Journal of Agricultural Science; Vol. 5, No. 3; 2013
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Cloning and Expression Analysis of an Ascorbate Oxidase (AO) Gene from Strawberry (*Fragaria×ananassa* cv. Toyonaka)

Yuanxiu Lin[1], Xinxin Gu[1], Haoru Tang[1], Yanxia Hou[1], Dingqun Yu[1] & Xiaonan Zhang[1]

[1] College of Horticulture, Sichuan Agricultural University, Ya'an, China

Correspondence: Haoru Tang, College of Horticulture, Sichuan Agricultural University, Ya'an 625014, China. Tel: 86-360-826-4028. E-mail: htang@sicau.edu.cn

Received: November 9, 2012 Accepted: December 14, 2012 Online Published: February 5, 2013

doi:10.5539/jas.v5n3p14 URL: http://dx.doi.org/10.5539/ jas.v5n3p14

Abstract

In this study, a total 1737 bp ascorbate oxidase (AO) gene encoding a 578 amino acid protein was cloned from strawberry (*Fragaria×ananassa* cv. Toyonaka). Real-time quantitative expression analysis was conducted on *FaAO* gene of strawberry fruits during 7 developmental stages and in 5 strawberry tissues. The results revealed that during 7 fruit developmental stages, *FaAO* expressing level gradually increased in yonger stages, and declined dramatically in maturing stages, in which it could barely be detected. According to experimental results, we can conclude that *FaAO* expresses at a higher level in younger fruits, and lower in latter stages. This could be related to the fact that younger fruits are growing actively, and *FaAO* has a significant effect on facilitating fruit growth. Results for expression in 5 tissues indicated the highest expression was detected in flowers, and the lowest in fruit tissue (at full red stage). One of the possible reasons is that intense respiration and high ethylene synthesis occur in the mature fruits, repressing the expression of *FaAO* gene in strawberry fruits. Also, it could be a reason why the ascorbic acid (AA) content in mature fruits is higher than that in other tissues and stages. The differences of expression results among 7 developmental stages and 5 tissues indicated a novel expression pattern of *FaAO*. We propose that *FaAO* is correlated with strawberry growth and development, the depression of the *FaAO* expression can lead to apparent accumulation of AA in strawberry plant.

Keywords: ascorbate oxidase, cloning, real-time quantitative PCR, expression analysis

1. Introduction

Ascorbate oxidase (EC 1.10.3.3, AO) is a cell wall localized copper-containing enzyme, belongs to multi-copper oxidases family (Sanmartin et al., 2007). It is also a key enzyme involved in Ascorbate-Glutathione cycle (AsA-GSH cycle) (Noctor et al., 1998). In this cycle, AO catalyzes the oxidation of AA into monodehydroascorbate (MDHA), which could be later reduced to AA again by monodehydroascorbate reductase (MDHAR). However, that is not unique reaction on MDHA, since it is unstable and rapidly disproportionates to dehydroascorbate (DHA) without enzyme catalyzing. Thus it is concluded that AO plays important role in modulating and maintaining the redox state and ratio of AA pool (Pignocchi et al., 2006). Besides, it was found that depressed expression of *AO* gene promoted AA accumulation in tomato fruit (Yamamoto et al., 2005).

