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Isolation, Restriction Mapping, and Promoter Sequence Analysis of an Isoperoxidase Gene from Korean-Radish, *Raphanus sativus* L.

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Abstract: A specific DNA fragment from Korean radish (*Raphanus sativus* L.) was amplified by performing PCR with oligonucleotide primers which correspond to the highly conserved regions of plant peroxidases. The size of the PCR product was ca. 400 bp, as expected from the known plant peroxidase genes. Comparison of the nucleotide and deduced amino acid sequences of the PCR product to those of other plant peroxidase-encoding genes revealed that the amplified fragment corresponded to the highly conserved region I and III of plant peroxidases. By screening a genomic library of Korean radish using the amplified fragment as a probe, two positive clones, named *prx*K1 and *prx*K2, were isolated. Restriction mapping studies indicated that the 5.2 kb *Sal*I fragment of the *prx*K1 clone and the 4.0 kb *Eco*RI fragment of the *prx*K2 clone encode separate isoperoxidase genes. Analyses of the promoter region of the *prx*K1 clone shows that putative TATA box, CAAT box, and TGA1b binding sequence (5′ TGACGT) are present 718 bp upstream from the start codon.

Key words: genomic DNA, promoter, restriction mapping, RT-PCR.

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

Determination of the gene structure and expression of peroxidases are absolutely required to understand their physiological functions. The complete amino acid sequences of the isoenzymes such as TP 7 from turnip and HRP-C from horseradish have been determined (Welinder, 1979; Mazza and Welinder, 1980). In addition, cDNA clones encoding peroxidases from several plant sources have been isolated (Buffard *et al.*, 1990; Morgans *et al.*, 1990; Bratonek-Roxa, *et al.*, 1991). Largrimini *et al.* (1987) first reported the complete primary structure of cDNA and the deduced amino acid sequence of the lignin-forming peroxidase from tobacco. Fujiyama *et al.* (1990) also reported the genomic structure of two horseradish peroxidase encoding genes.

In Korean radish (*Raphanus sativus* L.), there are at least eight distinguishable peroxidase isoenzymes as revealed by starch gel electrophoresis. These isoperoxidases were named A_1, A_2, A_3 and A_{3n} for anionic isoenzymes, and C_1, C_2, C_3 and C_{3n} for cationic isoenzymes (Lee and Kim, 1990). In our laboratory four anionic isoenzymes and two cationic isoenzymes were purified to near homogeneity from radish roots by chromatographic procedures, including ion exchange and gel filtration (Lee and Kim, 1994). In this report, we amplified the partial cDNA fragment of the Korean radish peroxidases by the PCR method. By screening radish genomic library using this partial cDNA as a probe, we isolated two genomic clones and constructed the restriction maps of the two genomic clones. Furthermore, the nucleotide sequence of the putative promoter region from one of the two genomic clones was determined in order to identify the regulatory sequences of the peroxidase gene.

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Materials and Methods

Plant material and isolation of total RNA

Korean-radish seedlings (*Raphanus sativus* L. cv Handsome Fall) were grown in the dark at 25℃. Young root tissues of 6 day-old seedlings were harvested and stored at −70℃ until they were used for the construction of the cDNA library (Okayama *et al.*, 1987). Total RNA was prepared by the method described by Logemann *et al.* (1987) with some modifications.

Preparation of primers and polymerase chain reaction (PCR)

In plant peroxidases, there are four highly conserved regions in the amino acid sequences. Two primers, named primer A and primer F based on acid/base catalysis and the helix F region of deduced amino acid sequences of several plant peroxidases (Fig. 1A), were synthesized using a DNA synthesizer (381A DNA synthesizer; Applied Biosystem Inc, CA, USA). Amplification by PCR was performed in a PTC-100TM Programmable Thermal Controller (MJ Research, Inc). The first strand cDNAs for isoperoxidases were generated by Molony Murine Leukemia Virus RNaseH$^-$ Reverse transcriptase (BRL) reaction with 5 μg total RNA (Krug *et al.*, 1987). The reaction mixture of 50 μl consisted of 5 μg first strand cDNAs, 50 pmol of each primer, 0.01% gelatin, 200 μM dNTP, and 2 U AmpliTaq™ polymerase in 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl and 1.5 mM $MgCl_2$. PCR amplification was completed through 35 cycles (94℃, 1 min; 37 to 50℃, 1 min; 72℃, 3 min). The PCR product was fractionated on a 1.2% SeaPlaque™ agarose gel (FMC BioProducts, USA), and putative bands were purified from the gel. Blunt-ended products were subcloned into the *Sma*I site of the pUC19 vector.

