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Molecular Cloning, Sequencing, and Expression of a cDNA Encoding Putative Phospholipid Hydroperoxide Glutathione Peroxidase from *Arabidopsis thaliana*

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A cDNA encoding *Arabidopsis* putative phospholipid hydroperoxide glutathione peroxidase (PHGPX) was cloned and sequenced by the reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends methods. The cDNA comprised 803 bp, and included an open reading frame which encodes a polypeptide of 169 amino acid residues with a molecular mass of 18,600 Da. The deduced amino acid sequence showed homology 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.

Key words : *Arabidopsis*, Phospholipid hydroperoxide glutathione peroxidase, Nucleotide sequence, Gene expression.

INTRODUCTION

Stressful conditions, such as Zn deficiency¹⁾, Fe toxicity²⁾, and Al toxicity³⁾, limit the growth of plant cells mostly by free radical peroxidation of membrane phospholipid and thiol groups⁴⁾. Phospholipid hydroperoxide glutathione peroxidase (PHGPX) is found in mammals⁵⁾ and catalyzes the reduction of hydroperoxides of phospholipids to protect biomembranes from oxidative damage. Recently, genes encoding homologs of mammalian PHGPX have been isolated from tobacco⁶⁾ and citrus cells⁷⁾. The putative PHGPX gene from tobacco was highly expressed in the fresh protoplasts, the leaves exposed to HgCl₂, and the leaves infected by green tomato atypical mosaic virus⁶⁾. The gene from citrus has been identified as a gene expressed in the presence of NaCl⁷⁾. NaCl stress has been suggested to generate superoxide- and hydrogen peroxide-mediated damage in chloroplasts of

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plants⁸⁾. If the putative PHGPX gene is induced by oxidative stress 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^{6,7,9)}, and its function is unclear.

Here we report the molecular cloning and the nucleotide sequence of a cDNA encoding a putative PHGPX from *Arabidopsis*. The deduced amino acid sequence of the protein was compared with those of PHGPXs. The cloned gene was expressed in *Escherichia coli* cells to produce the protein.

MATERIALS AND METHODS

1. RNA preparation

Total RNA was extracted from the leaves of *Arabidopsis* (*Arabidopsis thaliana*, ecotype Columbia) by the guanidium isothiocyanate method¹⁰⁾. Poly(A)⁺ RNA was isolated with Dynabeads Oligo(dT)₂₅ (Dynal).

2. Reverse transcription PCR (RT-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(AGCT)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.

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

RACE PCR was performed using the Marathon cDNA Amplification kit (Clontech). The primers for 3'- and 5'-RACE PCR were synthesized on the basis of the sequence of the RT-PCR product as follows: 5'-GGGCTTACCAACTCAAACACTACAC-3'; 5'-CTTCAAGAACTTATAGATTGGGG-3'. The PCR products of 489 and 462 bp for 3'- and 5'-RACE, respectively, were subjected to direct sequencing on both strands as described above with a series of synthetic primers.

4. Construction of expression plasmid for putative PHGPX

The PHGPX coding region of the cDNA was amplified by PCR with two primers: sense primer, 5'-CATATGGCTGCTTCTTCCGAACC-3', which creates an *NdeI* site (indicated by an underline) and includes the deduced start codon ATG (indicated in italics), and antisense primer, 5'-

GGATCCTTGCTTAAGCAGTAACT-3', which creates an *Bam*HI site (indicated by an underline) and includes the deduced stop codon TAA (indicated in italics). The 512 bp *Nde*I-*Bam*HI fragment obtained by PCR was subcloned into the pET-15b vector (Novagen). The resulting plasmid, pARGPX-EX1, was transformed into the host *Escherichia coli* BL21(DE3) cells.

5. Preparation of total cell protein

E. coli cells harboring pARGPX-EX1 were grown in an LB medium containing ampicillin (50 μ g/ml). When $A_{600\text{nm}}$ became 0.5, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM. The cultivation was continued as designated, then cells were harvested by centrifugation. They were suspended in SDS-polyacrylamide gel sample buffer¹¹⁾ and boiled for 10 min.

6. 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^{12,13)} and the malign program¹⁴⁾ using the E-mail network service at the National Institute of Genetics, Mishima, Japan.

