

Cloning and Expression Analysis of Ascorbate Peroxidase Gene During Fruit Development and Ripening in *Fragaria* × *ananassa* cv. Toyonaka

Hou Yan-Xia, Tang Hao-Ru, Zhang Yong, Luo Ya and Chen Qing

Horticultural College, Sichuan Agricultural University,
Ya'an, 625014, People's Republic of China

Abstract: A ascorbate peroxidase gene fragment named *Faapx-c* (GenBank number: FJ896040) was cloned from fruit of cultivated strawberry (*Fragaria* × *ananassa* cv. Toyonaka) by RT-PCR. The analysis showed that the fragment was 352 bp in length, encoding 116 amino acids, which were 97%~99% identical to the sequences of *Fragaria* × *ananassa* Duch. cv. Yoho cytosolic *apx* gene from the GenBank. Semi-quantitative RT-PCR analysis showed that the expression of *Faapx-c* was obviously diverged during fruit development and ripening. And the amount of expression of *Faapx-c* was lowest at the small green stage, gradually increased to the maximum at the full red stage, with the relative content of 0.4385, 0.5825, 0.7870, 0.9133, 1.2219, 1.3614 and 1.4055, respectively.

Key words: *Fragaria* × *ananassa* cv. Toyonaka • Ascorbate peroxidase gene cloning • Expression

INTRODUCTION

Ascorbate peroxidase (EC 1.11.1.11) is one of the key enzymes in the ascorbate-glutathione cycle that catalyses the conversion of H₂O₂ to H₂O and O₂ using ascorbate as the specific electron donor [1]. It plays an important role in scavenging and protecting cells against the toxic effects of H₂O₂ in higher plants [2]. APX isozymes are distributed in at least four distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX), mitochondrial membrane-bound APX (mitAPX) and cytosolic APX (cAPX) [3]. To date many reports on the purification, molecular cloning and physiological function of APX isoenzymes based on enzymological and molecular approaches have been published; these studies indicate that APX isoenzymes are critical components that prevent oxidative stress in photosynthetic organisms. In addition, Researchers have reported that the expression of *apx* gene is related to fruit ripening [4]. It is presumed that fruits have different physiological properties including changes of APX activities and kinds during development and ripening [5]. However, it is still very little about the research on *apx* gene in fruit during this physiological process.

Strawberry (*Fragaria* × *ananassa*) is one of the most economically important fruit tree, widely grown in all temperate regions of the world. Apart from its commercial importance, strawberry is becoming a model of choice for functional genomics approaches in the studying of Rosaceae genetics [6]. In this study, we use the most extensive cultivar (*Fragaria* × *ananassa* cv. Toyonaka) applied in forcing culture of strawberry and cloned a cDNA fragment encoding the cytosolic APX enzyme from its fruit. Further, we analyzed its expression during fruit developmental processes.

MATERIALS AND METHODS

Plant Material: Strawberry fruits (*Fragaria* × *ananassa* cv. Toyonaka) were harvested at different developmental stages: small green (SG), 7 days after fruit set. large green (LG), 15 days after fruit set; green ripe (GR), white fruits; turning red (TR), 1/4 red; half red(HR), 1/2 red; red ripe(RR), >1/2 red and full red (FR), immediately frozen in liquid nitrogen and stored at -80 °C until ready for use.

RNA Isolation and RT-PCR [7]: Total RNA was extracted from fruit samples independently using the Plant RNA_{OUT} kit, as per the manufacturer's instructions (TIANDZ, China). The first strand cDNA was synthesized

from 2µg of the total RNA by reverse transcriptase with Oligo-(dT) primer according to the instructions of the Easy-Go™ RT PreMix kit (SBS Genetech, China).

Primer Design and PCR Amplification Conditions [8]:

Based on the conserved regions of *apx* gene nucleotide sequences from *Fragaria × ananassa* (AF159630), *Malus × domestica* (EF528482) and *Vitis pseudoreticulata* (DQ150258), the gene-specific primers used for isolating *Fragaria × ananassa* cv. Toyonaka *apx* gene were designed as follows: Fa-F: 5'-GCCTGATGTTCTTCCCA-3', Fa-R: 5'-CTCTTTATGCGACGCCTA-3'. PCR was performed with a 20 µl reaction mixture containing 1 µl of first-strand cDNA, 2.0 µl of 10×PCR Buffer without Mg²⁺, 2.0mM MgCl₂, 200µM dNTPs, 0.5µM of each primers and 1.0 unit of *Taq* DNA polymerase (TIANGEN, China). The PCR procedure started with 94°C for 3min, then 35 cycles of 94°C for 1min, 53.5°C for 1min, 70°C for 2min and finally 72°C for 7 min. The PCR products were analyzed on a 1.0% agarose/EtBr gel and the corresponding DNA bands were 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).

