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Structure and function of a phospholipid hydroperoxide glutathione peroxidase-like protein from barley

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

A cDNA encoding barley phospholipid hydroperoxide glutathione peroxidase (PHGPX)-like protein was cloned and sequenced by the reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends methods. The cDNA comprised 846 bp, and included an open reading frame which encodes a polypeptide of 169 amino acid residues with a molecular mass of 18,532 Da. The deduced amino acid sequence showed significant identity to plant putative PHGPXs and mammalian PHGPXs. The cloned gene was expressed in *Escherichia coli* cells to produce an extra protein, which showed a molecular mass similar to the deduced one, and the clone cells were much more tolerant to NaCl stress than the host cells.

Keywords: Phospholipid hydroperoxide glutathione peroxidase; Barley; Salt stress

1. Introduction

Glutathione peroxidase (GPX) catalyzes the reduction of hydroperoxides using glutathione (GSH) as a reducing agent (Scheme 1). Phospholipid hydroperoxide glutathione peroxidase (PHGPX), found in mammals [1], belongs to GPX family and reduces hydroperoxides of phospholipids, which cannot be reduced by other GPXs [2]. Therefore, PHGPX has been considered one of the defense against oxidative destruction of biomembranes [3].

$$\mathbf{ROOH + 2\ GSH \longrightarrow ROH + GSSG + H_2O}$$

Scheme 1.

Genes encoding homologs of mammalian PHGPX have been isolated from tobacco [4], citrus [5], spinach [6], *Arabidopsis* [7], and tomato [8]. The putative PHGPX gene from tobacco was highly expressed in the fresh protoplasts, the leaves exposed to $HgCl_2$, and the leaves infected by green tomato atypical mosaic virus [4]. The gene from citrus has been identified as a gene expressed in the presence of NaCl [5] and *Escherichia coli* cells expressing citrus PHGPX gene was more tolerant to methyl viologen [9]. Expression level of *Arabidopsis* PHGPX was increased under NaCl and Al/Fe treatments [7]. NaCl stress has been suggested to generate superoxide- and hydrogen peroxide-mediated damage in chloroplasts of plants [10] and other stressful conditions, such as Zn deficiency [11], Fe toxicity [12], and Al toxicity [13], limit the growth of plant cells mostly by free radical peroxidation of membrane phospholipid and thiol

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groups [14]. If the putative PHGPX gene is expressed significantly and the protein encoded by the gene catalyzes the reduction of hydroperoxides, it would be a good candidate component of the antioxidant system in plant cells. However, the putative PHGPX gene has been cloned and characterized in only a few plants, and its function is unclear.

Here, we report the molecular cloning and the nucleotide sequence of a cDNA encoding PHGPX-like protein from barley. The deduced amino acid sequence of the protein was compared with those of PHGPXs and the cloned gene was expressed in *E. coli* cells. The clone cells produced the PHGPX-like protein and showed NaCl tolerance.

2. Materials and methods

2.1. Plant materials and growth condition

Seeds of barley (*Hordeum vulgare* L.), OUK305, were germinated and cultured in a hydroponic solution which consists of 4 mM KNO_3, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mg/ml Fe-citrate, pH 5.5 [15]. After 5 days, the roots were harvested, frozen in liquid nitrogen, and stored at −80 °C.

2.2. RNA preparation

Total RNA was extracted from the roots by the guanidium isothiocyanate method [16]. Poly(A)$^+$ RNA was isolated with Dynabeads Oligo(dT)$_{25}$ (Dynal).

