Isolation of Genomic DNA Containing a Cytosolic Ascorbate Peroxidase Gene (ApxSC) from the Strawberry (Fragaria x ananassa)

In-Jung Kim and Won-II Chung

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Kusong-dong, Yusong-ku, Taejon 305-701, Korea

Received January 13, 1998

We isolated a genomic DNA harboring a cytosolic ascorbate peroxidase gene (ApxSC) from a genomic library of the strawberry (Fragaria x ananassa). Restriction mapping and sequence analyses showed that the DNA is composed of 2.5 kb of the full-length ApxSC gene, 3.7 kb of the 5' upstream region, and 0.5 kb of the 3' downstream region. The ApxSC genomic DNA contains 10 exons and 9 introns, which is similar to the structure of pea ApxI. A primer extension analysis suggested that the transcription of ApxSC gene was started at three start sites with different degrees. The promoter region of ApxSC gene contains a sequence or structure distinct from other reported plant ascorbate peroxidase genes, though with several known functional elements such as a TATA box.

Key words: cytosolic ascorbate peroxidase; genomic DNA; promoter; strawberry (Fragaria x ananassa)

Ascorbate peroxidase (APX, EC 1.11.1.11), a hydrogen-peroxide removing enzyme in cytosol and chloroplasts of higher plants, has been found in higher plants, algae,19 and some cyanobacteria.20 APX is distinct from other peroxidases, such as guaiacol peroxidase and anionic peroxidase, in the primary structure and function and in its preference for ascorbate as a reductant.4,14 A number of cDNAs encoding cytosolic and chloroplastic APX isoforms were isolated and characterized from several plants including Arabidopsis,2,6 pea,7 pepper,8 and spinach.9,10 The primary structures of these cDNAs have significant similarity.

It was reported that the expression level of APX was controlled by growth conditions11,12 and environmental stresses such as chemicals,13 heat,13 ozone,14,16 and microbial infection.17 The expression of APX was known to increase more in its transcript than in the activity and the protein, and to be regulated in the posttranscriptional level.12,13 Particularly, in the bell pepper8 and banana,18 the APX expression is related to fruit ripening in the transcription level. Our previous study also showed that the transcription level of cytosolic APX in the strawberry changed, with some differences from that of bell pepper, according to developmental stages of the fruit, and that it was the strongest in fruit than other tissues.19 These results suggest that the transcription of APX was regulated by signals delivered from inside and outside of the cell.

The genomic DNAs of APX were also isolated from pea14 and Arabidopsis.20,21 They have a similar exon/intron organization. However, the nucleotide sequences of promoter regions are quite different, which may affect APX expression.

In this study, we present the isolation of a genomic DNA clone that contains the promoter region and the ApxSC gene of strawberry. The genomic structure of this DNA was also compared with those of pea and Arabidopsis.

Materials and Methods

Plant materials. Strawberry (Fragaria x ananassa Duch. cv. Yoho) was used throughout this work. Plants were cultivated under greenhouse conditions.

Construction of a genomic library and screening. Total DNA was isolated from strawberry leaves, by the method of Dellaporta.22 The DNA was digested with XbaI, ligated into the XbaI site of λ-ZapII vector, and packaged into GigapackIII gold cloning kits (Stratagene), according to the manufacturer's instruction. In vivo excision of pBluescript plasmids was done in the Escherichia coli SOLR strain. The library was screened with a radiolabeled full-length cytosolic ascorbate peroxidase cDNA probe (Genbank accession No. AFO22213 and AF039953) by standard plaque lift methods.23 The probe was prepared from cDNA labeled with [α-32P] dCTP by using the Prime-a-Gene labeling system (Promega). After treatment for 1–2 hr at 42°C in 30% formamide, 5x Denhardt's solution, 5x SSPE, and 100 µg/ml denatured salmon sperm DNA, the filters were incubated with a 32P-labeled probe for 24 hr under the same conditions. The filters were washed twice in 2x SSC and 0.05% SDS for 15 min at 42°C and twice in 0.2x SSC and 0.1% SDS for 15 min at 68°C.

DNA sequencing and analyses. Nucleotide sequencing using the dyeoxy chain termination method24 was done with the Sequenase 2.0 kit (United States Biochemical) for a double strand to avoid errors, with custom-made (DNA International) oligonucleotide primers for sequencing of internal sequences. Computer analyses

* Corresponding author.

Abbreviations: APX, ascorbate peroxidase; APX, gene coding for APX; ApxSC, a cytosolic APX of strawberry fruit; ApxSC, gene coding for a cytosolic ApxSC of strawberry; bp, base pairs; kb, kilobase pair(s); nt, nucleotides; UTR, untranslated region(s).
for the nucleotide and amino acid sequences were done by the PCGENE software ( IntelliGenetics Inc., Release 6.60).

