Radish Phospholipid Hydroperoxide Glutathione Peroxidase Gene Structure and Upstream Regulatory Sequence Analysis*

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Abstract A novel radish *RsPHGPx* cDNA, which encodes a functional phospholipid hydroperoxide glutathione peroxidase (PHGPx) protein, was identified in the previous work. In the study genomic organization and the upstream regulatory sequence analysis of this gene was presented. Southern blot analysis showed that *RsPHGPx* gene existed in radish genome in manner of single copy. Moreover, a 3.3 kb genomic DNA fragment of *RsPHGPx* gene was isolated by combination of common PCR and genome-walking method. Sequence analysis on this genomic fragment demonstrated that *RsPHGPx* gene consists of seven exons separated by six introns, and suggested that a short 5'-flanking sequence immediately before the exon 1 should be the putative *RsPHGPx* promoter region, which is proceeded by the upstream neighboring biotin synthase gene. *Cis*-acting elements search showed that the putative promoter contains elements responsive to hormones (eg. E-Box and W-Box), abiotic stresses (eg. MYB and MYC binding sites), and light (Box Ⅱ and I-Box), etc. Northern blot analysis indicated that the expression of *RsPHGPx* was subjected to up-regulation of chilling and down-regulation of ABA and successive illumination (in etiolated seedlings), implying the regulatory roles of some predicted elements. However the up-regulation effect of herbicide paraquat, which can induce oxidative stress, suggested the presence of some unknown elements in the promoter region. This is the first report on gene structure and upstream regulatory sequence analysis in reported plant *PHGPx* genes, which will be a prerequisite to understand regulatory mechanism of *PHGPx* gene expression in plants.

Key words radish, phospholipid hydroperoxide glutathione peroxidase, gene structure, upstream regulatory sequence, *cis*-element

Glutathione peroxidases (GPx) are a group of important antioxidant enzymes that catalyze the reduction of H_2O_2 and various hydroperoxides in the presence of glutathione as the hydrogen donor[1]. They have been found in a variety of organisms including yeast [2], plants [3,4] and animals [1,5]. In animals, they consist of four isoforms including cytosolic GPx (c-GPx), plasma GPx (p-GPx), gastrointestinal GPx (GPx-GI), and phospholipid hydroperoxide glutathione peroxidase (PHGPx). Among these isoforms, PHGPx is a distinct one in structure and substrate specificity. It is monomeric rather than tetrameric, and it is capable of reducing peroxidized phospholipids, cholesterol hydroperoxides, and thiamine hydroperoxides[1], which cannot be reduced by the other GPx enzymes. Thus, PHGPx is considered to be the primary enzymatic defense against oxidation for biomembranes in mammals[1, 2, 5].

In contrast to the intensive studies carried out on mammalian PHGPxs, only sporadic reports on PHGPx in plants were published [3]. Up to now, a number of *PHGPx* homologues have been isolated from some plant species [6~12]. Northern blot analyses of these homologues demonstrated that they were expressed in tissue-specific [6,7], development-dependent [8], or stress-responsive [7~9] manners. However, our knowledge about regulatory mechanisms involved in plant *PHGPx* gene expression is very limited.

Recently, a novel radish *PHGPx* gene (*RsPHGPx*) encoding a functional PHGPx enzyme was identified in our laboratory and suggested to be submitted to a complicated regulation [13]. To comprehensively understand the regulatory mechanisms, we isolated the genomic DNA of *RsPHGPx* gene and characterized its genomic organization and putative promoter region. Our results provide a prerequisite to probe into regulatory mechanisms and biological functions of

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PHGPx genes in plants.