2.3. Reverse transcription-PCR

Total RNA was reverse transcribed with an oligo(dT)$_{16}$ and the murine leukemia virus reverse transcriptase (Perkin-Elmer). The resulting cDNA was subjected to PCR with two primers designed on the basis of well-conserved amino acid sequences of tobacco and citrus putative PHGPX: sense from VNVASQCG, 5′-GT(AGCT)AA(CT)GT(AGCT)GC(AGCT)(AT)-(GC)(AGCT)CA(AG)TG(TC)GG-3′, and antisense from VDKEGNVV, 5′-AC(AGCT)AC(AG)TT(AG-CT)CC(CT)TC(CT)TT(AG)TC(AGCT)AC-3′. The PCR product of 328 bp was subjected to direct sequencing from both strands by using a Taq DyeDeoxy Terminator Cycle Sequencing kit, with an Applied Biosystems 377 DNA sequencer.

2.4. Rapid amplification of cDNA ends PCR (RACE PCR)

RACE PCR was performed using the Marathon cDNA Amplification kit (Clontech). The primers for 3′-RACE PCR and 5′-RACE PCR were synthesized on the basis of the sequence of the reverse transcription-polymerase chain reaction (RT-PCR) product as follows: 5′-GACCAATTCCAACTATACG-GAACTCG-3′; 5′-GAGAAGTTCCATTTGATGTTG-TCCCC-3′. The PCR products of about 700 and 500 bp for 3′-RACE and 5′-RACE, respectively, were subjected to direct sequencing on both strands as described above with a series of synthetic primers.

2.5. Construction of expression plasmid for putative PHGPX

The PHGPX coding region of the cDNA was amplified by PCR with two primers: sense primer, 5′-CC-CATATGGCCGCCGCCGCCTCTTCCG-3′, which creates an *Nde*I site (indicated by an underline) and includes the deduced start codon ATG (indicated in italics), and antisense primer, 5′-GGGGATCCAGC-ATAAAGAT*TTA*AGAAC-3′, which creates an *Bam*HI site (indicated by an underline) and includes the deduced stop codon TAA (indicated in italics). The 534 bp *Nde*I-*Bam*HI fragment obtained by PCR was subcloned into the pET-15b vector (Novagen). The resulting plasmid, pBarPHGPX, was transformed into the host *E. coli* BL21(DE3) pLysS cells.

2.6. Preparation of total cell protein

E. coli cells harboring pBarPHGPX were grown in an LB medium containing 50 μg/ml of ampicillin (Ap). When A_{600nm} became 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM. The cultivation was continued as designated, then cells were harvested by centrifugation. They were suspended in SDS-polyacrylamide gel sample buffer [17] and boiled for 10 min.

2.7. Growth condition under salt stress

E. coli cells cultured overnight in LB medium containing 50 μg/ml of Ap were diluted by one-tenth with

LB medium containing 50 μg/ml of Ap and cultured at 25 °C. After A_{600nm} became 0.3, IPTG was added to the culture at a final concentration of 0.5 mM and cultured at 25 °C for 1 h. Then NaCl was added to the culture to make various concentration (1, 3, 5 and 7%) and *E. coli* cells were continuously cultured at 25 °C.

2.8. Computer analysis

The handling, analysis, and translation of the nucleotide sequences were performed with the GENETYX-Mac (Software Development). Homology search and alignment of amino acid sequences were performed with the BLAST program [18,19] and the CLUSTAL W algorithm [20] using the network service at the National Institute of Genetics, Mishima, Japan (http://www.ddbj.nig.ac.jp/Welcome.html).

3. Results and discussion

3.1. Sequence analysis of putative PHGPX cDNA

The resulting nucleotide sequence, except for the poly(A) sequence, and its deduced amino acid sequence are shown in Fig. 1. A possible open reading frame from a start codon AUG at position 42 to a stop codon UAA at position 549 encodes a polypeptide of 169 amino acid residues with a calculated molecular mass of 18,532 Da.