Ascorbic acid (AA) is one of the most abundant antioxidants existing in all subcellular compartments including the apoplast (Sanmartin et al., 2007). It is important to keep plants from injuring under stressful environment (Smirnoff, 2000). AA participates in scavenging reactive oxygen species (ROS) directly (Foyer et al., 1983), and the apoplastic AA is considered as the first wall of resisting damage from exterior oxidants like NO_2 and ozone (Barnes et al., 2002; Plöchl et al., 2000). The apoplast contains no NAD(P)H or glutathione, so the redox state of AA pool reflects the redox status of whole apoplast (Foyer et al., 2005). Pignocchi et al. (2006) showed that the redox state of the AA pool in the leaf apoplast specifically influenced plant growth, glutathione pool and expression of genes. While the balance of redox state of apoplastic AA pool would be regulated by the enzyme ascorbate oxidase (AO). Over-expression *AO* in the apoplast of transgenic tobacco decreased the AA redox states and increased plant sensitivity to ozone (Sanmartin et al., 2003b). Pignocchi (2003) reported that the changes in apoplastic AO activity in transgenic tobacco plants, had little effects on whole leaf AA content but oxidized the apoplastic AA pool. Besides, AO had an important effect on controlling plant growth and development (Pignocchi, 2003), stress responses (Sanmartin et al., 2003a), fresh fruits storage (Yan et al., 2008), gene expression (Pignocchi et al., 2006) and florescence regulation (Yamamoto et al., 2005). It also has a similar

function with auxin on facilitating cell elongation and expansion, and highly expressed in growing tissues in many cucurbits (Sanmartin et al., 2007). Moreover, the enzyme activity is in connection with disease resistance in wheat (JianRong et al., 1998) and pepper (ZeGao et al., 2003) and *AO* gene expression may decrease with fruit ripening (De Tullio et al., 2004). Recently, *AO* genes were cloned in cucumber (Ohkawa et al., 1989), melo (Sanmartin et al., 2007), pumpkin (Esaka et al., 1990) and tabacco (Kato et al., 1996) and some other plant species.

Strawberry is presently one of the most widely planted fruit tree in modern agricultural era. However, they are often hurt by randomly short time low temperature due to poor protection measure and greenhouse chilly reversal phenomenon, and because of which, abundant loss appeared in strawberry industry. Furthermore, the fruit is ripening and growing old so fast and so intolerance storage that rots shortly after ripening. The period of stay fresh would last two to three days at most , which is a trouble in strawberry fruit storage. Therefore, in this research we have cloned *AO* gene of strawberry cultivated variety 'Fengxiang' (*Fragaria×ananassa* cv. Toyonaka), analyzed its expression pattern during fruit ripening and in different tissues through real-time quantitative PCR (RT-qPCR). This would be helpful to promulgating details of its expression model, providing the theoretical basis of strawberry and other horticultural plants genetic improvement to defense environmental stresses and ensure longer fruits storage.

2. Materials and Methods

2.1 Materials

The strawberry cultivated variety 'Fengxiang' (*Fragaria×ananassa* cv. Toyonaka) was cultivated in No. 7 research area greenhouse in Sichuan Agricultural University. Fruits were collected with random sampling method and divided into seven developmental stages: small green (SG); large green (LG); white fruits (W); turning red (TR); half red (HR); red ripe (RR) and full red (FR) by different characteristics according to Yan-xia Hou (Yan et al., 2009). Fresh roots, fresh stalks, immediately unfold leaves, fully expanded petalages and full red fruits were also collected with random sampling method. All these samples were washed in freshwater for about 5 minutes to remove the soil particles adhering then frozen in liquid nitrogen immediately to keep them under -80 °C low temperature, being ready for RNA isolation.

2.2 Isolation of RNAs and First-Strand cDNA Synthesis

Total RNA from fruit sample was used for cloning and extracted by improved cetyltrimethyl ammonium bromide (CTAB) method (Qing et al., 2012). The quality of RNA sample was assayed using spectrophotometer. First-strand cDNA was synthesized using cDNA first-strand synthetize kit (RevertAid™ First Strand cDNA Synthesis Kit, Fermantas), according to the manufacturers' instructions.