1 Materials and methods

1.1 Plant materials and treatments

Radish seeds (*Raphanus sativus* cv. Meinong) were sown in pots containing a mixture of organic substrate and vermiculite (3∶1) and grown in growth chamber set to (25±1)℃ under the light (16 h light /8 h dark cycle, 50 ~ 80 μmol photons·m^{-2}·s^{-1}) to prepare normal seedlings, or in the dark to prepare etiolated seedlings. Eight-day-old seedlings were directly sprayed with 2 mmol/L salicylic acid, 40 μmol/L paraquat, or 2 mmol/L ethephon, and kept in the light for 24 h, or carefully pulled out from pots, gently washed, and dipped separately in aqueous solutions containing 50 μmol/L NaCl, 100 μmol/L $AlCl_3$, 100 μmol/L abscisic acid (ABA), 10 mmol/L H_2O_2, and 0.1 mol/L sucrose, followed by incubation in the dark for 20 h. Chilling treatment was carried out by exposing seedlings to 4℃ in the dark for 6 h and then restoring them to room temperature in the light for 12 h. For successive illumination treatment, etiolated seedlings grown in the dark were successively kept under light for different time. Normal and etiolated seedlings untreated or treated with distilled water were taken as controls. All these prepared materials were immediately frozen in liquid nitrogen and stored at −70℃until use.

1.2 Genomic DNA extraction, RNA isolation and hybridization analyses

Total genomic DNA was extracted from 8-day-old radish seedlings by the method described previously [14]. 15 μg of genomic DNA was digested with restriction endonucleases, separated on 0.8% agarose gel, and transferred onto a nylon membrane (Hybond N^+, Amersham, USA) using techniques recommended by the manufacturer. Southern blot hybridization was performed with α-^{32}P labeled *RsPHGPx* cDNA probe by the standard method[15].

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, USA). RNA concentration and purity were determined spectrophotometrically, and the integrity as well as size distribution was checked by agarose gel electrophoresis. For Northern blot analysis, equal amounts of total RNA (25 μg) for each blot were loaded, electrophoresed in 1.2% formaldehyde agarose gel, and blotted to the nylon membrane as described above. Northern blot hybridization was performed as described previously [16].

1.3 Determination of *RsPHGPx* gene structure

To isolate the *RsPHGPx* genomic sequence and avoid infidelity resulted from long distance PCR, two-step amplification with the primers (P1/P2 and P3/P4) listed in Table 1 using the genomic DNA as a template was performed. The final PCR products were cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced. Exon/intron boundaries were determined by aligning the genomic sequence with the *RsPHGPx* cDNA.

Table 1 Primers used in this study

Name	Sequence (from 5′ to 3′)	Positions in cDNA
P1	ATGCCTAGATCAAGAAGTC	42～60
P2	TCACGTAGATGCCAATAGC	617～635
P3	TACAACATCACCTCTTGAGA	578～597
P4	CGTACAAAAAGATTTTGGTAATG	820～842
GSP1	AGAAGGCAGGTACCTGTACA	103～122
GSP2	CTTCTTGATCTAGGCATCGAGACAG	34～58
AP1	GTAATACGACTCACTATAGGGC	Outer adaptor primer
AP2	ACTATAGGGCACGCGTGGTCG	Inner adaptor primer

1.4 Cloning and analysis of promoter region

GenomeWalker™ Kit (Clontech, USA) was employed to isolate the 5′ - flanking sequence of *RsPHGPx* gene. Gene-specific primers (GSP) and adaptor primers (AP) are listed in Table 1. After complete digestion of genomic DNA with restriction enzyme *Eco*RⅤ, the digest with blunt ends was ligated to the GenomeWalker adaptor to construct a library. Using the library as template, primary PCR amplification was performed with the outer gene-specific primer GSP1 and the outer adaptor primer AP1. To improve the specificity of target PCR product, the primary PCR products were then diluted and used as templates for a secondary PCR reaction (nested PCR) with primer GSP2 and AP2. The second-round PCR products were cloned into pMD18-T vector and sequenced. Database search of the isolated sequence was carried out using Basic Local Alignment Search Tool (BLAST) program of the National Center of Biotechnology Information [17] to identify the putative promoter region and the upstream neighboring gene. The PLACE database[18] was used to search *cis*-elements contained in the putative promoter

region (http://www.dna.affrc.go.jp/htdocs/PLACE).