3.2. Amino acid sequence similarity

The deduced amino acid sequence encoded by the barley cDNA was compared with those of proteins in the SWISS-PROT and PIR data banks. The amino acid sequence of the barley protein was aligned with plant putative PHGPXs (Fig. 2A) and mammalian PHGPXs (Fig. 2B), respectively. Putative PHGPXs from *Arabidopsis*, citrus, spinach, and tobacco showed 86, 83, 78, and 77% identities with the barley protein, respectively. Among mammalian proteins, PHGPXs from human testis [21], pig heart [22], pig blastocyst [23], rat brain [24], and rat testis [25] showed 45, 44, 44, 44, and 44% identities with the barley protein, respectively. The number of conserved amino acid residues among three plant proteins and among the barley protein and five mammalian PHGPXs are 110 and 75, respectively. The barley protein has 169 amino acid residues which is in good agreement with

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TGCTCCGCCCGGTCGGCGCCCGATTCTCGCTGTCCAGCAGCATGGCCGCCGCCGCCTCTTCCGCCTCCTCCGTCC   75
                                          M  A  A  A  A  S  S  A  S  S  V  H   12

ACGACTTCACCGTCAAGGATGCAAGTGGAAAAGATGTCGATCTGAGCACCTACAAGGGGAAGGTTCTCCTCATTG  150
  D  F  T  V  K  D  A  S  G  K  D  V  D  L  S  T  Y  K  G  K  V  L  L  I  V   37

TCAATGTTGCATCCCAGTGTGGATTGACCAATTCCAACTATACGGAACTCGCTCAGTTGTATGAGAAGTACAAGG  225
  N  V  A  S  Q  C  G  L  T  N  S  N  Y  T  E  L  A  Q  L  Y  E  K  Y  K  D   62

ACCAGGGTTTTGAGATCCTTGCTTTCCCATGCAACCAGTTTGGTGGGCAGGAACCTGGCACTAATGAGGAAATTG  300
  Q  G  F  E  I  L  A  F  P  C  N  Q  F  G  G  Q  E  P  G  T  N  E  E  I  V   87

TTCAGTTTGCTTGCACTCGCTTCAAGGCCGAGTATCCAATTTTTGACAAGGTTGATGTCAATGGTGACAATGTTG  375
  Q  F  A  C  T  R  F  K  A  E  Y  P  I  F  D  K  V  D  V  N  G  D  N  V  A  112

CACCTGTCTACAAGTTTCTGAAGTCGAGCAAAGGCAGTCTCTTCGGGGACAACATCAAATGGAACTTCTCCAAGT  450
  P  V  Y  K  F  L  K  S  S  K  G  S  L  F  G  D  N  I  K  W  N  F  S  K  F  137

TCTTGGTTGACAAGGATGGGAATGTTGTGGATCGCTACGCGCCGACCACCTCCCCCCTCAGCATCGAGAAGGACA  525
  L  V  D  K  D  G  N  V  V  D  R  Y  A  P  T  T  S  P  L  S  I  E  K  D  I  162

TCAAGAAGCTGCTCGCGAGTTCTTAAATCTTTATGCTGGATCAACTCGACGCATCGGTACACCTGAAGCTTCAAT  600
  K  K  L  L  A  S  S  *                                                      169

AATGTTGTAATAAGGGTCGTGCTCGGAACTGGCTATGTTATGCGTGCTCAGCAGCCTCCGATACCTTGCGCATTC  675
CAATCTCGTATTGCCCGTCTCGTAGTATATATGACATGTAATGGATTTGGTGGAATGCATTACTTCTGTTGCAGA  750
GATATCTCCTCTATCTGTGTCTTTATTGTTGTTGTGAGATTCATGTTCCTGAGGAACAAGTTTGGAATGTGATGC  825
ATGCCTGTTGTTCGTGGCTCT                                                         846
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Fig. 1. Nucleotide and deduced amino acid sequences of a cDNA encoding barley PHGPX-like protein. The nucleotide and the amino acids are numbered from the 5′ end of the cDNA and from the initiation methionine residue, respectively. A stop codon is indicated by an asterisk. The nucleotide sequence data reported appears in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB096704.