RESULTS AND DISCUSSION

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 113 to a stop codon UAA at position 620 encodes a polypeptide of 169 amino acid residues with a calculated molecular mass of 18,600 Da. A putative polyadenylation signal, AAUAAA, at position 649 to 654 is found about 150 bp upstream from the 3'-end of the cDNA. The nucleotide sequence of the putative PHGPX gene from *A. thaliana* showed identity with those of *Arabidopsis* EST cDNA clones 118F4T7, 118F6T7, and 99N6T7¹⁵⁾, which may have a full length cDNA corresponding to the putative PHGPX gene.

2. Amino acid sequence homology

The deduced amino acid sequence encoded by the *A. thaliana* cDNA was compared with those of proteins in the SWISS-PROT and PIR data banks. The amino acid sequence of the *A. thaliana* protein was aligned with plant putative PHGPXs (Fig. 2A) and mammalian PHGPXs (Fig. 2B), respectively. Putative PHGPXs from tobacco⁶⁾, citrus⁷⁾, and spinach⁹⁾ showed 80, 82, and 82% homology with the *A. thaliana* protein, respectively. Among mam-

Putative phospholipid hydroperoxide glutathione peroxidase gene from *A. thaliana*

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CGCCGGGAGGTAACGATTCGATTCTGCGAAACCACTTTCAATTCTCATCGGATTATTCTCTTCCAATTTCTACAACGGGAGCTAAACTATCTAGATCGGAGCATT 111
AATGGCTGCTTCTCCGAACCCAAATCTCTTTACGATTTACCGTCAAGGATGCTAAGGAAACGATGTTGATCTAAGCATCTACAAAGGAAGGTTCTTTTGATTGTGAA 222
M A A S S E P K S L Y D F T V K D A K G N D V D L S I Y K G K V L L I V N 37

CGTTGCTTCTCAATGTGGCTTGACTAATCGAATATACTGAGCTTGCGCAGCTGTATGAGAAGTACAAGGCCATGGTTTCGAGATTCTTGCCTTCCGTGTAACCAAGTT 333
V A S Q C G L T N S N Y T E L A Q L Y E K Y K G H G F E I L A F P C N Q F 74

TGGGAATCAAGAGCCTGGTACTAATGAAGAGATTGTTCAAGTTGCTTACTCGTTTCAAGGCTGAGTACCCGATTTTCGACAAGGTTGATGTTAACCAGGTGACAAAGCTGC 444
G N Q E P G T N E E I V Q F A C T R F K A E Y P I F D K V D V N G D K A A 111

CCCAGCTACAAGTTTCTGAAATCAAGCAAAAGCGGGCTCTTTGGAGACGGCATTAAAGTGAAGTTCGCAAAAGTTCTTGGTTGACAAGATGGAATGTTGCGATCGTTT 555
P V Y K F L K S S K G G L F G D G I K W N F A K F L V D K D G N V V D R F 148

CGCACCACTACCTCACCTCTCAGCATTGAGAAGGATGTGAAGAAGTTGTTGGGGTACTGCTTAAGCAAGGCAAGATTGCATAATTAGACAAATAAAAGCTCATTAGTA 666
A P T T S P L S I E K D V K K L L G V T A * 169

TTGATTACCAATACTGTGTAGTAGCTGAGTTCGTGAGTGTGTCCTTTGGGACCGGTACAATTATAATCCGTTTTTTAGCGGCCAACTATGTAATAATCGTAGATCT 777
AAATATCAGATTTCCGTTACCTTAC 803

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Fig. 1. Nucleotide and deduced amino acid sequences of a cDNA encoding *A. thaliana* putative PHGPX. 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 and a putative polyadenylation signal is double-underlined. The nucleotide sequence data reported appears in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AD001568.

malian proteins, PHGPXs from human testis¹⁶⁾, pig heart¹⁷⁾, pig blastocyst¹⁸⁾, rat brain¹⁹⁾, and rat testis²⁰⁾ showed 50, 49, 49, 49, and 49% homology with the *A. thaliana* protein, respectively. The number of conserved amino acid residues among three plant proteins and among the *A. thaliana* protein and five mammalian PHGPXs are 125 and 79, respectively. The *A. thaliana* protein has 169 amino acid residues which is in good agreement with 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 gene¹⁶⁻²⁰⁾. The Cys-42 of the *A. thaliana* protein was identified with the Cys-43 and the Cys-41 of tobacco and citrus, respectively, which were suggested to correspond to the SeCys in mammalian PHGPXs^{6,7)}. The Cys-42 of the *A. thaliana* protein was also identified with the SeCys of mammalian PHGPXs. In these plant genes, the Cys was encoded by UGU. Therefore, these results suggest that the *A. thaliana* cDNA encodes a putative PHGPX, in which the Cys-42 might be a catalytic residue.

