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cDNA Cloning and Overexpression of an Isoperoxidase Gene from Korean-Radish, *Raphanus sativus* L.

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Abstract: A partial cDNA encoding a Korean radish isoperoxidase was obtained from a cDNA library prepared from 9 day old radish root. In order to obtain Korean radish isoperoxidase cDNA, 5′ RACE (rapid amplification cDNA end) PCR was performed and a cDNA (*prx*K1) encoding a complete structural protein was obtained by RT (reverse transcription)-PCR. Sequence analysis revealed that the length of the cDNA was 945 base pairs, and that of the mRNA transcript was ca. 1.6 kb. The deduced amino acid of the protein were composed of 315 amino acid residues and the protein was 92% homologous to turnip peroxidase, and 46% to 50% homologous to other known peroxidases. The 945 bp cDNA encoding Korean radish isoperoxidase was overexpressed in *Escherichia coli* up to approximately 9% of total cellular protein. The recombinant fusion protein exhibited 43 kDa on SDS-PAGE analysis and the activity level of the recombinant nonglycosylated protein was two fold higher in IPTG induced cell extracts than that of uninduced ones.

Key words: cDNA cloning, 5′ RACE (rapid amplification cDNA end) and RT (reverse transcription)-PCR, overexpression.

Plant peroxidases (EC 1.11.1.7) have been studied widely in several higher plants. This enzyme plays an important role in several physiological functions such as removal of peroxide, oxidation of toxic reductants, oxidation of indole-3-acetic-acid, wound healing, cell wall biosynthesis, catabolism of auxin and in the defense system against pathogen attack (Hammerschmidt *et al.*, 1982; Espelie *et al.*, 1986). Peroxidases are also involved in degradation and biosynthesis of lignin, and oxidation of phenolic pollutants (Strivastava and Huystee, 1977; Grisebach, 1981). In addition, peroxidase has been widely used for practical purposes such as enzyme immunoassays and diagnostic assays.

Determination of the gene structure and expression of peroxidases might be helpful in clarifying the physiological roles and catalytic functions of plant peroxidases. Several studies have been already performed to express peroxidase in yeast (Fishel *et al.*, 1987) and bacteria (Loprasert *et al.*, 1990). Recently, expression of an active HRP (horse radish peroxidase) was reported in insect tissue culture using a baculovirus transfer system (Hartmann and Ortiz de Montellano, 1992).

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In Korean radish (*Raphanus sativus* L.) there are eight distinguishable isoperoxidases according to the migration distance in starch gel electrophoresis; A_1, A_2, A_{3n}, A_3, C_1, C_2, C_{3n} and C_3 (Lee and Kim, 1990). In our laboratory, these isoperoxidases were purified to near homogeneity from Korean radish by chromatographic procedures including ion exchange and gel filtration (Lee and Kim, 1994). In the present work, the entire cDNA sequence of one cationic Korean radish isoperoxidase has been cloned by both 5′ RACE (rapid amplification cDNA end)- and RT (reverse transcription)-PCR techniques and then overexpressed in *E. coli* under the *lac* promoter.

Materials and Methods

Plant material and total RNA isolation

Korean-radish (*Raphanus sativus* L. cv Handsome Fall) seedlings were grown at 25℃ in the greenhouse. Harvested tissues were stored at −70℃ until they were used for construction of a cDNA library (Okayama *et al.*, 1987). Total RNA was isolated from Korean-radish radicles (6 days growth in dark condition at 25℃), with some modifications based on the method of Logemann *et al.* (1987). Radish roots (5 g) were powdered

with a homogenizer (Tektra) in liquid nitrogen. Total RNA was isolated from the powdered roots by guanidinium/cesium chloride centrifugation.