2.3 Amplification for Cloning

Partial *AO* gene sequence from NCBI database was used to blast in strawberry genome database in GDR database. Then specific primers for amplification of the full-length open reading frame (ORF) of *AO* were designed by primer 6.0 software. Primers are showed below (5' to 3'), the forward primer (AOF) was ATGGCGGAAGCAGGTCATAATAAC, the reverse primer (AOR) was TTAATTTCCAGTGAGACCGCAAGC. PCR reactions were performed with 1 μl respectively of primers (10 μM) AOF and AOR, 1μl cDNA (100 ng) as the template, 10 μl PCR mixture, add ddH_2O to volume 20 μl in total. Following one denaturation step (5 minutes at 94 ℃), 35 cycles of amplification (50 seconds at 94 ℃, 50 seconds at 58 ℃, 2 minutes at 72 ℃) and one elongation step of 10 minutes at 72 ℃ were carried out. Purified product was recovered, ligated with pMD 19-T Vector (Takara), and transferred into *E. coli* JM109 cells. Then positive clones were screened with white-blue plaque selection. At last, the PCR of recombinant plasmid identification was performed with the same procedure/volume as listed above. Squencing was done by Shanghai Sangon Biological E&T and CO. Ltd.

2.4 Sequence Analysis

Sequence analysis was performed using DNAMAN software. Amino acid sequence blasting was performed using NCBI blastp online tool (http://blast.ncbi.nlm.nih.gov/Blast) and alignment with clustal Omega-multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). Molecular weight and isoelectric point were estimated using ExPASy (http://web.expasy.org/compute_pi/). Signal pepetide was forecasted using SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Polygenetic tree was constructed using clustal Omega and MEGA 5.0 software.

2.5 Expression of FaAO Gene

Total RNAs were extracted from strawberry fruits and tissues samples using the same method as described above. We chose the strawberry FaGAPDH gene as a housekeeping gene. Specific primers (AOF-113, AOR-113 and FaGAF-135, FaGAR-135) for real-time quantitative PCR were designed using beacon designer 7.0 software, according to full length *AO* gene sequence (obtained in this research) and FaGAPDH gene sequence (from GeneBank). Primers were all synthesized by Shanghai Sangon Biological E&T and Co. Ltd. The used primers for expression experiment were listed below (5' to 3'), AO-F113: GGCATTCCACTGTCACATTG, AOR-113: TGAGACCGCAAGCAAGAG,

FaGAF-135: GAGTCTACTGGAGTGTTCA, FaGA-R135: CTTGTATTCGTGCTCATTCA.

PCR reactions were performed on 96-well plates with a CFX96 real-time PCR determination system (Bio-Rad) to detect amplification of *FaAO* gene using SYBR Green. Total 20 μl reaction volume consisted of 0.6 μl of each primer (10 μM), 10μl SYBR ExTaq Mix (Takara) and 2 μl cDNA template derived from strawberry fruits and tissues samples. Reactions followed the manufacturer recommended cycling parameters: 95°C for 30 seconds, 95°C for 5 seconds, 60°C for 30 seconds, after 40 cycles insert melting curve (65°C to 95°C, 0.5/°C), and three hole repeat. The results of PCR reaction were analyzed with $2^{-\Delta CT}$ method (CT: threshold cycle, ΔCT=CT *FaAO* - CT *FaGAPDH*). The expression of housekeeping gene *FaGAPDH* was used to normalize raw data and calculate relative transcript levels. The histogram represent the results of expression were constructed using sigmaPlot 12.0 software.

3. Results

3.1 Cloning and Sequence Analysis of FaAO Gene

An expected nearly 1800 bp product was obtained with PCR amplification using the specific primer (AO-F and AO-R), this fragment was then recovered and ligated with pMD 19-T Vector. Subsequently, the recombinant plasmids were identified also using PCR method (Figure 1).

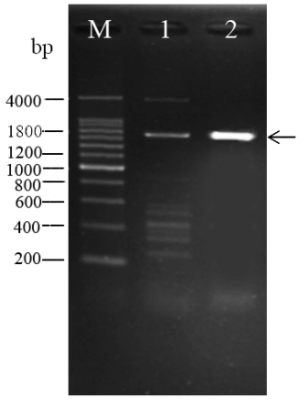

Figure 1. Products of RT-PCR amplification and recombinant plasmid PCR identification

Lane 1: product of RT-PCR. It is nearly 1800 bp product obtained by PCR amplification using the specific primers AOF and AOR. **Lane 2**: the product of plasmid PCR identification performed using the same primers (AOF, AOR) and the same PCR parameter after recovered the band of lane 1 and cloned with pMD19-T vector, transformed into *E. coli.* The plasmid used as PCR DNA template was extracted using conventional phenol-chloroform method. M: the molecular weight standard, what we used in this research is 200 bp DNA Ladder.