Semi-quantitative RT-PCR Analysis: The expression levels of *apx* gene at seven stages of strawberry fruits and ripening were determined by semi-quantitative RT-PCR using the primer Fa-F and Fa-R.

The strawberry *FaGAPDH2* gene was used as a reference, the primer, fwd: 5'-CAGACTTGAGAAGAAGGCCACCTA-3', rev: 5'-GATACCCTTCATCTTCCCTCAGA-3'.

RESULTS

Cloning and Identification of cDNA Encoding *Apx* Gene[9, 10]:

In this study, a pair of specific primers was used to amplify ascorbate peroxidase gene cDNA fragment from *Fragaria × ananassa* cv. Toyonaka. PCR amplification generated DNA products with the expected size of about 350 bp (Fig. 1). The DNA fragment was cloned into pMD19-T vector (TaKaRa, China) (Fig. 2) and was identified by plasmid PCR (Fig. 1), then it was sequenced with Big Dye Terminators using a PRISM 377 Sequencer (PE Applied Biosystems, Foster City, California, USA). Sequence analysis revealed this fragment was 352 bp in length, encoding 116 amino acids (Fig. 2), which was 97%~99% identical to the sequences of *Fragaria × ananassa* Duch. cv. Yoho cytosolic

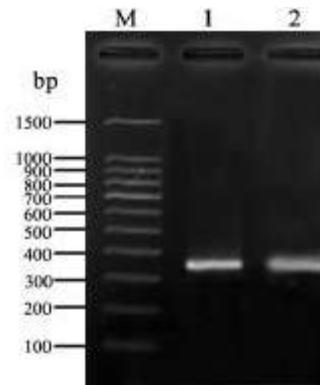


Fig. 1: Agarose gel electrophoresis of RT-PCR product and plasmid PCR product. M = DNA ladder; lane 1 indicates the RT-PCR product; lane 2 indicates the plasmid PCR product