Construction of cDNA library and screening by plaque hybridization

Poly(A)-enriched RNA was isolated from total RNA using an mRNA purification kit (Pharmacia LKB Biotechnology). Double-stranded cDNA was then synthesized from poly(A)-enriched RNA as described in the manufacturer's manual (Stratagene), and ligated into Uni-ZAP arms. These were *in vivo* packaged into phage particles using packaging extract purchased from Stratagene and transfected to *E. coli* XL-1 blue MRF'.

A screening probe was prepared from RT-PCR with degenerated primers based on acid/base catalysis (5' CAC/TTTC/TCAC/TGAC/TTGC/TTTC/TGT3') and helix F region (5'GGTGTGT/CGCA/GCCT/AGC/AT/AAGTGCA/CC/AC3'). The cDNA library in Uni-ZAP was screened with 393 bp PCR product as a probe. The probe was labeled with 110 TBq/mmol of [α-^{32}P] dCTP using a random primer labeling kit (Amersham). Plaques of 2×10^5 were transferred onto duplicate nylon membranes (HybondN$^+$, Amersham). The filters were added to the ^{32}P-labeled probe in hybridization solution (5×SSC, 1% blocking reagent, 0.02% SDS, 0.1% N-lauroylsarcosine) at 50℃. They were rinsed with 2×SSC and 0.1% SDS at room temperature for 20 min and then with 0.1×SSC and 0.1% SDS at 50℃ for 20 min. These filters were exposed to X-ray film (AGFA CURIX) for autoradiography.

Polymerase chain reaction

The first strand cDNA for isoperoxidase was generated by Molony Murine Leukemia Virus RNaseH$^-$ Reverse transcriptase (BRL) reaction with 5 μg total RNA (Krug *et al.*, 1987). For 5' RACE PCR, purified first strand cDNA was used directly for dG-tailing with tailing primer (5'CCCGAATTCGCGGCCGCCCCCCCCC-CCCCCCCCCCCCCC3'). The tailing mixture was composed of 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.25 mM $MgCl_2$, 50 μg/ml BSA, 0.2 mM dGTP, cDNA, and 10 U TdT (GIBCO BRL). The reaction mixtures were incubated for 5 min at 37℃, then 10 min at 65℃. Tailed cDNA was amplified from the TdT reaction mixture with both tailing primer and internal primer (5' GGCTAAGATATCAGCACAAG3') designed from the partial cDNA sequence. The DNA-denaturation step was at 94℃ for 1 min, the primer-annealing step at 50℃ for 1 min, the primer-extension step at 72℃ for 3 min. The reaction was run for 35 cycles.

Amplification by RT-PCR was performed in a PTC-100TM Programmable Thermal Controller (MJ Research, Inc). The reaction mixture of 50 μl consisted of 5 μg first strand cDNA, 50 pmol of two primers (5'CT-CACATATGGCTTCAAATATTGCC3' from nt −22 to +18 and (5'CGTGGATCCATTACTCAGTTAGTCCT-CCCACAG3', from nt+1030 to +1062), 0.01% gelatin, 200 μM dNTP, and 2 U AmpliTaq™ polymerase in 10 mM Tris-HCl buffer, pH 8.3 containing 50 mM KCl and 1.5 mM $MgCl_2$. Cycling condition was similar to 5' RACE PCR except primer-annealing step at 55℃ for 1 min.

DNA sequence analysis

DNA sequence was determined by the dideoxy chain termination method of Sanger *et al.* T7 DNA sequence Kit (Pharmacia) and 373A DNA Sequencer (ABI) were used as described in the manufacturer's description. DNA sequence was analyzed using the Mac3.2 DNAsis program.

Northern blot analysis

Total RNA (20 μg) was separated on a 1% formaldehyde agarose gel, blotted onto a nylon membrane, hybridized with the ^{32}P-labeled cDNA, and washed twice with a mixture of 2×SSC and 0.1% SDS at 37℃ for 15 min, and then with 0.1×SSC and 0.1% SDS at 42℃ for 20 min. The filters were subjected to autoradiography for 24 h at −70℃.

