

Cloning of 3' End cDNA of Ascorbate Peroxidase Gene from *Fragaria × ananassa* cv. Toyonaka

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Abstract

A fragment of 3' end cDNA of *apx* in *Fragaria × ananassa* cv. Toyonaka was cloned by rapid amplification of cDNA 3' end (3'RACE) PCR. By joining the sequence with the known fragment sequence which we had submitted to GenBank (FJ896040), a 3'-end partial cDNA of 672bp was obtained, encoding 140 amino acids. The sequence contained the corresponding coding sequence of 424bp long including a stop codon TAA, and the 3'-untranslated region(3'UTR) of 248bp including a potential polyadenylation signal (AAATAA) and poly(A) of 28bp long. Homology analysis of nucleotide sequence and deduced amino acid sequence on the *apx* gene showed that it shared high homology with different plants species, among which it shared the highest sequence homology with *Fragaria × ananassa* cv. Yoho.

Keywords: *Fragaria × ananassa* cv. Toyonaka, Ascorbate peroxidase gene, 3'-RACE, Cloning

1. Introduction

Ascorbate peroxidase (APX; EC 1.11.1.11) is a hydrogen peroxide-scavenging enzyme, found in higher plants, algae, and some cyanobacteria (Asada, 1992). It catalyses the conversion of H₂O₂ to H₂O and O₂ using ascorbate as the specific electron donor (Asada, 1999). APX has a presumed function of protecting cells from hydrogen peroxide accumulation, particularly under stressful conditions (Lin *et al.*, 2007). Researchers have reported that the activities and the mRNA expression levels of APX would increase when plants encounter environmental stresses such as high temperatures, drought, light, and so on (Tanaka *et al.*, 1990; Mittler *et al.*, 1994).

APX comprises a family of isoenzymes with different characteristics in higher plants (Chen and Asada, 1989; Bunkelmann and Trelease, 1996; Shigeru *et al.*, 2002). To date many researchers have cloned *apx* isozyme genes from a variety of plants. Strawberry is one of the most economically important fruit trees. Although *apx* gene fragment has been isolated from different strawberry cultivars, the full-length sequences of the genes have not been reported. However, the entire 3' sequence of a cDNA is very important because the non-coding terminal region often contains signals that regulate the stability or subcellular localization of mRNAs, and sometimes alternative genomic sites are used for cleavage and polyadenylation, which can alter these aspects or change the encoded protein. In this study, we obtained the 3' end cDNA sequence of *apx* gene from *Fragaria × ananassa* cv. Toyonaka by rapid amplification of cDNA ends (RACE) PCR.

2. Materials and methods

2.1 Plant material and RNA isolation

Strawberry (*Fragaria × ananassa*) cv. Toyonaka was used in this study. Total RNA was extracted from strawberry fruit using the Plant RNA_{OUT} kit, as per the manufacturer's instructions(TIANDZ, China).

2.2 Primer design

According the theory of classical RACE (Scotto-Lavino *et al.*, 2006), we synthesized three universal primers (Q_T, Q_O, Q_I) and two gene-specific primers(GSP1, GSP2). Q_T consisted of a unique 35-base oligonucleotide sequence and 17 Ts. Q_O and Q_I respectively contains part sequence of Q_T. GSP1 and GSP2 was designed according to the known fragment sequence which we had submitted to GenBank (FJ896040). The sequences of these primers are shown as follows: Q_T: 5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTT-3'; Q_O: 5'-CCAGTGAGCAGAGTGACG-3'; Q_I: 5'-GAGGACTCGAGCTCAAGC-3'; GSP1: CAAGCCCGAAC CACCACCAGA-3'; GSP2: 5'-GGAAGGGCACACAAGGAACGG-3'.

2.3 cDNA synthesis

The first strand cDNA was synthesized from 2 µg of the total RNA by reverse transcriptase with Q_T primer according to the instructions of the Easy-GoTM RT PreMix kit (SBS Genetech, China).

2.4 3'-RACE PCR amplification

The first round PCR was performed with a 20 µl reaction mixture containing 1 µl of first-strand cDNA (50 ng/µl), 2.0 µl of 10×PCR buffer without Mg²⁺, 2.0 mM MgCl₂, 200 µM dNTPs, 0.5 µM Q_O, 0.5 µM GSP1 and 1.0 unit of *Taq* DNA polymerase. The second round PCR template was 1/50 of the product from the first round PCR and PCR performed with the primer pair Q_I / GSP2. Others were the same with the first round PCR. The PCR procedure were all started with 94°C for 3 min, then 35 cycles of 94°C for 1 min, 64°C for 1 min, 70°C for 2 min, and finally 72°C for 7 min. The PCR products were analyzed on a 1.0% agarose/EtBr gel and the corresponding DNA band was recovered, then cloned into the pMD19-T Vector (TaKaRa, China) for sequencing. Sequence analysis was performed using the software DNAMAN (Version 3.0, Lynnon BioSoft).