**Primer extension.** An antisense 18-nucleotide primer corresponding to nucleotides 279-262 of the genomic sequence (see Fig. 2) was synthesized and end-labeled with [γ-32P]ATP by the T4 DNA polynucleotide kinase. Poly(A)⁺ RNA (1 μg) prepared by the PolyATtract mRNA Isolation System III (Promega) was hybridized with the oligonucleotide primer (5 × 10⁴ cpm). The cDNA synthesis was done with MMLV reverse transcriptase (Promega) and analyzed on a 6% (w/v) sequencing gel.

**Genomic DNA blot analysis.** Genomic DNA was isolated from young leaves of strawberry plants, by the method of Dellaporta. Genomic DNA was digested with EcoRI, HindIII, XbaI, and EcoRV, separated on 0.7% agarose gels, and then blotted onto a Hybond-N (Amersham). Hybridization and washing of filters were done as described in genomic library screening, except for high stringency washing (0.1 × SSPE and 0.1% SDS, 68°C).

**Results and Discussion**

**Isolation of genomic DNA clones**

We isolated a genomic DNA fragment containing a cytosolic ascorbate peroxidase (ApxSC) gene from the strawberry genomic library through hybridization with a cDNA encoding ApxSC (Genebank accession No. AF039953) of the strawberry. Sequence analysis and restriction mapping showed that the isolated genomic clone contains a 6.7 kb XbaI fragment harboring 3.7 kb of the 5'-upstream region, 2.5 kb of the full-length ApxSC gene and 0.5 kb of the 3'-downstream region. The restriction map and structure of the genomic DNA are shown in Fig. 1.

**Structure of the ApxSC gene**

By comparison of the gene sequence with previously isolated cDNA, the exon/intron organization was identified. The isolated ApxSC gene was composed of 10 exons interrupted by 9 introns (Fig. 1), which is similar to the structure of pea ApxT, but not of Arabidopsis APX1a and Arabidopsis APX1b. The splice site of introns followed the GT-AG rule. The first intron was in the 5'-untranslated region (UTR) of the mRNA (Fig. 2). A similar structure was observed in ApxF and APX1a gene. This feature is not common in plant genes, with some exceptions. Arabidopsis APX1a and a pea Apx1 gene with the intron in 5'-UTR were expressed in several tissues including leaves, while no transcript of Arabidopsis APX1b without the intron could be detected. These results suggest that a different expression could occur partly because of the intron in 5'-UTR. The introns were found to contain a high A+T content (average of 66.5%), which is common in APX and other genes of dicotyledonous species.

The transcription start site was identified by primer extension analysis as shown in Fig. 3. Three distinct products of different intensity were detected with 111, 109, and 108 nucleotides in length. The transcription start site (CTCTACTCT) derived from 108 nucleotides was well matched with the consensus sequence, while the other two start sites (CTACTCTC) derived from 111 and 109 nucleotides were not. Although transcription start sites deviating from the consensus sequence were observed from genes of some plants, such as the alcohol dehydrogenase gene of Arabidopsis and the extin gene of carrot, the third transcription start site derived from 108 nucleotides showed a stronger signal than the other two sites in this primer extension analysis, and we temporarily designated as position 1 the third start site.

Several potential regulatory elements were found between positions −620 and 1. A putative TATA and a CCAAT box were identified at positions −34 and −104, respectively. A putative ethylene response element (ERE, ATATCACA) was found at position −575. One potential heat-shock element (CTAGAGCCTTCTCT) and an enhancer element

---

**Fig. 1.** Genomic Structure of the ApxSC Gene.

Boxes represent exons, solid boxes indicate open reading frames, and open boxes refer to 5'- and 3'-untranslated regions. The restriction map of the genomic clone is depicted with several restriction enzymes, such as E, EcoRI; H, HindIII; X, XbaI; S, SacI.
Fig. 2. Nucleotide Sequence and Deduced Amino Acid Sequence for the ApxSC Clone of Strawberry.

Uppercase letters represent exons, and lowercase letters indicate introns. 5' upstream and 3' downstream regions. TATA and CCAAT boxes are represented by uppercase letters in bold. The potential promoter elements are represented both by underlines and letters above: HSE, heat-shock elements; ERE, ethylene responsive elements; CGE, β-conglycinin gene enhancer. The other underlined letters indicate repeat sequences (RPT1, RPT2) and the poly(A) signal. An antisense 18-nucleotide primer is shaded. Transcript start sites are represented by uppercase letters in bold with arrow.
the significance, if any, is not known.