Expression of recombinant isoperoxidase in *E. coli*

In order to obtain Korean radish isoperoxidase in *E. coli*, the cDNA clone, named *prx*K1 was overexpressed under the *lac* promoter of pBluescriptSK vector (Stratagene). The pBluescriptSK vector was introduced into *E. coli* DH5α and the transformant was grown aerobically in LB medium containing 50 μg/ml ampicillin. After reaching an A_{600} of 0.5, cells were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to 1.0 mM and then incubated for 3 h at 37℃.

Results and Discussion

Korean radish seedling peroxidase isozymes showed a tissue specific expression pattern according to the growth days (Lee and Kim, 1990). In the report, we showed that on 3 days after germination, cationic isoperoxidase C_{3n} was expressed intensively in roots, hypocotyl and cotyledon but C_3 didn't appear in these tissues. In mature radish, isoperoxidase C_3 was the major protein in all tissues. Also, anionic isoperoxidases were not expressed in all tissues until 4 days. On 5 days, anionic isoperoxidase A_3 was expressed in roots, and on 7 days, anionic isoperoxidases A_1, A_2 and A_3 were expressed in all tissues as mature radish roots.

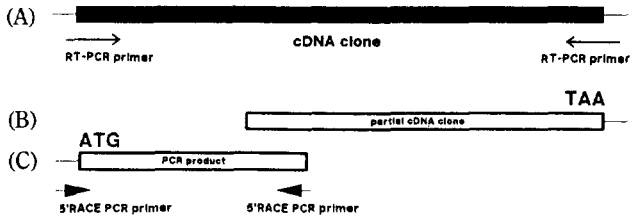

Fig. 1. Schematic diagram of the *prx*K1 cDNA. A composite of the isolated *prx*K1 partial cDNA and RT-PCR product equivalent to 5′ region of cDNA was shown. (A) cDNA was amplified with two primers. (B) partial cDNA gene was isolated from cDNA library as described under Material and Methods. (C) 5′ region of the cDNA was obtained by 5′ RACE-PCR.

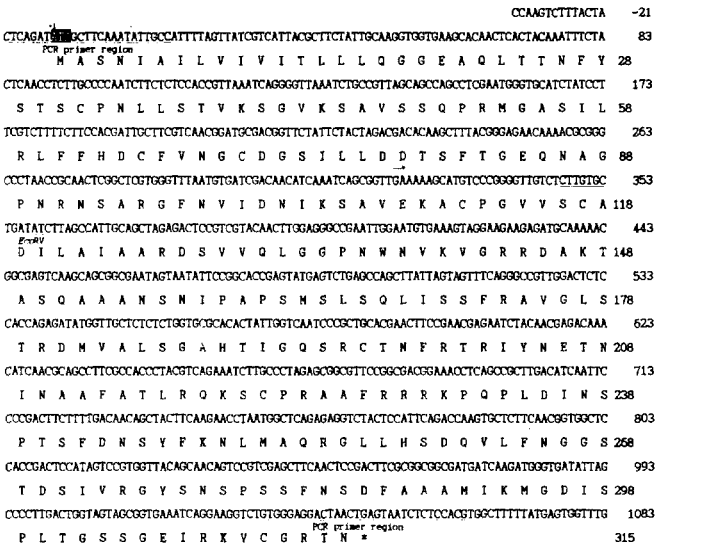

Fig. 2. Nucleotide sequence of the *prx*K1 cDNA. Deduced amino acid sequence is shown below the nucleotide sequence. Each primer was underlined. The arrow indicate the 5′ end of the partial cDNA gene. A consensus polyadenylation signal (TAATAAG) at 28 bp nucleotides before the addition of the most possible poly(A) was shown.

Therefore, radish radicles of 9 days old seedling were chosen in order to extract total RNA containing isoperoxidase mRNAs.