3. Results and analysis

3.1 The cloning of 3' RACE

The agarose gel electrophoresis showed that no specific band but a smear was found for the first PCR product (Fig.1, Lane 1). However, a very specific band of about 500 bp was obtained in the second PCR product using inner primer pair Q_I / GSP2 (Fig.1, Lane 2). After the RACE-PCR product was recovered and cloned into pMD19-T vector, plasmid PCR was then performed for rapidly screening APX cDNA clone with primers Q_I and GSP2. Finally, positive cDNA clones were picked out and sequenced.

3.2 Sequence analysis of 3'-end partial cDNA

Sequence analysis revealed that 3' RACE product was 506 bp long, and the sequence showed a high sequence homology with *apx* of many kinds of plants. Therefore, we presumed that it was the target fragment. By joining the sequence with the known fragment sequence which we have submitted to GenBank (FJ896040), a 3'-end partial cDNA of 672 bp was obtained, encoding 140 amino acids, named *Faapx*. The sequence contained the corresponding coding sequence of 424 bp long including a stop codon TAA, and the entire 3'-untranslated region (3'UTR) of 248 bp including a potential polyadenylation signal (AAATAA) and poly(A) of 28 bp long (Fig.2).

3.3 Similarity analysis of *apx* gene

The similarity analysis of nucleotide sequence and deduced amino acid sequences of *apx* genes within different plants species (Table 1) revealed that they shared high homology (Fig.3). *Faapx* shared a similarity of the nucleotide (62.8–98.1%) and deduced amino acid sequences (79.9–98.6%) with *apx* genes of other plants, among which it shared the highest sequence homology with *Fragaria* × *ananassa* cv. Yoho. Both nucleotide phylogenetic tree and amino acid homology tree showed that the two strawberry cultivars fell into one cluster. The nucleotide homology tree clustering analysis revealed that strawberries and *Malus* × *domestica* then clustered together, but they did not cluster directly in the amino acid homology tree. Although there were some differences in the cluster results between nucleotide sequence and amino acid sequence, the homology of them were respectively over 65%. These results indicate that *apx* genes are relatively conserved in different plants.

4. Discussions

As a key enzyme in decomposing H₂O₂, APX is not only closely related to plant growth and resistance to environmental stress, but also one of the enzymes in the metabolism of vitamin C (Schantz *et al.*, 1995). Therefore, cloning full length cDNA encoding APX is very important to have a better understanding of its functions. RACE technology has become a common strategy to cloning full cDNA sequence of unknown genes, from reverse transcription through to amplification, and took less a day to perform. In this study, we used a classic RACE technique to clone 3' end cDNA encoding APX. The result showed that the nucleotide and deduced amino acid sequence shared high homology with those of other plants APX reported to date. Thus, the 3' end cDNA fragment obtained is the target gene which we needed. However, the technique is particularly sensitive to amount of cDNA template used in the research. Very little substrate is required for the PCR, because too much starting material will lead to the production of large amounts of non-specific product. We obtained successfully the 3' end cDNA sequence of *apx* from *Fragaria* × *ananassa* cv. Toyonaka by the means, only using 1 µl of first-strand cDNA (50 ng/µl). Now we are cloning 5' end cDNA encoding APX of *Fragaria* × *ananassa* cv. Toyonaka in order to make a great foundation for researching of its structure and function.

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Table 1. The *apx* genes of different plants species in this study

Code	Scientific name	Code	Scientific name
A	<i>Fragaria × ananassa</i> cv. Toyonaka	G	<i>Lycopersicon esculentum</i>
B	<i>Fragaria × ananassa</i> cv. Yoho	H	<i>Solanum tuberosum</i>
C	<i>Malus × domestica</i>	I	<i>Capsicum annuum</i>
D	<i>Cucumis sativus</i>	J	<i>Litchi chinensis</i>
E	<i>Cucumis melo</i>	K	<i>Dimocarpus longan</i>
F	<i>Citrus maxima</i>	L	<i>Vitis pseudoreticulata</i>