Preparation of radish genomic DNA

Genomic DNA was prepared from Korean-radish youngishroots (9 days growth in the dark at 25℃), with some modifications based on the method of Murray *et al.* (1980). Radish roots (5 g) were powdered with a homogenizer (Tektra) in liquid nitrogen, and then genomic DNA was isolated from the powdered roots after they were lysed in 10 mM Tris-HCl (8.0), 100 mM NaCl, 0.5% SDS, 25 mM EDTA (pH 8.0) containing 0.1 mg/ml of proteinase K for 16 h at 50℃. The mixture was extracted twice with buffer-saturated phenol, twice with chloroform-isoamyl alcohol (24:1), precipitated with ethanol, and washed in 70% ethanol. The samples were dried and dissolved in 10 mM Tris, 1 mM EDTA (pH 8.0).

Screening of genomic DNA library by plaque hybridization

Korean-radish genomic DNA library was constructed in a lambda EMBL3/*Bam*H1 vector kit according to the manufacturer's directions (Stratagene, USA) and screened by Woo's method (Woo *et al.*, 1979) with a slight modification, using the partial cDNA 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 (Hybond N$^+$; Amersham). The filters were hybridized with the ^{32}P-labeled partial cDNA probe in 5×SSC, 1% blocking reagent, 0.02% SDS, 0.1% N-lauroylsarcosine at 55℃ for 16 h. Filters 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.

Southern blot analysis

The recombinant plasmids of the 5.2 kb *Sal*I fragment of *prx*K1 clone and the 4.0 kb *Eco*RI fragment of *prx*K2 digested with various restriction endonucleases (Boehringer Mannheim, Germany), were fractionated on a 0.8% agarose gel. After denaturation with 0.5 M NaOH and neutralization with 1 M Tris-HCl (pH 8.0) containing 1.5 M NaCl, the DNA fragments were transferred to Hybond N$^+$ (Amersham, USA) according to the procedure of Southern (1975). After UV-cross-linking (Fluo-Link, USA), hybridization was performed with a Dig-11-dUTP labeled probe overnight at 55℃ in 5×SSC, 0.1% (W/V) N-lauroylsarcosine, 0.02% (W/V) SDS, and 1% (W/V) blocking reagent (Boehringer Mannheim, Germany). The filter was first washed in 2×SSC, and 0.1% (W/V) SDS for 15 min at room temperature, and then washed in 0.5×SSC, and 0.1% (W/V) SDS for 15 min at 55℃, twice, respectively. The membrane was equilibrated for 2 min in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The color reaction was developed with NBT (nitroblue tetrazolium salt) and X-phosphate solution in the dark at room temperature. With isotope labeled probe, the autoradiograms were exposed for 12 h at −70℃.

DNA sequence analysis

The DNA sequence of the amplified fragment was determined by the dideoxy chain termination method of Sanger *et al.* (1977). A 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 Mac DNAsisPro version 3.2.

A 0.7 kb and 0.9 kb *Hin*dIII fragment of the 5.2 kb *Sal*I fragment derived from a *prx*K1 clone were subcloned into the pUC19/*Hin*dIII vector and the nucleotide sequence of these fragments was determined using pUC/M13 forward and reverse sequencing primers (forward, 5'GTAAAACGACGGCCAGT3'; reverse, 5'GGAAACAGCTATGACCATG3'). Additionally, two kinds of nucleotides (5'GACGCCGGAAGCATATACTTGCCAAAG3' and 5'ATATAAGCACTACAAGC3') were synthesized in order to sequence the internal region of the 0.7 kb and 0.9 kb *Hin*dIII fragments.