2 Results and discussion

2.1 Genomic organization of the radish *RsPHGPx* gene

Previously, we identified a full-length *PHGPx* cDNA (*RsPHGPx*) from radish and showed that the cDNA encodes a functional PHGPx protein [13]. To investigate the copy number of *RsPHGPx* in radish genome, total genomic DNA was digested with restriction enzymes, including *Bam*HⅠ, *Eco*RⅠ, *Hin*dⅢ, and *Xba*Ⅰ. Southern hybridization result showed that only one band was detected for each DNA digest (Figure 1a), indicating that *RsPHGPx* is a single-copy gene in radish genome.

To obtain genomic sequence of *RsPHGPx* gene, PCR amplification was carried out using radish genomic DNA as template. A 5′-fragment of 1 427 bp and a 3′-fragment of 643 bp were cloned with primers P1/P2 and primers P3/P4, respectively (Figure 1b). As shown in Figure 2 and Table 2, *RsPHGPx* gene consists of seven exons separated by six introns. The size of exons ranges from 49 (exon 6) to 191 bp (exon 7), and the introns vary in size from 73 bp (intron 3) to 441 bp (intron 1). As seen from Table 3, all exon/intron junctions match the consensus GT-AG rule [19] at the intron acceptor and donor sites, and amino acid codons are split by introns 2 and 3 at the junctions of their adjacent exons. The genomic sequence of *RsPHGPx* gene has been deposited in the GenBank under accession number AY919316.

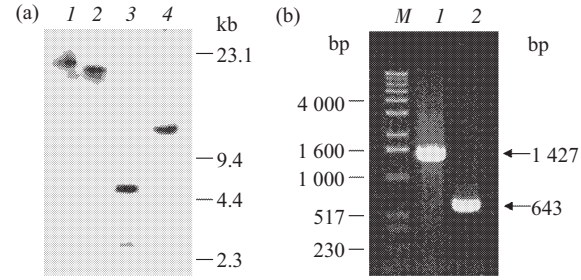

Fig.1 Southern blot analysis and isolation of the *RsPHGPx* genomic DNA fragment

(a) Genomic DNA (15 μg) was separately digested with restriction endonuclease *Bam*HⅠ (*1*), *Eco*RⅠ (*2*), *Hin*dⅢ (*3*), and *Xba*Ⅰ (*4*) and hybridized with α-^{32}P labeled *RsPHGPx* cDNA. DNA sizes are indicated on the right. (b) Two-step PCR amplification using genomic DNA as template was performed and a 1 427 bp (*1*) and a 643 bp (*2*) fragments were obtained. Molecular markers (*M*) are indicated on the left.

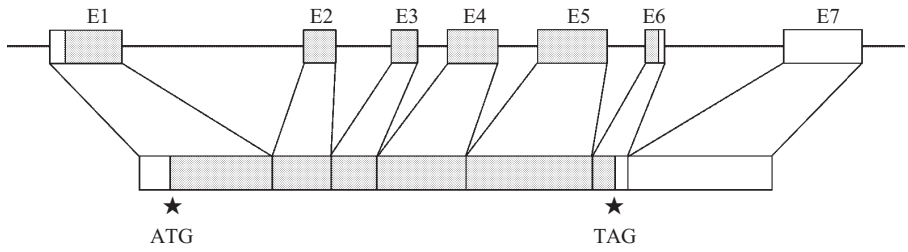

Fig.2 Genomic organization of *RsPHGPx* gene

Boundaries between exons (box E1~E7) and introns (lines spaced by boxes) were determined by aligning the genomic sequence with the corresponding *RsPHGPx* cDNA. Dark boxes represent the open reading frame of *RsPHGPx* gene, and the open boxes represent untranslated regions. Asterisks indicate the translation start codon (ATG) and stop codon (TAG).