**Genomic DNA blot analysis**

The genomic DNA isolated from strawberry leaves was digested with EcoRI, HindIII, XbaI, and EcoRV, and then separated on 0.7% agarose gels. As shown in Fig. 4, the digested DNA fragments were hybridized with the ApxSC cDNA probe and washed at high stringency (0.1× SSPE and 0.1% SDS, 68°C). The genomic blot pattern showed 4–6 bands in each DNA sample. *Fragaria x ananassa*, the plant used in this study, is a hybrid of two parents, *Fragaria virginiana* and *Fragaria chiloensis*. It has a polyploidic genome, which makes it difficult to interpret the number of ApxSC genes per chromosome set. To confirm the number, it may be necessary to isolate several APX clones from the genomic library and to compare their nucleotide sequences.

**Comparison of ApxSC genomic structure with Arabidopsis APX1a and APX1b, and pea ApxI**

The structures of ApxSC genes are similar[4,20,21] to those of the other reported APX genes, as shown in Fig. 5. The latter five exons of four genes are highly similar, while the others are more variable. The highest similarity was found between ApxSC and ApxI. The sequence of the exons in the ApxSC gene is very similar to those of other plant APX genes. However, no significant homology was detected in length and nucleotide se-

Fig. 3. Primer Extension Analysis. Autoradiogram of the primer extension products separated on a 6% sequencing gel. Lanes: primer extension products, P; sequencing ladders, G, A, T, C. Sequencing ladders were obtained by priming sequencing reactions with the same 18-nucleotide primer and the isolated genomic clone as a template. Sizes of primer extension products are indicated on the left.

Fig. 4. Genomic Blot Analysis of ApxSC. Genomic DNA (10 μg) was digested with EcoRI (EI), HindIII (HIII), XbaI (XI), and EcoRV (EV) for each DNA sample. Size markers (kb) are indicated on the left. The blot was done with previously isolated ApxSC cDNA probe labeled with [α-32P]dCTP.

Fig. 5. Comparison of ApxSC with Other APX Genes in Genomic Structure. ApxSC, strawberry ApxSC gene in this study; ApxI, pea ApxI gene[19]; APX1a, Arabidopsis APX1a gene[20]; APX1b, Arabidopsis APX1b gene[21]. Solid boxes represent exons.
quence of introns, except in high A+T content.

The promoter region of \( \textit{ApxSC} \) showed no significant similarity to that of the \( \textit{APX1a}, \textit{APX1b}, \) or \( \textit{APx1} \) genes, with exceptions of several common features. That is, all isolated promoters of \( \textit{APX} \) genes have the putative TATA and CCAAT boxes. However, their locations in 5'-UTR are different: position -34 for TATA box and -104 for CCAAT box in \( \textit{ApxSC} \), -25 and -147 in \( \textit{APx1} \), -26 and -236 in \( \textit{APX1a} \), -42 and -236 in \( \textit{APX1b} \). The putative heat-shock element found in \( \textit{ApxSC} \) genes has been identified, if at different positions, in other \( \textit{APX} \) genes\(^{3,20,21}\) and many heat-shock genes.\(^{37,39,40}\) The promoter of the \( \textit{ApxSC} \) gene has a very high A+T content (average of 70%) between positions -580 and -350, which feature is also found in those of many heat-shock genes.\(^{37,39,40}\) That is, although some common regulatory elements were located at promoter regions, the exact location and the number of those elements were different. The promoter of the \( \textit{ApxSC} \) gene of the strawberry has a distinct nucleotide sequence and some different putative regulatory elements from other plant \( \textit{APX} \) genes.

The different expressions of \( \textit{ApxSC} \) gene according to tissues, developmental stages, or environmental stresses may be regulated by various regulatory elements in the promoter region. These expressions are different from those of other \( \textit{APX} \) genes, though they have similar cDNA structures. For complete understanding of the regulation of expression, it is necessary to analyze and compare the function and structure of the \( \textit{APX} \) gene promoters. Expression analysis of the \( \textit{ApxSC} \) promoter of the strawberry is in progress.

Acknowledgments
This work was supported by research grants from the Rural Development Administration of Korea.