Results and Discussion

Peroxidase isoenzymes of Korean radish seedling were reported to show tissue specific expression patterns depending upon the growth days (Lee and Kim, 1990). After 5 days growth all isoperoxidases expressed in seedling were the same isoenzymes in mature radish roots. We chose 6 day-old radish radicles for the extraction of total RNAs since there are too many carbohydrates in tissues after this period of seedling. However, we used 9 day-old root tissues for the genomic DNA preparation. The first strand cDNA for isoperoxidase was generated by Molony Murine Leukemia Virus RNaseH$^-$ reverse transcriptase (BRL) reaction with 5 μg total RNA. The amplification products with primer A and F (Fig. 1A) were heterogeneous, as expected, since there should be several different cDNAs that encode peroxidases with highly homologous sequences. The amplified fragments were subcloned into the pUC 19 vector and nucleotide sequence was determined. The DNA fragment of approximately 393 bp was amplified with primer A and F from a PCR mixture containing 5 μg of 1st cDNA (Fig. 1B), and it's nucleotide sequence was shown in Fig. 2.

The deduced amino acid sequence of the partial cDNA was compared with those of peroxidases from other plants such as Arabidopsis (Intapuruk *et al.*, 1991), tobacco (Lagrimini *et al.*, 1987), horseradish (Fujiyama *et al.*, 1988), turnip (Mazza and Welinder, 1980), and potato (Roberts and Kolattukudy, 1988) (Fig. 3). The amino acid sequence of the partial cDNA showed 92% homology to the turnip peroxidase and 46% to 50% homology to other peroxidases by computer analysis (Clustalw, KTCC). These results suggested that the amplified fragment corresponded to the highly conserved region I and III of plant peroxidases.

The radish genomic DNA library in EMBL/*Bam*HI vector containing 2×10^{12} pfu/ml was constructed as described in Materials and Methods. The genomic DNA library consisting of approximately 2×10^5 recombinant phages was screened using the partial cDNA as a probe, and five positive clones were isolated. Bacteriophage DNA of these clones was isolated by the liquid culture method (Leder *et al.*, 1977) for restriction mapping and subcloning of inserted fragments. Four clones were the same in restriction maps of inserted DNA and tentatively named clone 1. The other was different in restriction maps from clone 1 and was designated clone 2. The size of the inserted DNA in the EMBL3 /*Bam*HI vector of clone 1 and clone 2 was about 16 and 18 kbp, respectively. Southern hybridization studies indicated that a 5.2 kb *Sal*I fragment from clone 1 and a 4.0 kb *Eco*RI fragment from clone 2 were hybridized to the partial cDNA. These fragments were subcloned into the pGEM3zf(+) (Promega, USA) and designated pGEMP5.2 and pGEMP4.0. Isoperoxidase genes coded by the 5.2 kb *Sal*I and the 4.0 kb *Eco*RI fragments were named *prx*K1 and *prx*K2, respectively. The *prx*K1 and *prx*K2 fragments were subjected to Southern hybridization after they were digested with var-

(A)

Primer A:

5' CACTTCCACGACTGCTTCGT 3' (20 mer)
T T T T T T

Primer F:

5' GGTGTGTGCACCTGCTAGTGCACC 3' (24 mer)
C G A AA CA

(B)

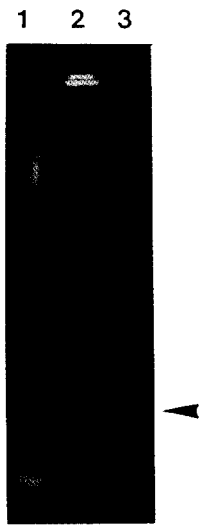

Fig. 1. (A) The oligonucleotide degeneracy primers used for PCR amplification of cDNA encoding Korean radish isoperoxidase. The design of two primers, primer A and B, based on the conserved region (acid/base and helix F region) of plant peroxidases. (B) Amplification of first strand cDNA from total RNA of Korean radish roots. Total RNA was isolated from 5 g of Korean radish roots. Electrophoresis was performed in 1% NuSieve agarose gel (FMC). Lane 1 and 2 are DNA molecular weight markers (123 bp DNA ladder and 1kb DNA ladder, Bio-Rad, respectively). lane 3, the amplification product of cDNA encoding Korean radish isoperoxidase.