Table 2 *RsPHGPx* gene exons and introns

Exon				ORF			Intron			
Number	Start	End	Size	Start	End	Size	Number	Start	End	Size
1	1	176	176	42(ATG)			1	177	617	441
2	618	694	77				2	695	826	132
3	827	888	62				3	889	961	73
4	962	1 080	119				4	1 081	1 176	96
5	1 177	1 344	168				5	1 345	1 435	91
6	1 436	1 484	49		1 466(TAG)		6	1 485	1 771	287
7	1 772	1 962	191							
Total /bp			842			594				1 120

Table 3 Sequence at exon-intron junctions and the interrupted codens by introns

5′-Donor exon	Intron	3′-Acceptor exon	Codon interrupted/Amino acid
CCGTTAAG	GTATATAT.....AATTGCAG	GACATTGA	AAG/GAC (K45/D46)
TCTAAGTG	GTAAGCTA.....TTGTGCAG	TGGTCTAA	TG/T (Cys71)
AACTAAGG	GTACGCTC.....CACGGCAG	GGCTTGAG	G/GG (Gly82)
TTGACAAG	GTTGGTTC.....ATGTTAAG	ATTGAAGT	AAG/ATT (K131/I132)
AGATTGAG	GTTAGTAG.....TTTCACAG	AAGGACAT	GGG/AAG (E187/K188)
AATTCAAG	GTGATTTG.....TGGTGCAG	GACAGGAG	3′-UTR

Underlined sequences indicate consensus sequences of exon-intron junctions, and double underlined letters indicate the codons interrupted by introns.

The overall organization of the radish *RsPHGPx* gene is very similar to the *Arabidopsis AtGPX3* gene, which also has seven exons and was predicted to encode a mitochondrion-localized protein of 206 amino acid residues [20]. Comparison of the two genes demonstrated that all exons, except exons 1, 6, and 7, have the same size, and all introns, although different in size in nucleotide, have similar relative length. However, comparison of the radish *RsPHGPx* with the well-known human *PHGPx* gene [21], encoding also a mitochondrion-targeting protein of 197 amino acid residues, indicated that they contains identical exon/intron numbers, but varies in size of each exon and intron. These results embody not only the relative conservation but also the variation of *PHGPx* genes during the course of genetic evolution.

2.2 Characterization of *RsPHGPx* promoter region

Generally speaking, *cis*-acting DNA elements in promoters are responsible for interacting with corresponding transcription factors to control transcription of related genes in response to a variety of developmental and environmental signals. To get promoter sequence of *RsPHGPx* gene, we employed genome-walking technique to isolate its 5′-flanking region. When the primary PCR amplification was performed with the primers AP1/GSP1, a weak band of approximately 1.5 kb was amplified from the *Eco*R Ⅴ library (Figure 3, lane *1*). A nested PCR amplification using the primary PCR product as template was subsequently carried out, and a more specific fragment was obtained (Figure 3, lane *2*). Sequencing analysis revealed that this fragment (excluding the primer sequence) contained an expected 33 bp 5′-UTR and a 1 321 bp upstream sequence. Undoubtedly, this is the expected genomic sequence beginning from the known sequence at the 5′-end of *RsPHGPx* gene and extending into unknown adjacent genomic DNA. This nucleotide sequence of the 5′-flanking region has been deposited in the GenBank under accession number AY919315.

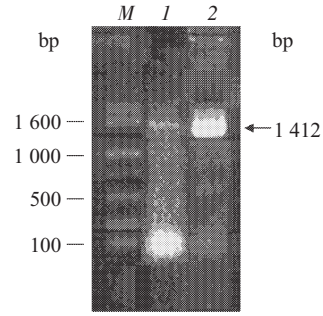

Fig.3 Isolation of the 5′-flanking sequence of *RsPHGPx* gene by genome-walking technique

The first- and second-order PCR products are shown in the lane *1* and *2*, respectively. Molecular markers (*M*) are indicated on the left.