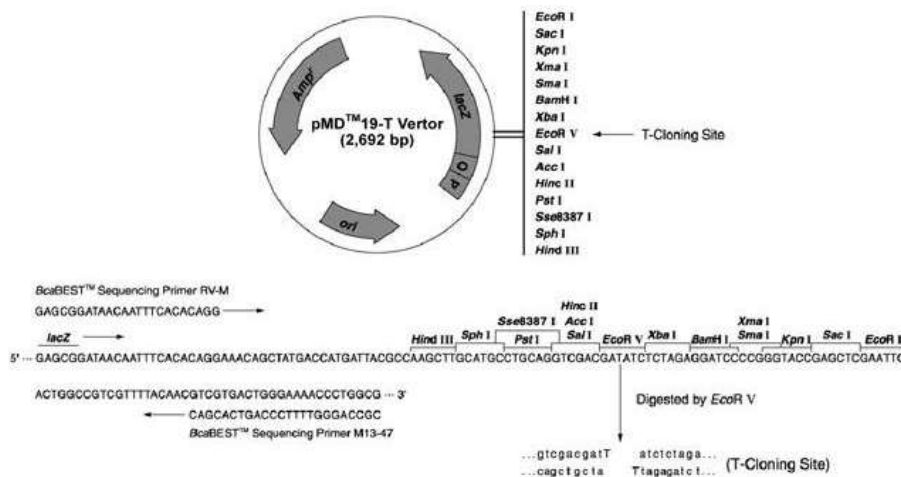


Fig. 2: The structure of pMD 19-T vector

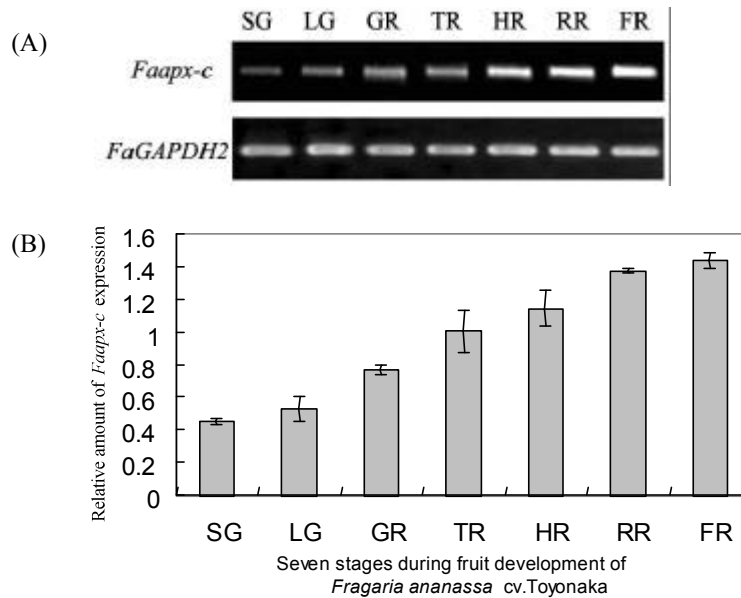


Fig. 3: Expression patterns of *Faapx-c* gene during fruit development and ripening of *Fragaria* × *ananassa* cv. Toyonaka. Fruits were sampled at the following developmental stages: SG, small green; LG, large green; GR, green ripe; TR, turning red; HR, half red; RR, red ripe and FR, full red. (A) semi-quantitative RT-PCR detection of *Faapx-c* expression in different development stages of fruit; (B) relative amount of *Faapx-c* expression in different development stages of fruit.

apx gene and 84%, 82%, 81% identity respectively with that of *Malus domestica* (EF528482), *Cucumis sativus* (D88649) and *Glycine max* (AB082931) cytosolic *apx* gene from the GenBank. Therefore, we presumed that it is a cytosolic *apx* from *Fragaria* × *ananassa* cv. Toyonaka, named *Faapx-c* and GenBank number: FJ896040.

Expression of *Faapx-c* During Fruit Development and Ripening:

To understand the expression patterns of *Faapx-c* in the process of fruit development and ripening, we selected fruit sampling of seven developmental stages, which covered the development and ripening stage of strawberries and the main parameters considered were fruit size and colour. Then semi-quantitative RT-PCR is used to analysis the expression of *Faapx-c* gene and the strawberry *FaGAPDH2* gene was used as a reference. As shown in Fig. 3, the expression of *Faapx-c* was differences obviously during fruit development and ripening. The amount of expression was lowest at the small green stage, gradually increased to the maximum at the full red stage, with the relative content of 0.4385, 0.5825, 0.7870, 0.9133, 1.2219, 1.3614 and 1.4055, respectively.

DISCUSSION

APX has been found in higher plants, algae and some cyanobacteria [11]. It is reported that the conservatism of APX is high in the process of evolution [12]. The homology of APX isoenzymes in higher plants reach to 70~90% within each group. Furthermore, the four groups of APX isoenzymes show 50~70% homology with each other [13]. In this study, we have isolated a cDNA fragment encoding cytosolic *apx* gene from *Fragaria* × *ananassa* cv. Toyonaka and also discovered that its sequences shared high similarity at nucleotide and polypeptide level with the sequences from the cytosolic *apx* gene of other plants. For instance, the nucleotide sequences of *Faapx-c* is reach up to 97%~99% identical to the sequences of *Fragaria* × *ananassa* Duch. cv. Yoho cytosolic *apx* gene. It is clear that cytosolic *apx* gene is highly conserved in the different cultivar of strawberry.

Fruit development and ripening is known to be a complex process since it undergo many physiological and chemical changes. This process is affected inevitably by oxidative stress. However, oxidative stress can induce the expression of *apx* gene. Semi-quantitative RT-PCR has

shown that the amount of expression of *Faapx-c* was lowest at the small green stage, gradually increased to the maximum at the full red stage (the amount of expression is exceed 3 fold as compared with the small green stage). This result is different from the expression patterns of *Fragaria × ananassa* Duch. cv. Yoho cytosolic *apx* gene by northern blots, in which the expression level was lowest at the small green stage, markedly increased to the maximum at the turning stage and declined a little at the full red stage [14]. And in Bell pepper, the transcript level was lowest in young green fruits, the signal remained almost constant during fruit development in mature green and orange fruits and increased about 3-4 fold up to the fully ripe stage [4]. These disparities may be derived from the differences of plant species and cultivar, as well as different growing environment and conditions.