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  1 CATTTCCATGATTGCTTTGTCAACGGATGCGACGGTTCTATTCTACTAGACGACACATCAAGCTTTACGGGAGAACAAAACGCGGGCCCT  90
     H  F  H  D  C  F  V  N  G  C  D  G  S  I  L  L  D  D  T  S  S  F  T  G  E  Q  N  A  G  P

 91 AACCGCAACTCGGCTCGTGGGTTTAATGTGATCGACAACATCAAATCAGCGGTTGAAAAAGCATGTCCGGGGGTTGTGTCTTGTGCTGAT 180
     N  R  N  S  A  R  G  F  N  V  I  D  N  I  K  S  A  V  E  K  A  C  P  G  V  V  S  C  A  D

181 ATCTTAGCCATTGCAGCTAGAGACTCCGTCGTACAACTTGGAGGGCCGAATTGGAATGTGAAAGTAGGAACAAGAGATGCAAAAACGGCG 270
     I  L  A  I  A  A  R  D  S  V  V  Q  L  G  G  P  N  W  N  V  K  V  G  T  R  D  A  K  T  A

271 AGTCAAGCAGCGGCGAATAGTAATATTCCGGCACCGAGTATGAGTCTGAGCCAGCTTATTAGTAGTTTCAGGGCTGTTGCACCCTCCACC 360
     S  Q  A  A  A  N  S  N  I  P  A  P  S  M  S  L  S  Q  L  I  S  S  F  R  A  V  A  P  S  T

361 AGAGATATGGTTGCACTTTCAGGCGCACACACC                                                          393
     R  D  M  V  A  L  S  G  A  H  T
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Fig. 2. Nucleotide and deduced amino acid sequence of the putative Korean radish isoperoxidase partial cDNA obtained from PCR amplification with primer A and F.

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                      I

prxK1     HFHDCFVNGCDGSILLDD|TSS-FTGEQNAGPNRNSARGFNVIDNIKSAVEKACP-GV|VSC  60
Turnip    FFHDCFVNGCDGSILLDD|TSS-FTGEQNAGPNRNSARGFTVINDIKSAVEKACP-GV|VSC
ARPCa     HFHDCFVNGCDASILLDN|TTS-FRTEXDALGNANSARGFPVIDRMKAAVERACP-RT|VSC
HRPC1a    HFHDCFVNGCDASILLDN|TTS-FRTEKDAFGNANSARGFPVIDRMKAAVESACP-RT|VSC
Potato    HFHDCFVDGCDGGILLDD|INGTFTGEQNSPPNANSARGYEVIAQAKQSVIDTCPNIS|VSC
Tobacco   HFHDCFVNGCDGSILLD-|TDG-TQTEKDAPANVG-AGGFDIVDDIKTALENVCP-GV|VSC
           ****** ***  **** |       *  .  * . * *. ..   * ..   **   |***

                      II

prxK1     ADILAIAARDSVVQLGGPNWNVKVGTRD|AKTASQAAANSNIPAPSMSLSQLISSFRAVAP  120
Turnip    ADILAIAARDSVVQLGGPNWNVKVGRRD|AKTASQAAANSNIPAPSMSLSQLISSFSAVGL
ARPCa     ADMLTTAAQQSVTLAGGPSWKVPLGRRD|SLQAFLDLANANLPAPFFTLPQLKANFKNVGL
HRPC1a    ADLLTTAAQQSVTLAGGPSWRVPLGRRD|SLQAFLDLANANLPAPFFTLPQLKDSFRNVGL
Potato    ADILAIAARDSVAKLGGQTYNVALGRSD|ARTANFTGALTQLPAPFDNLTVQIQKFNDKNF
Tobacco   ADILALASEIGVVLAKGPSWQVLFGRKD|SLTANRSGANSDIPSPFETLAVMIPQFTNKGM
          **.*. *.   *    *    *  *  *|.  *    * . .*.*   *      *