3. Expression of putative PHGPX gene in *E. coli* cells.

The time course of the putative PHGPX protein synthesis in *E. coli* cells harboring pARGPX-EX1 induced by IPTG was analyzed by SDS-polyacrylamide gel electrophoresis¹¹⁾ (SDS-PAGE) (Fig. 3). An extra protein band of about 22,000 Da was detected 1 h after IPTG induction, and expression of the protein in *E. coli* cells harboring pARGPX-EX1 reached a maximum of about 12% of the total cellular protein based on the intensities

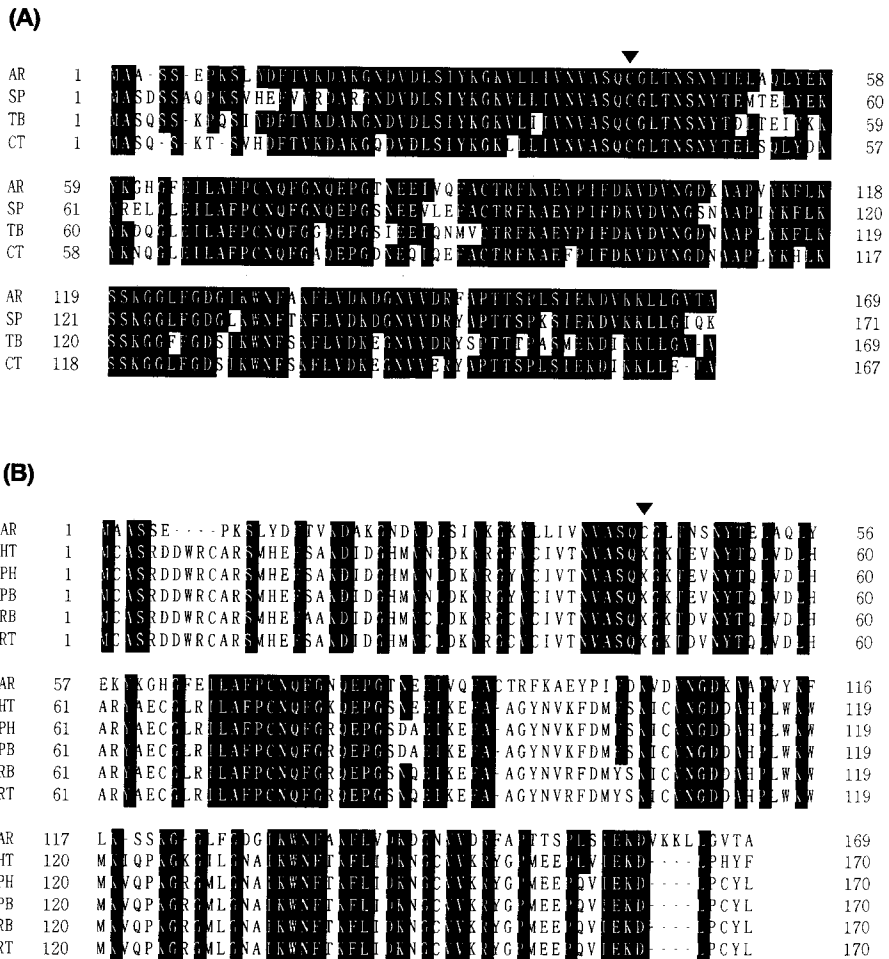


Fig. 2. Alignment of the amino acid sequence of *A. thaliana* putative PHGPX with those of plant putative PHGPXs (A) and mammalian PHGPXs (B). Gaps, indicated by dash, are introduced in the sequences to maximize the homology. Amino acid residue identical with that of *A. thaliana* putative PHGPX is represented 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 al²⁰. AR, *A. thaliana* putative PHGPX; TB, tobacco putative PHGPX⁶; CT, citrus putative PHGPX⁷; HT, human testis PHGPX¹⁶; PH, pig heart PHGPX¹⁷; PB, pig blastocyst PHGPX¹⁸; RB, rat brain PHGPX¹⁹; RT, rat testis PHGPX²⁰.

