Plant Material Preparation

P. terminalis cuttings were purchased from Yoder Brothers, Inc. (www.yoder.com/). These cuttings were growing in a greenhouse for 2 weeks, when new leaves grew out into full size. These new leaves were collected and used for total RNA extraction. Before cold treatments, these plants were incubated in a growth chamber at a photon flux density of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a 24-hour photoperiod under a constant temperature of 24 to 25 °C for 5 d. Plants were then transferred to a temperature regime of 4 °C with the same light conditions. Leaf tissues were sampled at 5 h and 12 h of treatment. Leaves collected after 5 d of incubation at 25 °C were considered controls. Collected leaf tissues were frozen in liquid N_2 and stored at -80 °C.

Total RNA Isolation

Total RNA was extracted using RNA pure kit (Genhunter, Nashville, Tenn.) following

the same procedure as Zhou and colleagues (2005). Briefly, the leaf tissues were homogenized in the extraction buffer (1:5, w/v). After adding chloroform (15% volume), the homogenates were mixed and incubated on ice for 2 h. After centrifugation at 13,000 g and 4 °C for 10 min, the upper phase containing RNA was removed to a clean tube, mixed with 2-isopropanol (1:3, volume/volume), and stored at -20 °C for a minimum of 2 h. Total RNA was precipitated by centrifugation following the same procedure as previously described. The RNA pellets were washed with 75% ethanol, air-dried, and dissolved in DEPC- H_2O .

Complementary DNA (cDNA) Library Construction and Screening

A cDNA library was constructed with messenger RNA purified from total RNA extracted from leaves after cold treatments. The procedures of synthesis of cDNA, size selection, addition of linkers, insertional ligation, and packaging into λ gt11 phage vector were performed following the manufacturer's instructions in the ZAP-cDNA Synthesis and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, Calif.). The total primary

titer of the library in recombinant plaque-forming units was 7.1×10^6 . After amplification, the cDNA libraries were lifted onto N^+ -nylon membrane (Promega, Madison, Wis.) and hybridized with the probes prepared from gene fragments previously identified (Zhou et al., 2005). The conditions for membrane hybridization were prehybridization at 42 °C overnight, hybridization at 42 °C overnight, followed by two 20-min washes in $1\times$ SSC buffer (Sambrook et al., 1989) (7.5 mM NaCl, 0.75 mM Na_3 citrate; pH, 7.0) and in $0.25\times$ SSC and 0.1% sodium dodecyl sulphate (SDS) at 50 °C. The prehybridization and hybridization buffers contained 50% formamide and 0.1% SDS, $5\times$ Denhardt (Sigma, St. Louis, Mo.), $5\times$ SSPE (0.75 M sodium chloride, 50 mM sodium hydrogen phosphate, 5 mM ethylenediamine tetraacetic acid (EDTA); pH, 7.4) and 100 mg-L herring sperm DNA (Sigma). After hybridization, signals were detected on radiographic film by overnight exposure at -70 °C. The positive clones were cured from the original NYZ plates and the cDNA library clones were used to subclone inserts by single-clone excision for conversion of λ phage to pBlueScript SK-phagemid vector. The resulting phagemid libraries were plated at low density on Luria Bertani agar plates containing ampicillin 100 mg-L. Single-strand cDNA was rescued from the phagemid and sequenced from both ends using M13 primers.

Real-time qRT-PCR

Real-time qRT PCR assay was performed using two-step procedures following the instruction in the SYBR-Green PCR Mix/RT kit (Applied Biosystems, FosterCity, Calif.). Genomic DNA was removed from total RNA with DNaseI (Genhunter). The DNA-free RNA was then quantified with a spectrophotometer and was loaded on a denaturing agarose gel to control concentration and integrity. Five micrograms of the RNA was reverse transcribed into cDNA using the MultiScribe Reverse Transcriptase (Applied Biosystems) according to the manufacturer's instructions.