                      III

prxK1     ST--RDM|VALSGAHT|  135
Turnip    ST--RDM|VALSGAHT|
ARPCa     DRPSDDL|VALSGAHT|
HRPC1a    NR-SSDL|VALSGGHT|
Potato    TL--REM|VALAGAHT|
Tobacco   DL--TDL|VALSGAHT|
               ..|***.* **|
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Fig. 3 Comparison of the derived amino acid sequence of the partial cDNA with those of other known peroxidases. The conserved regions are indicated by boxes. Histidine amino acid residues are shown in the dark-shadow.

ious restriction enzymes. The partial cDNA was hybridized to the 0.3 kb *Hin*dIII fragment from the *prx*K1 gene and the 0.75 kb *Eco*RI/*Sph*I fragment from the *prx*K2 gene (Data not shown). Fig. 4 shows the restriction maps of the putative isoperoxidase *prx*K1 and *prx*K2 gene.

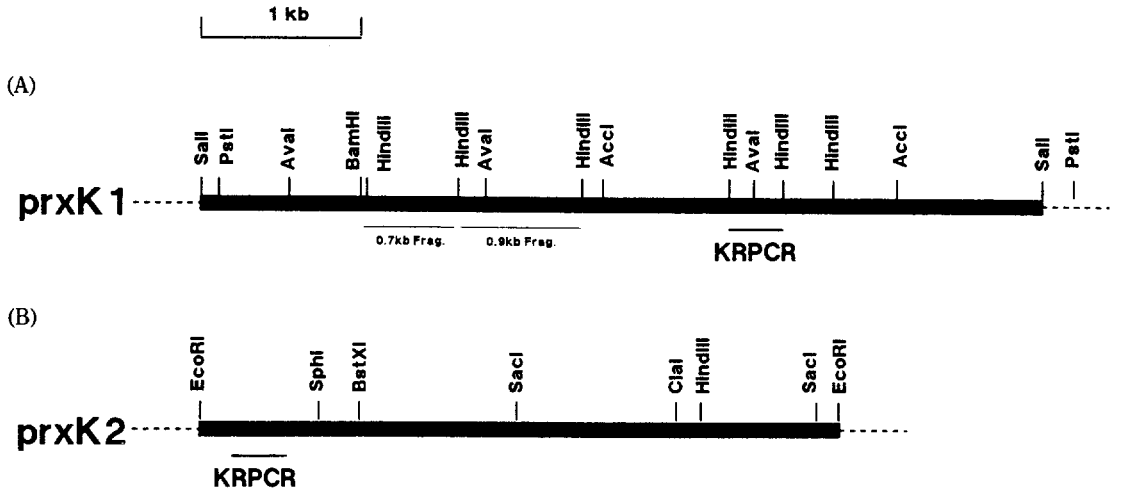

Fig. 4. Restriction map of the *prx*K1 and *prx*K2 genomic clones. (A) *prx*K1 genomic clone. (B) *prx*K2 genomic clone. Bold dash indicates the partial cDNA probe.