Sequencing result indicated the fragment we obtained was 1737 bp and encoded 578 amino acids (Figure 2). The molecular weight (MW) of this polypeptide is 63871.45 KDa and its isoelectric point (pI) is 7.74 showing alkalescency. What's more, it is an excretion protein that has a signal peptide about 27 bp (1-27) in length, and a multiple copper family signature sequence - GVWAFHCHIEPHLHMGMGVVF (536-556) - indicating it is a member of multi-copper oxidases superfamily. Further, amino acid sequence alignment was performed using NCBI blastp tool in nr database and results showed that it shared high identical with the various AO amino acid sequences of other species. The similarities were all above 70%, and 78% identity with that of *Vitis vinifera* (XP_002275678) is the highest, 75%, 73%, 72% identity respectively with that of *Populus trichocarpa*

(XP_002312838), *Ricinus communis* (XP_002530197), *Cucumis melo* (AAF35911) and *Cucumis sativus* (CBY84386) *AO* genes from GenBank. Consequently, we concluded that it is a fragment of *AO* gene cDNA from strawberry cultivated variety 'Fengxiang', named *FaAO* and the accession number is JX998186.

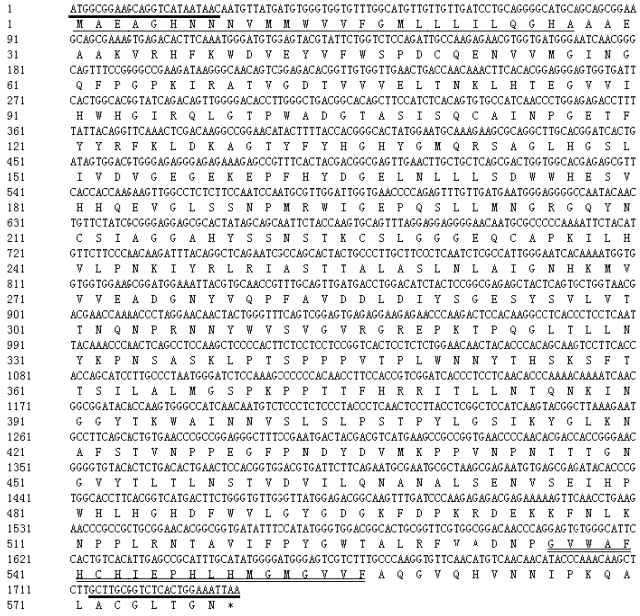

Figure 2. *FaAO* cDNA sequence and its putative amino acids

Upper: cDNA sequence of *FaAO* corrected artificially according to the map of sequencing result. Lower: deduced amino acid sequence that was 578 in length putatively translated using DNAMAN software, the "*" is a standard of terminator codon. Single regular underline: the transit peptide sequence MAEAGHNNNVMMWVVFGMLLLILQGHA (1-27). Single bold underline: the positions and sequences of primers for cloning. Double underline: the multiple copper family signature sequence GVWAFHCHIEPHLHMGMGVVF.

The alignment of 25 AO amino acid sequences was obtained by clustal W program. As shown in Figure 3, significant regions including the copper binding sites and neighbor equivalent amino acids are highly conserved. What's more, all amino acid sequences have high conserved Cys residues important to form the disulfide bridges between those domains. In strawberry, these Cys residues are Cys-50, Cys-112, Cys-211, Cys-226 and Cys-573. The phylogenetic tree of these AO amino sequences was constructed (Figure 4). As the results suggest, *FaAO* gene clustered with that from *Vitis vinifera* and tabacco was consistent with the results of homology blast in NCBI. These three species may have a nearer genetic relationship with each other, possibly because they are evolutionarily near. Since *Fragaria×ananassa* and *Vitis vinifera* are both higher plant, their *AO* genes are seemly closer in evolution than other species and plausibly familiar in response to environmental changes.