References
1) Shigeoka, S., Nakano, Y. and Kitaoaka, S., Metabolism of hydro-


2) Tel-Or, E., Huffjel, M. and Packer, L., Hydroperoxide


3) Chen G. X. and Asada, K., Ascorbate peroxidase in tea leaves:
ocurrence of two isozymes and the differences in their enzymatic


4) Patterson, W. R. and Poulos, T. L., Crystal structure of recombinant


5) Kobo, A., Saji, H., Tanaka, K., Tanaka, K., and Kondo, N.,

Cloning and sequencing of a cDNA encoding ascorbate peroxidase


6) Jespersen, H. M., Kjersgård, I. V. H., Østergaard, L., and

Weilinder, K. G., From sequence analysis of three novel ascobrate

peroxidases from \textit{Arabidopsis thaliana} to structure, function


7) Mittler, R. and Zilinskas, B. A., Molecular cloning and nucleo-
tide sequence analysis of a cDNA encoding pea cystolic ascor-

8) Schantz, M. L., Schreiber, H. S., Guillermuat, P., and Schantz, R., Changes in ascorbate peroxidase activities during fruit ripen-

9) Ishikawa, T., Sakai, K., Takeda, T., and Shigeoka, S., Cloning and

expression of cDNA encoding a new type of ascorbate peroxi-

10) Ishikawa, T., Sakai, K., Yoshimura, K., Takeda, T., and

Shigeoka, S., cDNAs encoding spinach stromal and thylakoid-
bound ascorbate peroxidase, differing in the presence or absence


11) Gilham, D. J. and Dodge, A. D., Chloroplast superoxide dis-

mutase and hydrogen peroxide scavenging systems from pea


12) Mittler, R. and Zilinskas, B. A., Regulation of pea cystolic ascor-
brate peroxidase and other antioxidant enzymes during the
progression of drought stress and following recovery from

13) Mittler, R. and Zilinskas, B. A., Molecular cloning and charac-
terization of a gene encoding pea cystolic ascorbate peroxidase.

14) Mohihorn, H., Cottam, D. A., Lucas, P. W., and Wilburn, A. R.,
Induction of ascorbate peroxidase and glutathione reductase
activities by interactions of mixtures of air pollutants. \textit{Free Radic.

15) Kubo, A., Tanaka, K., and Kondo, N., Expression of Arabidop-
sis cystolic ascorbate peroxidase gene in response to ozone or

16) Örvar, B. L., McPherson, J., and Ellis, B. E., Pre-activating
wounding response in tobacco prior to high-level ozone exposure

17) Hammond-Kosack, K. E. H. and Jones, J. D. G., Resistance
gene-dependent plant defense responses. \textit{Plant Cell}, 8, 1773-

18) Clendennen, S. K. and May, G. D., Differential gene expression in

19) Kim, I. J. and Chung, W. I., Molecular characterization of a
cystolic ascorbate peroxidase in strawberry fruit. \textit{Plant Science},
in press.

20) Kubo, A., Saji, H., Tanaka, K., and Kondo, N., Genomic DNA
structure of a gene encoding cystolic ascorbate peroxidase from

21) Santos, M., Gousseau, H., Lister, C., Foyer, C., Creissen, G.,
and Mullineaux, P., Cystolic ascorbate peroxidase from
\textit{Arabidopsis thaliana} I. is encoded by a small multigene family.

22) Dellaporta, S. L., Wood, J., and Hicks, J. B. A., Plant DNA

23) Sambrook, J., Fritsch, E. F., and Maniatis, T. in “\textit{Molecular
Cloning: A Laboratory Manual},’’ 2nd Ed., Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, New York, pp. 8.60-

24) Sanger, F., Nicklen, S., and Coulson, R., DNA sequencing with

25) Verwoerd, T. C., Dekker, B. M., and Hoekema, A., A small-
scale procedure for the rapid isolation of plant RNAs. \textit{Nucleic

26) Brown, J. W. S., A catalogue of splice junction and putative
branch point sequences from plant introns. \textit{Nucleic Acids Res.},
14, 9549-9559 (1986).

27) Werr, W., Frommer, W. B., Maas, C., and Starlingere, P., \textit{Struc-
ture of the sucrose synthase gene on chromosome 9 of \textit{Zea mays

28) Christensen, A. H. and Quail, P. H., Structure and expression of a
maize phytochrome-encoding gene. \textit{Gene}, 85, 381-390
(1989).

29) Pearson, L. and Meagher, R. B., Diverse soybean actin
transcripts contain a large intron in the 5'-untranslated leader:
structural similarity to vertebrate muscle actin genes. \textit{Plant Mol.

30) Christensen, A. H., Sharrack, R. A., and Quail, P. H., Maize
polyubiquitin genes: structure, thermal perturbation of expres-
sion and transcript splicing, and promoter activity following
transfer to protoplasts by electroporation. \textit{Plant Mol. Biol.}, 18,