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AAGCTTTCCTCTTCGTTTACCACAAAGGTACGTCCGAGCCAATTCTTTGGTAGTATTTATGGTAGAATCACAAGCGTAGATCATTTCCCCAAGTGTATCGCAGTGCAAGAAGCCGTAGAA -1068
CTGAGGAAAAATAAAATTAGTATCCGGAGACTCACCAACGTTAAGGAATCGGAAGGCAAATGGAGGGCTAACATCTAATATCGCTCGAGTGAGAGCTGAGTGCACTTTGTTTTCTATTAC -0948
TGTATGTTAATATAATTAAGCACCATCAACTGTAGAAAGTATAGTCAAAAATAATGATAAAAATAAACTTTAGACCGTTTATACTTTTTGCGTTGTTGTTTGCCAGGTCTCGATCAATTA -0828
                                                SEFBS                                                    *  *
TATGGACAGAAGTCACTCGACTGAAATGAGATGAGCAAAGTCATTCTTATAATTATGTTTTAAACAGTGGAGATATGCTAAAAACAGTGGAGATGAACACATTTCGATTTGACGTACAGA -0708
                                                                                                                TGA1b
AATTACGTATATGCTTCTTTGGCAAGTATATGCTTCCGGCGTCTATTTTAAAGAAGACGTGTATTTTGCAAATAGTTACTTCTTTTGCCTGCTAGTAAAGATAAATTCACTAACAATATC -0588
                                                Enh
GAATAAAGACATTGAGATGGTTAACAAAATGTTTGAGATTTTAATTTAAATGAAAACAGAGTAAAAAAAGCTTTCAAACAATCACAATGATATTGACATGAATTAAAAGAGTCTTTTGCC -0468
GCCTCTCAGTTACTTATTATACACAATCTTTGCAATAAATTTAGATGATACAGAACTAACAATTGCTTCTTAGATATTGTCACGCAAAACATATAAGCACTACAAGCTAATTTATTTTTT -0348
TCCATTAATTTAATCTCTTATTTCTTATCATCTTCCAAGTTAACTATATGAAGTCTTCGACTTAATTTGATTGTATACTAATTACTTGATACAAAATTATTACCGCTGACAGAGTTTGTT -0228
TTGGCTTCTCTCACCGACGGAGATATATACAAAACAAATTCGATGCAAAAATGAAGATTAAAACCTAAAAGCAAAATAAATAACACATTTTTCAACGCGTTAGTTTACCTGTCTATCACT -0108
                                                                                                                +1
TTAACTAGGGCATCCTTCACGTCTCTATATAAACCTCACTCCGTTATGCATTATTCTCCAAGTACAATAATCAACTCTAAGTTTAACCAAGTCTTTACTACTCAGATATG
```

Fig. 5. Nucleotide sequence of the 5′-noncoding region of *prx*K1. Putative TATA and CAAT boxes are underlined (CAAT boxes is indicated by italic letter). Two transcription start points are shown by asterisks. SEFBS, soybean embryo factor binding site; TGA1b, the sequence related to TGA1b binding site; Enh, conserved enhancer sequence found in several animal genes. The numbering scheme for the nt sequence specifies the A (reverse shading) of the start codon as +1.

In order to obtain promoter sequence we proceeded sequencing the 5′ noncoding region of the *prx*K1 genomic clone instead of the *prx*K2 genomic clone, to which the probe hybridized at the far end, and may not contain the whole genomic sequence and/or promoter sequence. Therefore, the 0.7 and 0.9 kb *Hin*dIII fragments of the 5.2 kb *Sal*I fragment were subcloned into the pUC19 vector for promoter sequence analysis. Fig. 5. shows the putative promoter sequences, TATA and CAAT box, in the 5′ noncoding region. The putative SEFBS (soybean embryo factor binding site) and Enh (conserved enhancer sequence) were found 890 and 642 bp upstream from the initiation codon, respectively. Especially, the 5′ TGACGT sequence closely related to the TGA1b protein binding site was found 718 bp upstream. Previously, Lam *et al.* (1989) reported that the 35S promoter of the cauliflower mosaic virus (CaMV) contained a tandem repeat of the TGACG sequence in the region −83 to −63. This 21 bp sequence, called as−1, was involved in root expression of the 35S promoter. The presence of the TGACG motif in the *prx*K1 promoter region suggests that the 5′ TGACGT sequence may also have a regulatory role in *prx*K1 gene expression. The possible transcription start point was determined by the primer extension method and indicated by asterisks in Fig. 5.