To isolate the cDNA of Korean radish peroxidase, degeneracy primers based on the conserved region of deduced amino acids of several plant peroxidases were used. We screened a cDNA library consisting of approximately 2×10^5 recombinant phages with ^{32}P-labeled cDNA obtained from RT-PCR as a probe at a plate density of 3×10^4 phages/150 mm plate. At the first screening, eight positive clones were selected after washing the hybridized filter twice at 50℃ for 15 min. Through secondary and tertiary screenings at 55℃ for 15 min, three clones turned out to be final positive clones. These clones were plaque-purified and pBluescript phagemids were excised from the positive phage DNAs for identification of the inserted fragments and sequence analysis. Unfortunately, sequence analysis revealed that the three cDNA clones isolated from the cDNA library were a partial cDNA clone (Fig. 1). The length of the partial cDNA clone was 702 base pairs, and the 74 bp 3′-untranslated region contained the consensus polyadenylation signal (AATAAG) at 28 bp nucleotides before the addition of the most possible poly(A). The deduced amino acids were 208 residues and didn't contain acid/base catalysis region, which was conserved well in most plant peroxidases.

In order to obtain cDNA encoding Korean radish isoperoxidase, 5′ RACE-PCR was performed using tailing primer (Materials and Methods). The amplified product was about 400 bp in length and subcloned into pGEM 3zf(+)/*Sma*I for sequencing. Based on the nucleotide sequence determined, we designed a PCR primer for N-terminal region based on putative ATG initiation codon. By RT-PCR with N-terminal and C-terminal region primers (Materials and Methods), the cDNA, *prx*K1, was amplified and the entire nucleotide sequence was determined (Fig. 2). Its size was 945 bp, and the deduced amino acids were 315 residues (putative molecular weight of cDNA gene product: 33.6 kDa), with estimated isoelectric point of 9.97. These results suggest that the *prx*1 cDNA may represent a basic isozyme (Korean radish isoperoxidase C_1, C_2, C_3, or C_{3n}). And the 21 amino acid peptide for *prx*K1 in N-terminal region might be signal peptide. Computer analysis (clustalw, KTCC) showed that the deduced amino acid sequence of *prx*K 1 cDNA was 92% homologous with turnip peroxidase (Mazza *et al.*, 1980), and 46% to 50% homologous to other peroxidases (tobacco, horseradish, and potato) (Lagrimini *et al.*, 1987; Fujiyama *et al.*, 1988; Roberts *et al.*, 1989) (Fig. 3). Two histidine residues, His^{40} and His^{42}, are known to be present in the acid/base catalysis region in all known plant peroxidases except turnip TP7, where Phe^{40} and His^{42} are present (Lobarzewski *et al.*, 1991). The *prx*K1 cDNA also has Phe^{40} and His^{42} in the acid/base catalysis region like turnip TP7. Interestingly, the number of total histidine residues represented in *prx*K1 cDNA were unchanged. The other histidine residue (His^{240}) was located between the conserved regions III and IV.

By Northern hybridization analysis using *prx*K1 cDNA clone as a probe, the length of the mRNA was found to be ca. 1.6 kb (Fig. 4). Therefore, the size of the 5′ UTR (untranslated region) region of *prx*K1 cDNA clone may be approximately 500 bp from the transcription start point to ATG initiation codon. However, this full length 5′ UTR region was not obtained by 5′ RACE PCR probably because the mRNA transcript which was purified from total RNA may be shorter than that of