BLAST analysis of the 1 321 bp upstream sequence identified a 3′-end of a putative biotin synthase (BS) gene sharing high homology with the known *Arabidopsis* BS gene. Comparison of the two BS genes suggested that in the 1 321 bp upstream sequence the triplet TAA from −363 to −361 is the termination codon, and the hexad AATAAT from −291 to −286 is the predicted polyadenylation signal of the putative BS gene (Figure 4). Therefore, the promoter of *RsPHGPx* gene should be located in the short region from −285 to −1. Surprisingly, the identity of upstream neighboring gene and the putative promoter size of *RsPHGPx* gene are also similar to the counterpart of *Arabidopsis AtGPX3* gene[20].

To find out *cis*-acting elements in the promoter region, PLACE database search with the 285 bp sequence was performed. As shown in Figure 4, this promoter region contains a lot of *cis*-elements for response to hormones, including E-BOX [22] and

RAV1 [23] (ABA), DOF (gibberellin), ARR1 (cytokinin), NTBBF1 (auxin) as well as W-Box 1 (salicylic acid). Additionally, elements regulating the transcription of genes in response to abiotic stresses abound in this sequence. Besides recognition sites of transcription factor MYB[24] and MYC[24,25] regulated by water and cold stresses, multiple transcription factor RAV1-recognized elements related to both ABA and cold responses exist in different ways. Two copies scatter, and interestingly the other five copies are arranged in tandem near the potential transcription initiation site. Finally, elements required for light responsiveness (Box Ⅱ [26] and I-Box [27]) and a sugar-responsive element (W-Box 2) are present in the putative promoter, too. The presence of various *cis*-acting elements gives a broad hint that the expression of *RsPHGPx* gene may be regulated in response to those signals described above.

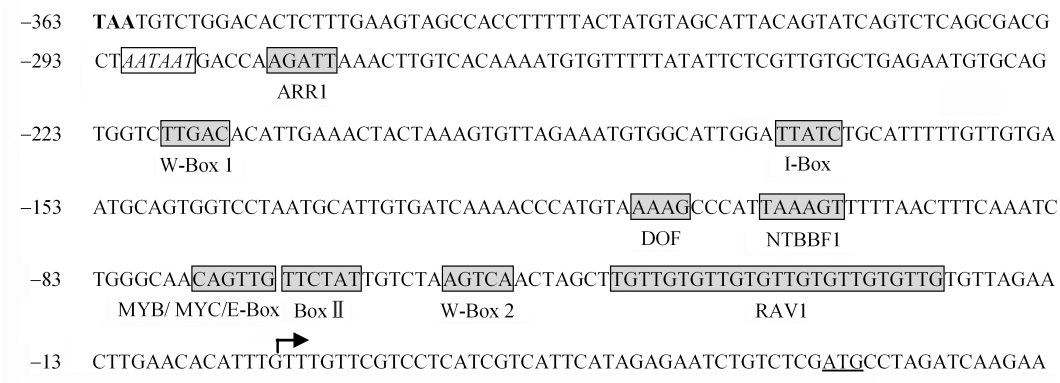

Fig.4 Nucleotide sequence of the 5′-flanking region of the *RsPHGPx* gene

Part of exon 1 (+1 to +57), the putative promoter (−285 to −1), and the 3'-end of the upstream neighboring biotin synthase gene (−363 ～−286) are shown. The potential transcription initiation site (+1) is indicated by an arrow, and the start codon is underlined. Predicted *cis*-elements (see the text for explanations) are indicated by grey boxes. The bold sequence is the stop codon, and the boxed italic sequence is the putative polyadenylation signal of the biotin synthase gene.

2.3 Relationship between expression patterns and the putative *cis*-elements

To examine the expression patterns of *RsPHGPx* gene, Northern blot analysis was performed using radish seedlings treated with various stresses, including hormones (ABA, salicylic acid, and ethephon, an ethylene releasing compound), chilling (4℃), successive illumination (on etiolated seedlings), and exogenously applied sugar (sucrose). In addition, oxidative-related stresses (ROS-forming herbicide paraquat and H_2O_2) as well as other commonly used stress treatments, such as high salinity (NaCl) and heavy metal ($AlCl_3$), were also included.