Faapx-c was detectable at all stages of fruit development and ripening studied and the amount of expression was gradually increased as fruit ripening. It is interesting that the content of AsA is also gradually increased during strawberry fruit development and ripening [15]. There is perhaps some relationship between AsA and the expression of *apx* gene. Because APX is an capital enzyme involved in the metabolism of AsA in plant and AsA is very important to maintain stability of APX [16]. Molecular analyses of APX isoenzymes described in the AsA synthetic pathway and the AsA regenerate cycle in plant cells, has greatly increased within the past few years [17]. The combined results of studies of APX isoenzymes together with advances in knowledge of AsA metabolism will provide a fuller understanding of the physiological function of APX in plant.

ACKNOWLEDGEMENT

The present research is supported by National Natural Science Foundation of China (30671454) and Educational Commission of Sichuan Province, China (07ZZ023; 2006ZD004).

REFERENCES

1. Asada, K., 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Ann. Rev. Plant Physiol. Plant Molecular Biol.*, 50: 601-639.
2. Shigeoka, S., Y. Nakano and S. Kitaoka, 1980. Metabolism of hydrogen peroxide in *Euglena gracilis* Z by L-ascorbic acid peroxidase. *Biochemical J.*, 186: 377-380.
3. Kawakami, S., Y. Matsumoto, A. Matsunaga, S. Mayama and M. Mizuno, 2002. Molecular cloning of ascorbate peroxidase in potato tubers and its response during storage at low temperature. *Plant Sci.*, 163: 829-836.
4. Schantz, M.L., H. Schreiber, P. Guillemaut and R. Schantz, 1995. Changes in ascorbate peroxidase activities during fruit ripening in *Capsicum annum*. *FEBS Lett.*, 358: 149-152.
5. Biale, J.B., 1964. Growth, maturation and senescence in fruits: Recent knowledge on growth regulation and on biological oxidations has been applied to studies with fruits. *Sci.*, 146: 880-888.
6. Mathieu, R.G., L.K. Estelle, B. Laure, J.S. Daniel, M. Amparo, S. David, A. Pere, G. Guy and D.R. Be'atrice, 2008. Comparative Genetic Mapping Between Octoploid and Diploid *Fragaria* Species Reveals a High Level of Colinearity Between Their Genomes and the Essentially Disomic Behavior of the Cultivated Octoploid Strawberry. *Genetics Society of America*, 179: 2045-2060.
7. Jiang, H., H.R. Tang, Y.H. Gu, Y. Zhang and Y. Luo, 2008. Cloning and sequence analysis of *PGIP* gene from pear dwarf stock *S₂* (*Pyrus ussuriensis*). *J. Fruit Sci.*, 25(4): 462-466. (in Chinese).
8. Zhang, Y., H.R. Tang, Y. Luo, H. Jiang and Y.X. Hou, 2009. Cloning and sequencing of strawberry chitinase gene *CHI2*. *Res. J. Biol. Sci.*, 4(5): 624-628.
9. Su, X., J.K. Zhang and F.W. Ma, 2007. Cloning of ascorbate peroxidase cDNA from apple. *J. Northwest A and F University (Nat Sei Ed)*, 35(10): 77-80. (in Chinese).
10. Lin, K.H., H.F. Lo, C.H. Lin and M.T. Chan, 2007. Cloning and expression analysis of ascorbate peroxidase gene from eggplant under flooding stress. *Botanical Studies*, 48: 25-34.
11. Mathews, M.C., C.B. Summers and G.W. Felton, 1997. Ascorbate peroxidase: a novel antioxidant enzyme in insects. *Archive of Insect Biochem. Physiol.*, 34: 57-68.
12. Li, H.H. and Z.X. Lai, 2006. A review of progress in ascorbate peroxidase in plants. *Subtropical Plant Sci.*, 35(2): 66-69. (in Chinese).
13. Shigeoka, S., T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta and K. Yoshimura, 2002. Regulation and function of ascorbate peroxidase isoenzymes. *J. Experimental Botany*, 53: 1305-1319.
14. Kim, I.J. and W.I. Chung, 1998. Molecular characterization of a cytosolic ascorbate peroxidase in strawberry fruit. *Plant Sci.*, 133: 69-77.

15. Yao, F.R. and Y. Ren, 2007. Measure the content of vitamin C in strawberry at different ripening stages. *J. Bijie University*, 25(4): 84-88. (in Chinese).
16. Hossain, M.A. and K. Asada, 1994. Inactivation of ascorbate peroxidase in spinach chloroplasts on dark addition of hydrogen peroxide: its protection by ascorbate. *Plant Cell Physiol.*, 25: 1285-1295
17. Smirnoff, N., 2000. Ascorbic acid: metabolism and functions of a multi-faceted molecule. *Current Opinion in Plant Biol.*, 3: 229-235.