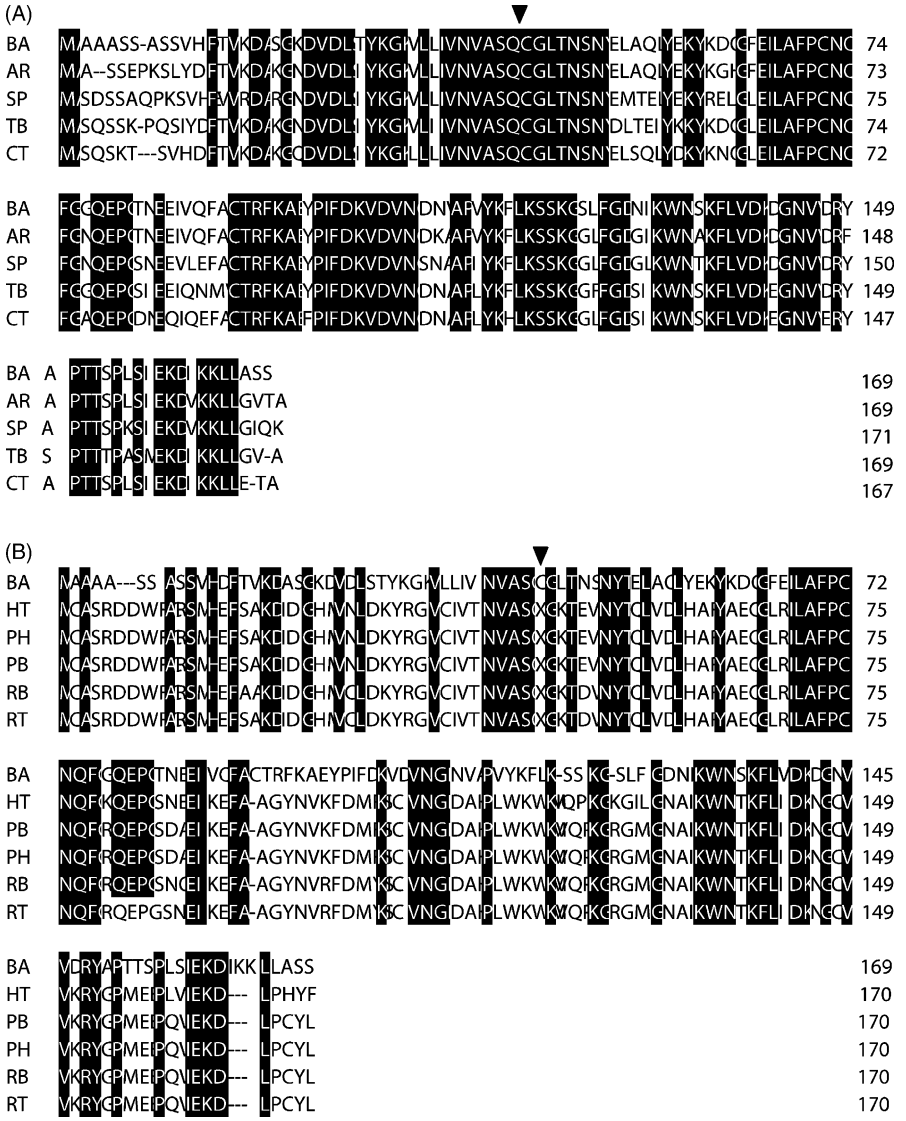

Fig. 2. Alignment of the amino acid sequence of barley PHGPX-like protein with those of plant putative PHGPXs (A) and mammalian PHGPXs (B). Gaps, indicated by dash, are introduced in the sequences to maximize the homology. Amino acid residue identical with that of barley PHGPX-protein is represented by reversal letters. The Cys residue corresponding to the SeCys in mammalian PHGPXs is indicated by an arrowhead. The SeCys is indicated by X. Amino acid sequences of mammalian PHGPXs are the mature form suggested by Pushpa-Rekha et al. [23]. BA, barley PHGPX-like protein; SP, spinach putative PHGPX; AR, *A. thaliana* putative PHGPX; TB, tobacco putative PHGPX; CT, citrus putative PHGPX; HT, human testis PHGPX; PH, pig heart PHGPX; PB, pig blastocyst PHGPX; RB, rat brain PHGPX; RT, rat testis PHGPX.