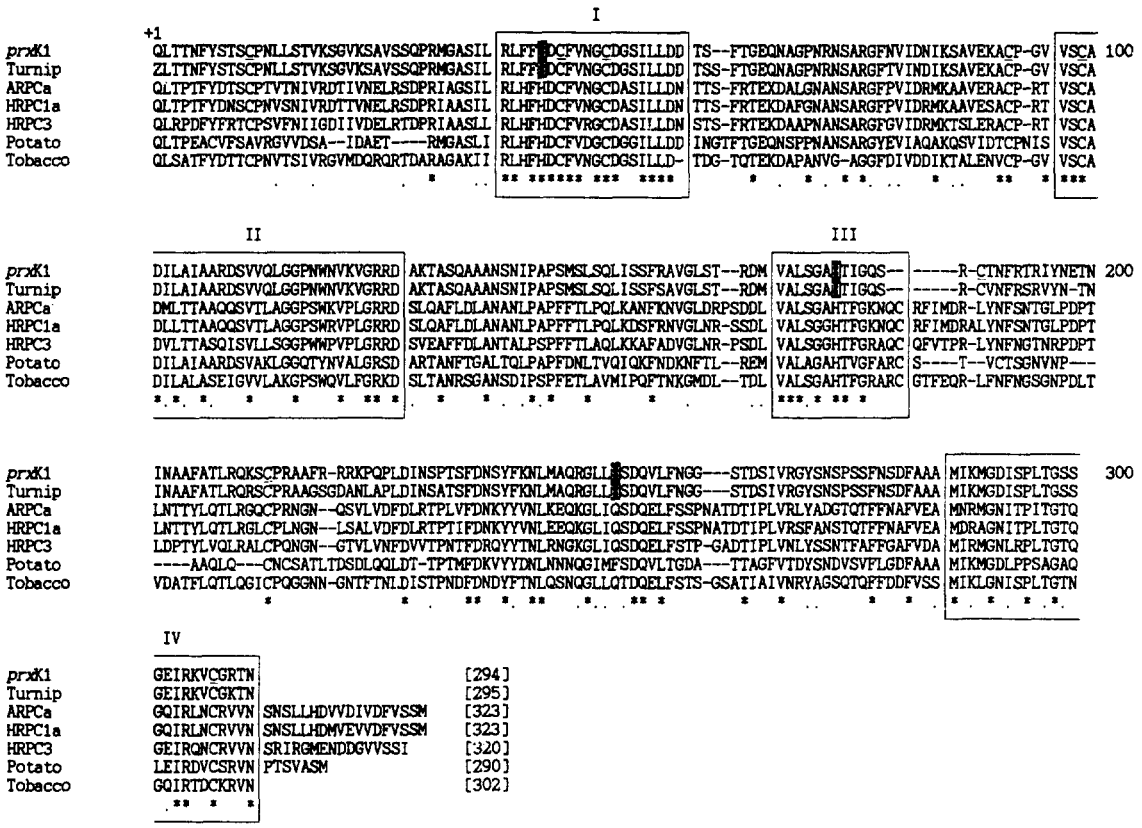

Fig. 3. Comparison of the derived amino acid sequence of Korean radish isoperoxidase *prx*K1 with other known peroxidases. The conserved regions are indicated by boxes.

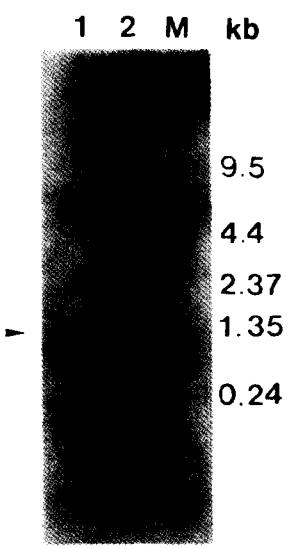

Fig. 4. Northern blot analysis of total RNA from radish radicles (9 days growth) of Korean radish. The *prx*K1 cDNA was used as a probe. Lane 1 : control (without total RNA); lane 2 : 20 μg total RNA; lane M : 0.24~9.5 kb RNA ladder.