To perform the PCR amplification, gene-specific oligonucleotide primers were designed using OligoPerfect™ Designer (Invitrogen, Carlsbad, Calif.; www.invitrogen.com/). 18S rRNA forward and reverse primers were designed based on the sequence of Genbank accession NC-003071. The position of these oligonucleotides had been chosen so that the size of the PCR product ranged between 50 bp and 150 bp. The genes, the sequence of their specific oligonucleotide primers, and other information are presented in Table 1. The cDNA was amplified on a 7000 Real Time PCR System (Applied Biosystems) using a program of 40 cycles of 94 °C, 30 seconds, and 60 °C, 1 minute.

Real-time PCR results were detected as cycle threshold (Ct). Ct is the cycle at which a significant increase in amount of PCR product (measured by increase in fluorescence) occurs, generally the middle of the exponential phase of amplification. The higher Ct value means there is less cDNA

Table 1. Real-time PCR primers of glycolate oxidase, NADH-dependent hydroxypyruvate reductase and Rubisco activase genes in *Pachysandra terminalis*.

Genes	Accession no.	Plant species	Primers forward/reverse	Amplicon, bp
18S rRNA	NC-003071	<i>Arabidopsis thaliana</i>	5'-CATCAGCTCGCGTTGACTAC-3' 5'-CACTTCACCGGATCATTCAA-3'	110
GOX	DQ442286	<i>Pachysandra terminalis</i>	5'-TCCAAATGCTTCACGATGAG 5'-GTGAGCGGCATCCACTTAAT	100
HPR	DQ442287	<i>P. terminalis</i>	5'-CCATTTGGTGCAAAATCAAA-3' 5'-ACACTGTTGGCAGCTTTCCT	80
RA ²	779769	<i>P. terminalis</i>	5'-GTTTCAGCTCCCGGTATGTA-3' 5'-CGGATAAGAGGGGCATACAA	95

²Rubisco activase.

Table 2. Characteristics of selected cDNA clones from *Pachysandra terminalis*.

Clones accession no.	Conserved domain	Groups (known homolog)	Identity (%) of deduced amino acid	Predicted peroxisome targeting Signal
DQ442286 GOX	GOX_like_FMN	Q9LRR9 (<i>Arabidopsis</i>)	81	-ARL
Bankit779769 RA ²	AAA	P10896 (<i>Arabidopsis</i>)	85	n/a
DQ442287 NADH-dependent HPR	2-Hacid_dh_C	BAA19751 (<i>Arabidopsis</i>)	88	-SKL

²Rubisco activase.

sequence at the start of PCR. Because real-time PCR is a procedure of high sensitivity (Charrier et al., 2002), each real-time PCR was repeated twice to minimize the potential bias introduced by handling during the preparation process. The Ct values from the two repeats were averaged and considered the Ct value for the PCR. To minimize the effect of variables in RNA extraction on the Ct value, the housekeeping gene 18S rRNA was used for RNA normalization. The relative Ct was used to measure the transcript level in each sample. It was calculated as

$$\text{Relative Ct} = A \div B$$

where A is the average of real-time PCR replicates of each sample and B is the average of two values of 18S rRNA. Four repeats were included in each independent temperature treatment. The specificity of real-time PCR was validated by separation of the amplicons on 2% agarose gels.

Sequence Analysis and Database Search

DNA sequence analysis was performed on a 3100 Avant Genetic Analyzer (Applied Biosystems). The DNA sequences were translated into peptide using the ExPASy translation tool (www.expasy.ch/tools/dna.html). Protein and DNA sequence data were searched against the database on the NCBI Web site (www.ncbi.nlm.nih.gov). PTS prediction was performed using the PTS1 predictor (<http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp>).

