Cloning and Characterization of Glycolate Oxidase and NADH-Dependent Hydropyruvate 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 hydropyruvate reductase gene (HPR) 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′-ribofuranosyl-adenine dinucleotide (NAD) binding domain (2-Hacid, DH C). C-terminal peroxisome targeting signal was predicted to be -AARL for DQ442286 and -SKL for DQ442287. Both genes encoded enzymes 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 486095) was also cloned and analyzed following the same procedure.

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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 Canvin, 1971), it is considered to be an essential metabolic pathway (Somerville and Ogren, 1982; Wu et al., 1991). Inhibitors of photorespiration enzymes 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 imidates for other metabolic processes, such as for the synthesis of glutathione, which is also involved in stress protection (Wingler et al., 1999, 2000).

Photorespiration results from the oxygen 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 is immediately dephosphorylated into glyceraldehyde. The glycated 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 hydroxypropionate, which is reduced by NADH to glyceraldehyde. Glyceraldehyde leaves the peroxisomes and is converted to sugars in the chloroplasts (Kisaki and Tolbert, 1969).

Glycolate oxidase (GOX) and NADH-dependent hydropyruvate 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; Lazazr 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.) (Tsugae 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 glacio- lis (Stebc 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 treatment was compared through real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Results from this research may...
Plant Material Preparation

P. terminalis cuttings were purchased from Yoder Brothers, Inc. (www.yoder.com/). These cuttings were grown 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 1227 μmol m⁻² 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 N₂ 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-H₂O.

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, insertionional ligation, and packaging into Agt11 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...
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

\[
\text{Relative Ct} = \frac{A}{B}
\]

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). Predicted peroxisome targeting signal was performed by 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

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession no.</th>
<th>Plant species</th>
<th>Primers forward/reverse</th>
<th>Amplicon, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>NC-003071</td>
<td>Arabidopsis thaliana</td>
<td>5’-CATGACGTCGCGTGACACCTC-3’</td>
<td>110</td>
</tr>
<tr>
<td>GOX</td>
<td>DQ442286</td>
<td>Pachysandra terminalis</td>
<td>5’-CTCAATATGGTCACGATGAG</td>
<td>100</td>
</tr>
<tr>
<td>HPR</td>
<td>DQ442287</td>
<td>P. terminalis</td>
<td>5’-ACACATGCGACGAGTCTTCCT</td>
<td>80</td>
</tr>
<tr>
<td>RA1</td>
<td>779769</td>
<td>P. terminalis</td>
<td>5’-GGTCAGCTCCCGGATAGA-3’</td>
<td>95</td>
</tr>
</tbody>
</table>

1Rubisco activase.

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

<table>
<thead>
<tr>
<th>Clones accession no.</th>
<th>Conserved domain</th>
<th>Groups (known homolog)</th>
<th>Identity (%) of deduced amino acid</th>
<th>Predicted peroxisome targeting signal</th>
<th>Predicted signal peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ442286 GOX</td>
<td>GOX_like – FMN</td>
<td>Q9LRR9 (Arabidopsis)</td>
<td>81</td>
<td>–ARL</td>
<td>n/a</td>
</tr>
<tr>
<td>Bankit779769 RA1</td>
<td>AAA</td>
<td>P10896 (Arabidopsis)</td>
<td>85</td>
<td>–SKL</td>
<td>n/a</td>
</tr>
<tr>
<td>DQ442287 NADH-dependent HPR</td>
<td>2/Hacid_DH_C</td>
<td>BAA19751 (Arabidopsis)</td>
<td>88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Rubisco activase.
transcription regulation of these genes by cold stress. The relative Ct 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 Ct 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 Ct 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 Ct values and a decrease of gene transcripts. One of the possible mechanisms is that short-term (4-h) chilling stress activated or enhanced the photosrespiration 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 Ct values. Real-time qRT-PCR also showed that the Rubisco activase gene had the same expression pattern as the peroxisomal 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.

Literature Cited