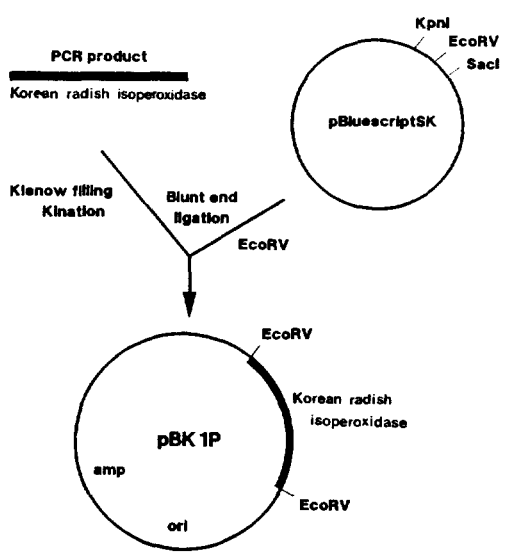

Fig. 5. The scheme of the expression plasmid pBK1P construction. Amplified cDNA products were ligated to the pBluescriptSK vector digested with *Eco*RV.

the putative intact region.

In order to obtain the recombinant radish isoperoxidase protein, the cDNA encoding the putative Korean radish isoperoxidase enzyme was ligated to the pBluescriptSK vector. The ligated vector was named pBK1P. A construction map of the expression vector is shown in Fig. 5. Cells harboring this vector were grown in LB-broth, induced with final 1.0 mM IPTG, and total protein was analyzed on SDS-PAGE (Laemmli, 1970)

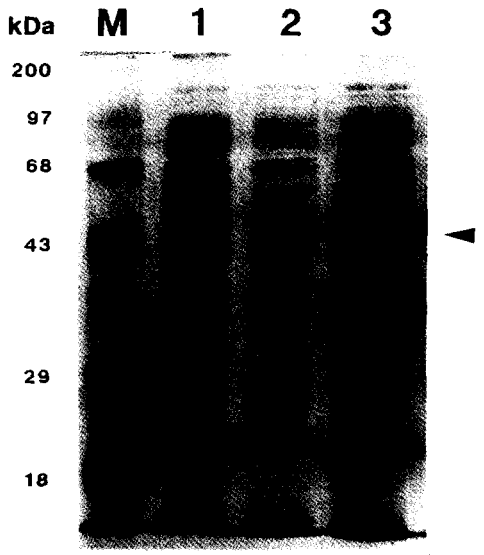

Fig. 6. SDS-PAGE analysis of the expressed isoperoxidase. Lane M : prestained standard proteins (200, 97, 68, 43, 29, 18 kDa) (BRL); lane 1 : host cell extracts; lane 2 : uninduced cell extracts; lane 3 : induced cell extracts. The arrow indicates the recombinant fusion-protein of Korean radish isoperoxidase.

Table 1. Isoperoxidase activity of the expressed Korean radish isoperoxidase in the presence of 5 mM H_2O_2 and 15 mM guaiacol

Strain	Specific activity (ΔOD at 470 nm/min/mg)	
	+hemin	−hemin
Host strain (DH5α)	0.136	0.010
DH5α/pBK1P (uninduced)	0.164	0.004
DH5α/pBK1P (induced)	0.306	0.014

The cell extracts were prepared by sonication under PBS (pH 7.0) buffer.
The activity of isoperoxidase was assayed by a modification procedure of Schafer *et al.* (1971).

(Fig. 6). Recombinant nonglycosylated fusion-protein, which was 43 kDa in size, was produced as approximately 9% of total cellular protein. When peroxidase activity of the recombinant Korean radish isoperoxidase was determined in the presence of 5 mM H_2O_2 and 15 mM guaiacol, the activity was two fold higher in IPTG induced cell extracts than that of uninduced ones after adding 20 μM hemin (Table 1). The relatively low activity of the recombinant Korean radish isoperoxidase was probably caused by inaccurate folding and/or absence of carbohydrate moiety. Therefore, cloning and expression of the cDNA gene in yeast, transgenic plant, or mammalian expression vector may be necessary to obtain a fully active form of peroxidase.

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