Shinmyo *et al.* (1993) reported that the nucleotide sequence of the 5′ noncoding region of the horseradish peroxidase *prx*C2 gene has several consensus motifs such as an Enh, SEFBS and UV *cis* element. In this study we also found Enh and SEFBS in the 5′ noncoding region of *prx*K1. However, no UV *cis* element

was found in the present study. Instead of the UV cis element, the TGACG motif that was known to be a response element of the signal pathway in the mammalian system (Katagri *et al.*, 1989) was found in the promoter region of the *prx*K1. The result suggests that the TGACG motif may also be involved in similar signal transduction pathways in higher plants. Thus, further studies are needed to clarify the role of these consensus motifs in the regulation of *prx*K1 gene expression under various physiological states of radish.

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References

Bartonek-Roxa, Eriksson E. H. and Mattiasson, B. (1991) *Biochim. Biophys. Acta* **1088**, 245.

Buffard, D. C., Breda, R. B., van Huystee, Asemota, O., Pierre, M., Dang Ha, D. B. and Esnault, R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8874.

Espelie, K., Franceschi, V. R. and Kolattukudy, P. E. (1986) *Plant Physiol.* **81**, 487.

Fujiyama, K., Takemura, H., Shbayama, S., Kobayashi, K., Choi, J., Shinmyo, A., Takano, M., Yamada, Y. and Okada, H. (1988) *Eur. J. Biochem.* **173**, 681.

Fujiyama, K., Takemura, H., Shinmyo, A., Okada, H. and Takano, M. (1990) *Gene* **89**, 163.

Grisebach, H. (1981) in The biochemistry of plants (E. E. Conn, ed.), Vol. 7, pp. 457-478, Academic press, New York.

Hammerschmidt, R. E., Nuckles, E. and Kuc, J. (1982) *Physiol. Plant Pathol.* **20**, 73.

Intapuruk, C., Higashimura, N., Yamamoto, K., Okada, N., Shinmyo, A. and Takano, M. (1991) *Gene* **98**, 237.

Katagiri, F., Lam, E. and Chua, N.-H. (1989) *Nature* **340**, 727.

Krug, M. and Berger, S. L. (1987) *Methods Enzymol.* **152**, 316.

Lagrimini, L. M., Burkhart, W., Moyer, M. and. Rothestein, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7542.

Lam, E., Benfey, P. N., Gilmartin, P., Fang, R-X. and Chua, N.-H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7890.

Leder, P., Tiemeier, D. and Enquist. L. (1977) *Science* **196**, 175.

Lee, M. Y. and Kim, S. S. (1990) *Kor. Biochem. J.* **23**, 503.

Lee, M. Y., Choi, Y. and Kim, S. S. (1994) *Plant Physiol & Biochemistry,* **35**, 287.

Logemann, J., Schell, J. and Willmitzer, L. (1987) *Anal. Biochem.* **163**, 16.

Lee, M. Y. and Kim, S. S. (1994) *Phytochemistry* **35**, 287.

Mazza, G. and Welinder, K. G. (1980) *Eur. J. Biochem.* **108**, 481.

Morgans, P. H., Callaan, A. M., Dunn L. J. and Albeles, F. B. (1990) *Plant. Mol. Biol.* **14**, 715.

Murray, M. G. and Thompson, W. F. (1980) *Nucl. Acids Res.* **8**, 4321.

Okayama, H. (1987) *Methods Enzymol.* **154**, 3.

Roberts, E. and Kolattukuby, P. E. (1988) *Plant Mol. Biol.* **11**, 15.

Sanger, R. S., Nicklen, A. and Coulson, R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463.

Shinmyo, A., Fujiyama, K., Kawaoka, A. and Intapuruk, C. (1993) in Biochemical, Molecular, and Physiological aspects of Plant Peroxidases (Lobarzewski, J., Greppin, H., Penel, C., GasparTh eds) p. 221, University of Geneva, Switzerland.

Southern, E. M. (1975) *J. Mol. Bioc.* **98**, 503.

Strivastava, O. P. and van Huystee, R. B. (1977) *Can. J. Bot.* **55**, 2630.

Welinder, K. G. (1979) *Eur. J. Biochem.* **96**, 483.

Woo, S. L. C. (1979) *Methods Enzymol.* **68**, 389.