Results and Discussion

Characterization of the cDNA Clones from *P. terminalis*

The NCBI database was searched for DNA sequences that were homologous to those previously identified (Zhou et al., 2005). Three of the clones were identified

and they shared high homology with the C-terminal sequences of GOX, NADH-dependent HPR, and Rubisco activase. The cDNA fragments from these clones were used to screen the cDNA library constructed in this study. The positive clones from the cDNA library hybridization were sequenced and the cDNA sequences were translated into amino acid sequences. These sequences were also compared with other sequences in the NCBI database. The results are summarized in Table 2. The deduced amino acid sequences of all the cloned genes showed high (>80%) identity with reported genes and had the conserved domains predicted for the encoded enzyme. The deduced peptide of

bankit779769 had the predicted conserved domain of AAA (ATPases associated with a variety of cellular activities) (Neuwald et al., 1999), which designated it to be an ATPase enzyme. The amino acid sequence shared 85% identity with *Arabidopsis* Rubisco activase at the C-terminal sequence. Based on these results, the bankit779769 was predicted as a putative Rubisco activase gene.

The deduced polypeptide of DQ442286 was predicted to have the conserved domain of GOX-like-FMN, which is the core sequence for GOX. The PST tripeptide at the C-terminal of DQ442286 was -ARL, which is conserved in *Arabidopsis* (Q9LRR9) and spinach (P05414). The deduced peptide sequence of DQ442287 had a conserved D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain (2-Hacid_DH_C). It had the tripeptide of -SKL at the C-terminal. The prediction of the targeting signal peptide using the PTS1 predictor located this enzyme protein in the peroxisome (Neuberger et al., 2003). Based on its possible function, location, and homology (88%) with other NADH-dependent HPR (BAA19751 from *Arabidopsis*), this gene was predicted to encode a putative NADH-dependent HPR (Table 2).

Expression of the Gene Clones at Low Temperature in the Leaves of *P. terminalis*

When the real-time PCR products were separated on 2% agarose gels, one specific band with the expected size (80–110 bp) was observed for each gene (Fig. 2). This result validated the specificity of the real-time PCR and the reliability of the Ct values.

qRT-PCR was performed to compare the number of the gene transcripts contained in the RNA extracts of each treatment to predict

Fig. 2. Real-time PCR products of glycolate oxidase (GOX DQ442286), NADH-dependent hydroxypyruvate reductase (HPR DQ442287) and Rubisco activase genes (RA Bankit779769) in *Pachysandra terminalis* and housekeeping gene (18S rRNA) separated on 2% agarose gel.

Fig. 3. Real-time qRT-PCR of glycolate oxidase, NADH-dependent hydroxypyruvate reductase, and Rubisco activase genes in *Pachysandra terminalis*. The relative $C_t = A \div B$, where A is the average of two real-time PCR replicates of each RNA extraction and B is the average of two values of 18s rRNA. Four replicates were included in each treatment.

transcription regulation of these genes by cold stress. The relative C_t values and changing patterns are presented in Figure 3. The standard deviation (SD) values were between 8% and 11% of the average value (data not shown). Because the relative C_t value was calculated as the ratio of the absolute transcript level of each gene to the absolute transcript level of 18s rRNA, the sds are not displayed (Charrier et al., 2002). Figure 3 shows that for all three genes, the relative C_t values were the lowest in the 4 °C treatment for 4 h. This indicated that more gene transcripts were accumulated in the leaf tissues with this treatment. Extending the cold treatment to 12 h resulted in an increase of the C_t values and a decrease of gene transcripts. One of the possible mechanisms is that short-term (4-h) chilling stress activated or enhanced the photorespiration process and thus upregulated expression of the GOX and HPR genes, and resulted in more gene transcripts accumulated in leaf tissues. When the cold stress lasted for a longer period of time (12 h), more enzyme molecules were needed because of the accumulation of substrates and the decrease of the enzyme activity at lower temperature (Rocha et al., 2003; Streb et al., 2005). The increased translation of messenger RNA into protein would consume some gene transcripts, leading to the decrease in the number of the transcripts and the increase of C_t values. Real-time qRT-PCR also showed that the Rubisco activase gene had the same expression pattern as the peroxisome genes in response to chilling stress in *P. terminalis*. Further study is needed for investigating the stress regulation of these genes at the protein and enzyme activity levels.

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