In the treatments with hormones, ABA decreased the expression of *RsPHGPx* gene (over 50% decrease, Figure 5a), whereas the others did not significantly change the expression level (Figure 5b). By comparison of the down-regulation effect of ABA with our previous observation that *RsPHGPx* gene was expressed at a higher level in younger tissues, in which gibberellin, auxin, and cytokinin are dominant, than in senescing tissues, where ABA predominates [13], we propose that *RsPHGPx* gene expression may be positively regulated by growth-promoting hormones but negatively regulated by senescence-promoting hormone (s). Probably, this type of regulation contributes to efficient elimination of reactive oxygen species easily accumulated during fast metabolism in younger tissues.

As expected, chilling treatment increased the expression of *RsPHGPx* (approximately 2-fold increase, Figure 5b), which supports the prediction of cold-associated *cis*-elements. Interestingly, successive illumination on etiolated seedlings remarkably down-regulated the transcription of *RsPHGPx* gene (over 50% decrease after 48 h, Figure 5c). Taking into consideration that successive darkness also decreased the expression level in normal seedlings[13], we suppose that *RsPHGPx* gene might be regulated by dark/light switch although the molecular mechanism is not known.

In the oxidative stresses investigated, the up-regulation effect of paraquat (approximately 2.5-fold increase, Figure 5b) and the negligible effect of H_2O_2 (Figure 5a) on *RsPHGPx* expression can be seen. This result suggests a linkage of *RsPHGPx* gene

transcription to some oxidative stresses and the possible presence of some unknown elements associated with such stresses. Additionally, despite the presence of corresponding *cis*-elements, sugar as well as salicylic acid did not significantly affect the expression of *RsPHGPx* gene (Figure 5a). This implies that only core *cis*-element might not be enough for the induction of response to certain factor, and other sequence (eg. the fit context sequences) may be necessary. Finally, no significant effects of NaCl and $AlCl_3$ treatments on the *RsPHGPx* expression (Figure 5a) seemed reasonable because of the absence of known corresponding *cis*-elements in the promoter region.

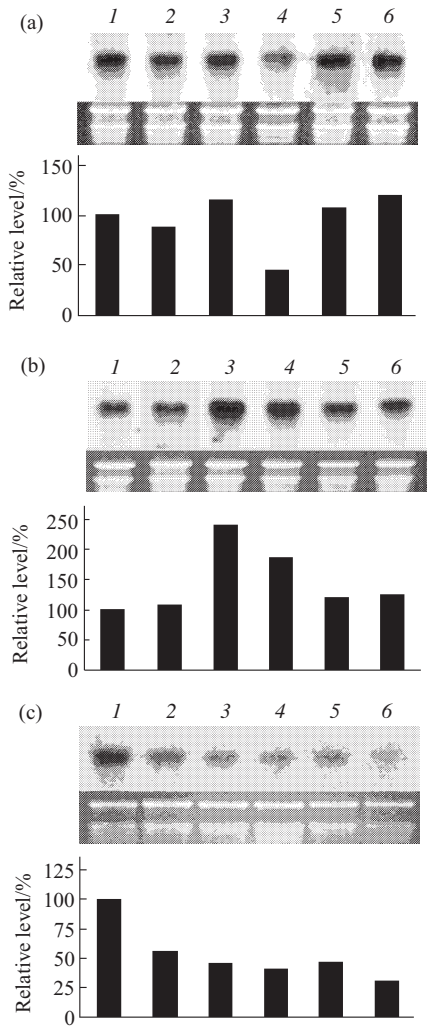

Fig.5 Expression of *RsPHGPx* gene in radish seedlings treated with various stress conditions

(a) Normal seedlings were treated with distilled water (*1*), NaCl (*2*), $AlCl_3$ (*3*), ABA (*4*), H_2O_2 (*5*) and sucrose (*6*). (b) Normal seedlings were untreated (*1*) or treated with salycilic acid (*2*), paraquat (*3*), chilling (*4*), ethphon (*5*) and distilled water (*6*). (c) Etiolated seedlings were exposed to illumination for 0 (*1*), 6 (*2*), 12 (*3*), 24 (*4*), 36 (*5*) and 48 (*6*) h. Total RNA (25 μg for each lane) extracted from these seedlings was blotted onto nylon membranes and hybridized with α-^{32}P labeled *RsPHGPx* cDNA. Ethidium bromide staining of rRNA under each blot indicates equal RNA loading, and the relative values of expression intensity are indicated at the bottom.