those of plant putative PHGPXs and mammalian PHGPXs, respectively. The mammalian PHGPX is a selenoenzyme, in which the active site is a selenocysteine (SeCys) encoded by a termination codon, UGA, on the gene [21–25]. The Cys43 of the barley protein was identified with the Cys43 and the Cys41 of tobacco and citrus, respectively, which were suggested to correspond to the SeCys in mammalian PHGPXs [4,5]. The Cys was encoded by UGU in these plant genes, suggesting that the barley cDNA encodes a

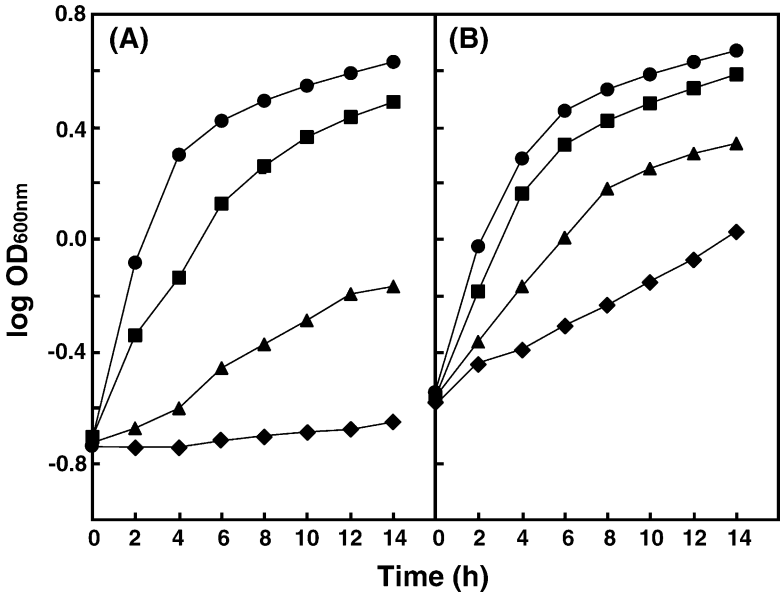

Fig. 3. Growth of *E. coli* cells harboring pBarPHGPX (A) and pET15b (B). *E. coli* cells were cultured in LB medium containing 50 μg/ml of Ap, 0.5 mM of IPTG, and 1% (●), 3% (■), 5% (▲), and 7% (◆) of NaCl at 25 °C. Cell growth after the addition of NaCl was monitored by measuring the absorbance at 600 nm of the culture.

putative PHGPX, in which the Cys43 corresponds to the active site, SeCys, in mammalian PHGPXs.