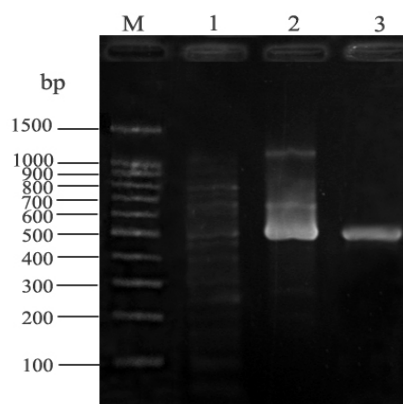


Figure 1. Agarose gel electrophoresis of PCR products and plasmid PCR product

M = DNA ladder; lane 1 indicates the first PCR product; lane 2 indicates the second PCR product; lane 3 indicates the plasmid PCR product

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1   GCCTGATGTTCTTTCCACCCAGGAAGAGAGGACAAAGCCCGAACCACCACAGAAAGGCCGTCTTCTGATGCTGG
1   P D V P F H P G R E D K P E P P P E G R L P D A G
76  AAAGGGTTCTGACCACTTGAGGGAAGTGTGGCAAAACCATGGGTCTCAGCCACCAGGACATTGTTGCTCTCTC
26  K G S D H L R E V F G K T M G L S H Q D I V A L S
151 TGTTGGTTCACACCTTGGGAAGGGCACACAAGGAACGGTCTGGATTTCGAGGGACCTGGACTCCCAACCCCTTAT
51  G G H T L G R A H K E R S G F E G P W T P N P L I
226 CTTTGACAACCTCATATTTCACTGTGCTGTTGAGTGGAGAGAAGGAAGGCCTTCTACAGCTTCCAACCTGACAAGGC
76  F D N S Y F T V L L S G E K E G L L Q L P T D K A
301 TCTTCTGTCAGACCCTGTCTTCCGCCCTCTTGTGAGAAATACGCTGCGGATGAAGATGCTTTCTTTGCTGATTA
101 L L S D P V F R P L V E K Y A A D E D A F F A D Y
376 TGCTCTAGCTCATCAGAGGCTCTTTGAGCTTGGTTTTGCTGAAGCTTAAgcagtggaactttactaaggataaag
126 A L A H Q R L F E L G F A E A *
451 gatgatgccaatgccaatgcctgcgtgccttgcctttgtattttgtattcctcagttctgggggttttaggcagtt
526 ggtgtgtgtttttattggttaggaaagtggatttcattttcagtttgatggatgtgttgaattggatagaatgat
601 ttggtgatcatccttttagataaataattacacatcctttactttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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Figure 2. Nucleotide sequence and deduced amino acid sequence of *apx* gene

The lower-case characters indicate noncoding regions; arrow indicates primer (GSP1 and GSP2) locations; single underline indicates poly(A); double underline indicates potential polyadenylation signal; TAA indicates the stop codon

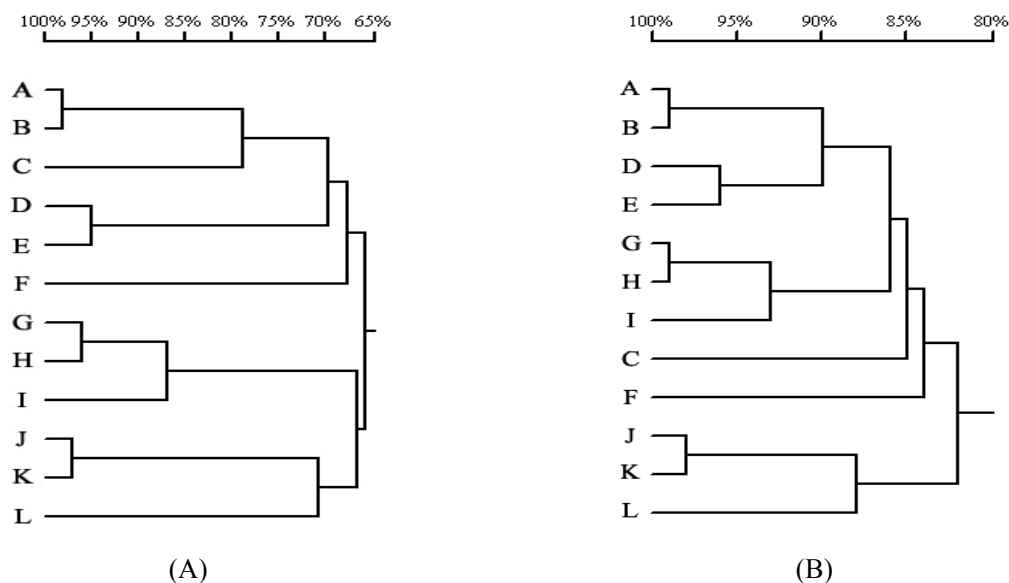


Figure 3. Homology tree of nucleotide sequences (A) and its deduced amino acid sequences (B) of the *apx* gene from twelve plants species(as in Table 1)