To conclude, a number of plant *PHGPx* genes have been isolated and characterized so far, however, there are no report providing a detailed description of gene structure and upstream regulatory elements. In this study, we isolated a 3.3 kb genomic DNA fragment of the *RsPHGPx* gene by genome-walking technique and characterized the gene structure and upstream regulatory elements. Additionally, we also confirmed *RsPHGPx* gene expression patterns by Northern blot analysis in response to stress treatments such as hormones, chilling, successive illumination, and oxidative-related stresses. The results demonstrated that the predicted *cis*-elements may contribute to a complicated regulation in response to these signals. This is the first report on characterization of genomic organization and promoter region of a plant *PHGPx* gene at molecular level. Furthermore, comparison of genomic organization and upstream regulatory region revealed the high similarity between radish *RsPHGPx* and *Arabidopsis AtGPX3* genes, suggesting the relative conservation of plant GPx genes during the course of genetic evolution. It is expected that this work will provide a valuable prerequisite to probe into exact regulatory mechanisms and biological functions of plant *PHGPx* genes.

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萝卜磷脂氢谷胱甘肽过氧化物酶基因结构及其调控序列分析 *

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摘要 磷脂氢谷胱甘肽过氧化物酶 (PHGPx) 是谷胱甘肽过氧化物酶 (GPx) 家族的重要一员，是目前已知能直接保护生物膜免受过氧化损伤的唯一酶类. 此前的研究表明，萝卜磷脂氢谷胱甘肽过氧化物酶基因 (*RsPHGPx*) 编码一个有生理功能的过氧化物酶, 并且 *RsPHGPx* 基因的表达可能受发育和环境胁迫信号的复杂调控. 要深入了解该基因的表达调控机制首先必须阐明 *RsPHGPx* 基因的结构及其上游调控序列. DNA 印迹表明萝卜 *RsPHGPx* 基因以单拷贝的形式存在于基因组中. 以基因组 DNA 为模板，通过常规 PCR 与染色体步行相结合的方法克隆到了一段 3.3 kb 长的 *RsPHGPx* 基因组序列. 分析发现，该基因由 7 个外显子和 6 个内含子组成，所有内含子的剪切位点均符合真核生物 GT-AG 规则. 另外还发现该基因的上游基因是生物素合成酶基因；位于 *RsPHGPx* 基因上游的调控序列只有不足 300 bp. 这些结构特征与拟南芥 *AtGPX3* 基因极其相似. 顺式作用元件的数据库搜索发现 *RsPHGPx* 基因的上游调控序列含有多个响应激素 (如 E-Box 和 W-Box)、胁迫 (如转录因子 MYB 和 MYC 的结合位点) 和光 (如 Box Ⅱ和Ⅰ-Box) 信号的元件. RNA 印迹分析表明 *RsPHGPx* 基因的表达受到脱落酸 (ABA) 和连续光照 (在黄化苗中) 处理的负调控，受到冷胁迫(4℃)的正调控，这暗示了预测的顺式作用元件的调控作用. 然而，除草剂

paraquat对该基因表达的正调控作用，暗示了某些与氧化胁迫相关的未知元件的存在. 这些结果进一步印证了 *RsPHGPx* 基因的表达受发育和环境胁迫信号复杂调控的推测. 这是迄今为止首个关于植物 *PHGPx* 基因结构和上游调控序列的系统报道，为今后全面认识植物 *PHGPx* 基因的表达调控机制奠定了必要基础.

关键词 萝卜，磷脂氢谷胱甘肽过氧化物酶，基因结构，上游调控序列，顺式作用元件

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