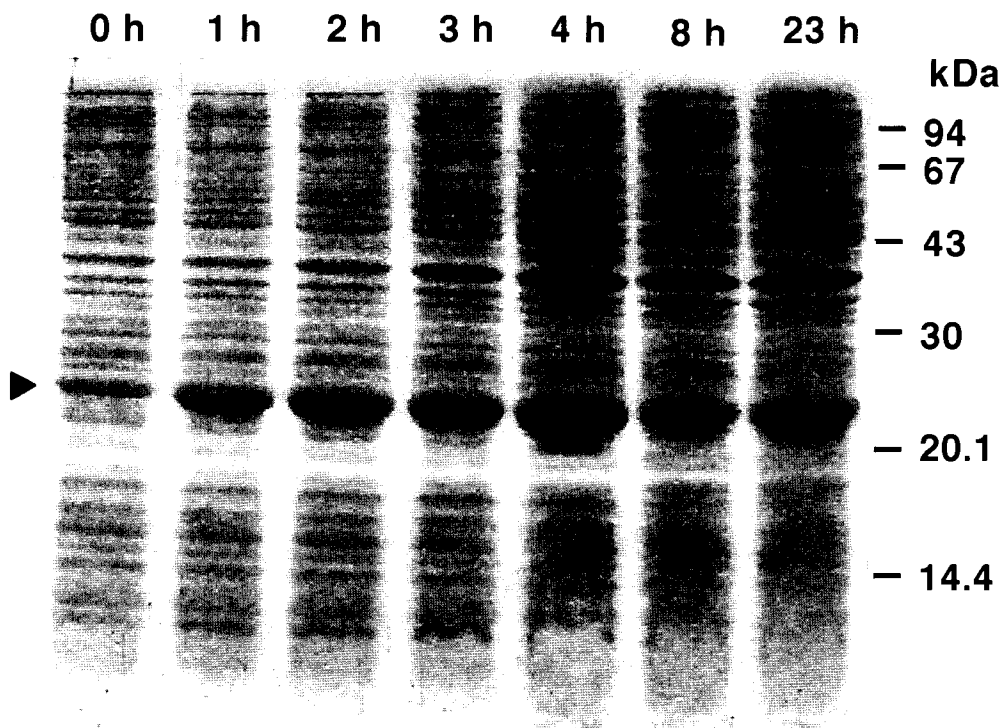


Fig. 3. SDS-polyacrylamide gel (15%) electrophoresis of *E. coli* cells. *E. coli* cells harboring pARGPX-EX1 were cultured for 0, 1, 2, 3, 4, 8, and 23 h after addition of IPTG. Protein was stained with Coomassie brilliant blue R-250. An extra band of 22,000 Da is indicated by an arrowhead. Marker protein bands are indicated on the right.

of protein bands. The pARGPX-EX1 plasmid expresses the PHGPX protein with N-terminal fusion of His-Tag oligopeptide (about 2,200 Da), showing the difference of molecular mass between the deduced PHGPX and the expressed protein.

Recently, 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 Cys²¹). The enzyme has activity toward phospholipid hydroperoxide and linolenic acid hydroperoxide but no significant activity toward hydrogen peroxide, whereas mammalian SeCys-dependent glutathione peroxidase has activity toward hydrogen peroxide and linolenic acid hydroperoxide but not toward phospholipid hydroperoxide²²). From these results, we speculated that the *A. thaliana* putative PHGPX might have different substrate specificity from mammalian PHGPXs, and it is important to purify the *A. thaliana* putative PHGPX to evaluate the enzymatic function. Purification of the *A. thaliana* PHGPX

protein is in progress to clarify the enzymatic function and the physiological role in plant cells exposed to oxidative stress.

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シロイヌナズナ由来過酸化リン脂質 グルタチオンペルオキシダーゼ様 遺伝子のクローニングと発現

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シロイヌナズナから過酸化リン脂質グルタチオンペルオキシダーゼと高い相同性を示すタンパク質をコードする cDNA を単離し、その塩基配列を決定した。本遺伝子は全長803bp かなり、169アミノ酸残基のタンパク質をコードしていた。アミノ酸配列は植物由来過酸化リン脂質グルタチオンペルオキシダーゼ様タンパク質と約80%の相同性を、哺乳類由来過酸化リン脂質グルタチオンペルオキシダーゼと約50%の相同性を示した。本遺伝子を大腸菌中で発現させた結果、遺伝子から予測される分子量をもつタンパク質が新たに生産された。

キーワード：シロイヌナズナ、過酸化リン脂質グルタチオンペルオキシダーゼ、
塩基配列、遺伝子発現