3.3. Contribution of putative PHGPX gene to NaCl tolerance

E. coli cells were assayed for their ability to grow in the medium containing NaCl (Fig. 3). In the 1% of NaCl (standard concentration in LB medium), there was no difference between the growth of *E. coli* cells harboring pBarPHGPX and that of *E. coli* cells harboring pET15b as a control. The growth of the cells harboring pBarPHGPX in the 3% of NaCl was similar to that in 1% of NaCl, while that of the control cells was slightly decreased than that in the 1% of NaCl. In the 5% of NaCl, the control cells showed a slight growth and aggregated after 10 h, however, the cells harboring pBarPHGPX kept growing and reached about 45% of the growth in the 1% of NaCl after 14 h. In the 7% of NaCl, the cells harboring pBarPHGPX showed a slight growth, of which the ratio was about 23% of that in the 1% of NaCl after 14 h, while no growth was observed in the control cells. These results indicate that the survival rate of *E. coli* cells harboring pBarPHGPX is higher than the control cells. Furthermore, the time course of the putative PHGPX protein synthesis in *E. coli* cells harboring pBarPHGPX induced by IPTG was analyzed by SDS-polyacrylamide gel electrophoresis [17] (Fig. 4). An extra protein band of about 22,000 Da was detected 1 h after IPTG induction, and production of the protein in the cells harboring pBarPHGPX reached a maximum of about 12% of the total cellular protein based on the intensities of protein bands, showing that a sufficient amount of the barley PHGPX-like protein was produced in the cells. From these results, barley PHGPX-like protein encoded by pBarPHGPX could enhance the tolerance of *E. coli* cells against NaCl stress, which generates oxidative stress.

SeCys-independent glutathione peroxidase gene was isolated from filarial nematode. The deduced amino acid sequence showed 42% homology with human liver SeCys-dependent glutathione peroxidase and SeCys in the active site was substituted by Cys [26]. The enzyme has activity toward phospholipid hydroperoxide and linolenic acid hydroperoxide but no significant activity toward hydrogen peroxide, whereas mammalian SeCys-dependent glutathione

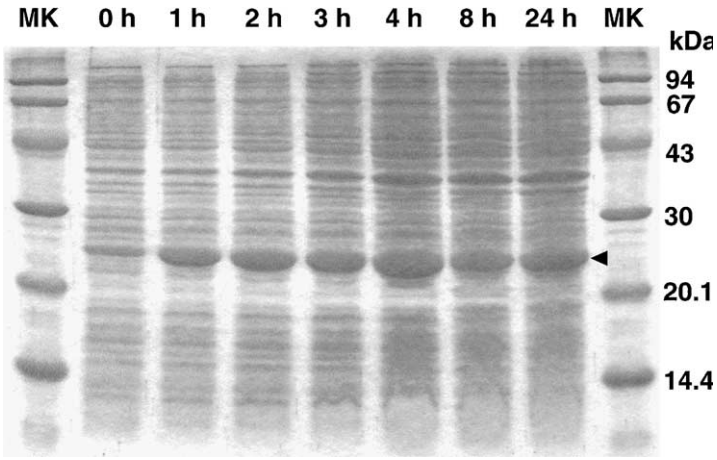

Fig. 4. SDS-polyacrylamide gel (15%) electrophoresis of *E. coli* cells. *E. coli* cells harboring pBarPHGPX were cultured for 0, 1, 2, 3, 4, 8, and 24 h after addition of IPTG. Protein was stained with Coomassie brilliant blue R-250. An extra band of 22,000 Da is indicated by an arrowhead. MK, marker proteins.

peroxidase has activity toward hydrogen peroxide and linolenic acid hydroperoxide but not toward phospholipid hydroperoxide [27]. It was reported that the mutant of human SeCys-thioredoxin reductase, in which SeCys was replaced with Cys, showed about 5% of the wild type enzyme [28] and the recombinant citrus PHGPX, in which the Cys41 was replaced with SeCys displayed a fourfold enhanced peroxidase activity [29]. We assayed the PHGPX activity of the cell-free extract prepared from *E. coli* cells harboring pBarPHGPX, however, no activity was detected when *tert*-butyl hydroperoxide, cumene hydroperoxide, and hydrogen peroxide were used as a substrate. Although it has not been clear that barley putative PHGPX might have different substrate specificity from mammalian PHGPXs or have other functions, our results suggest that the PHGPX-like protein would be a good candidate component of anti-salt/oxidative enzyme in plants. It is expected that transgenic plant overexpressing barley PHGPX-like gene would show salt tolerance and improve plant growth and agriculture productivity on the cultivated land, about 20% of which is affected by salinity [30].

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