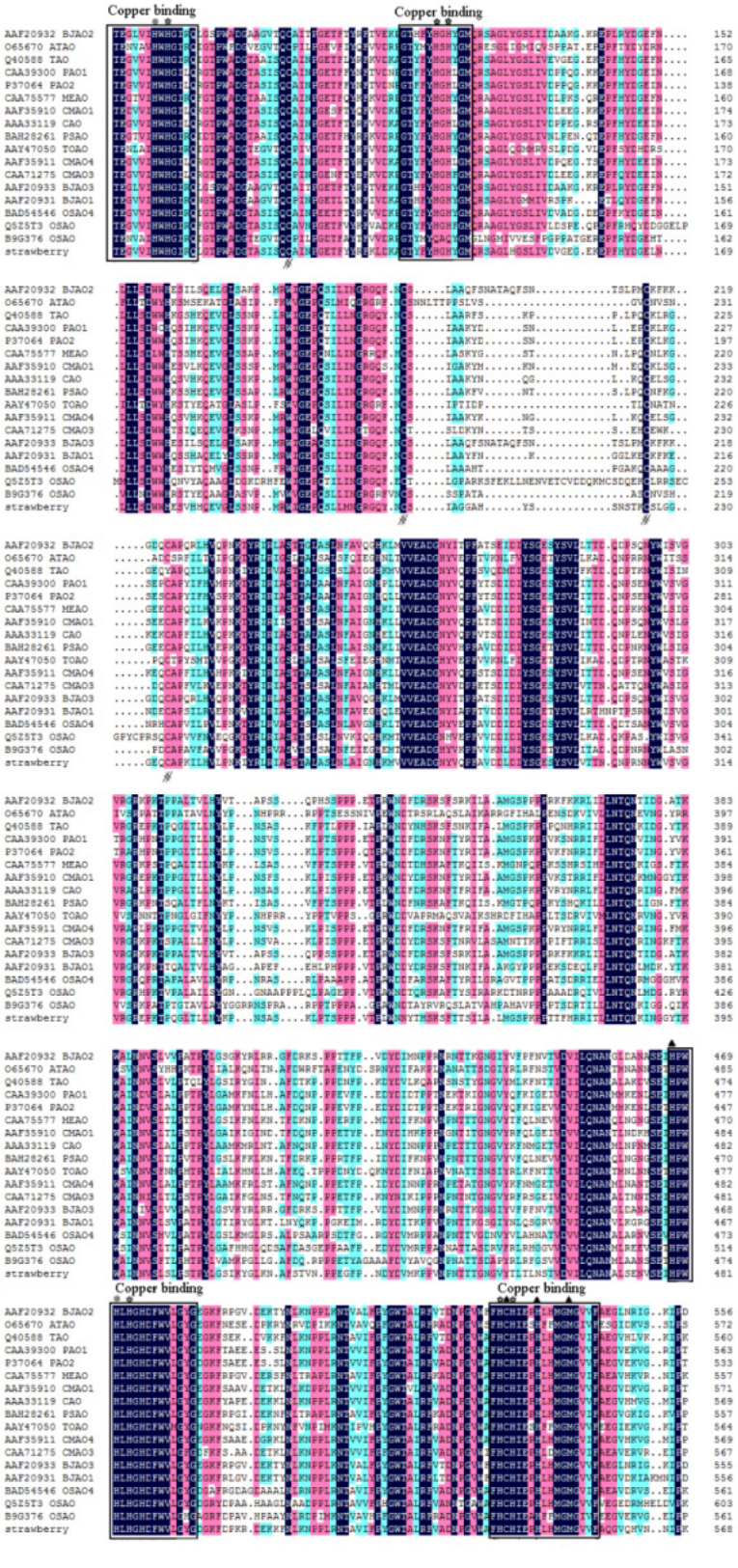

Figure 3. Comparison of AO amino acids sequences from varieties of plants

The highly conserved copper binding sites are indicated in hollow rectangle. Filled circles upon the alignment show the ligands of the type-1 copper. Asterisks upon the alignment show the ligands of the type-2 copper. Triangles upon the alignment show the ligands of the type-3 copper. Pound sign under the alignment show the Cys residues important to inform the disulfide bridges.

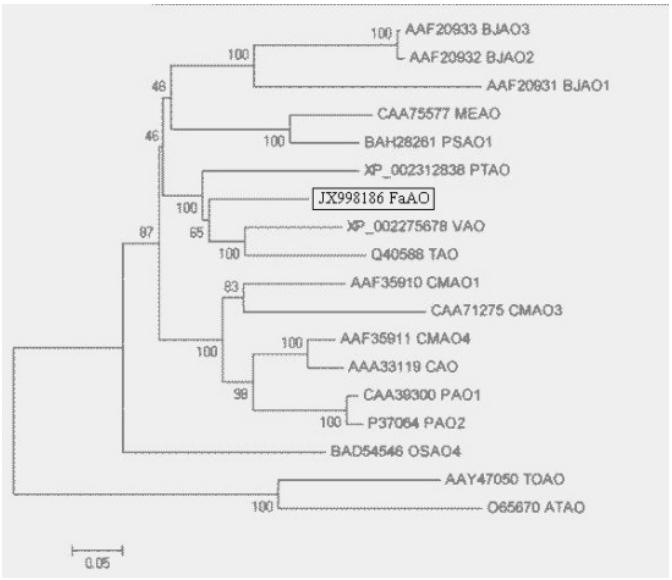

Figure 4. A phylogenetic tree was constructed with maximum likelihood method, after alignment of 25 AO amino acid sequences including different kinds of AO isoenzymes from NCBI database

The format in the picture is the accession number followed the Osmamthus fragrans. BJAO1: *brassica juncea* AO1. BJAO2: *brassica juncea* AO2. BJAO3: *brassica juncea* AO3. MEAO: *Medicago sativa* AO. PSAO1: *Pisum sativum* AO1. PTAO: *populus trichocapa* AO. VAO: *Vitis vinfera* AO. TAO: *Nicotiana tabacum* AO. CMAO1: *Cucumis melo* AO1. CMAO3: *Cucumis melo* AO3. CMAO4: *Cucumis melo* AO4. CAO: *Cucumis sativus* AO. PAO1-2: Pumpkin AO1-2. OSAO4: *Oryza sativa* AO4. TOAO: *Solanum lycopersicum* AO. ATAO: *Arabidopsis thaliana* AO. FaAO: *Fragaria×ananassa* CV. Toyonaka AO

3.2 Expression of FaAO Gene

To make it clear how *FaAO* gene express during strawberry fruits ripening and among different tissues, real-time quantitative PCR was executed to analyze the expression of *FaAO* gene using the total RNA samples extracted from five different tissues and seven developmental stages of strawberry fruits. A strawberry *FaGAPDH* gene was selected as a reference. Relative expression was calculated with $2^{-\Delta CT}$ (CT: threshold cycle, ΔCT=CT *FaAO* - CT *FaGAPDH*) method.

We can infer from the Figure 5, *FaAO* expressed differently during fruits development and ripening stages, as well as the relative expression in the different tissues. During the fruit developmental stages, *FaAO* gene fluctuates in expression level, which increased at the younger stages and reached the peak (at white fruit stage), then decreased at turning red stage, continuously at low expression level to mature stages which almost cannot be detected (Figure 5A). In the different tissues, the highest expression was detected at flowers tissue, followed the leaves, stems, roots successively and the lowest expression at full red fruits tissue (Figure 5B).

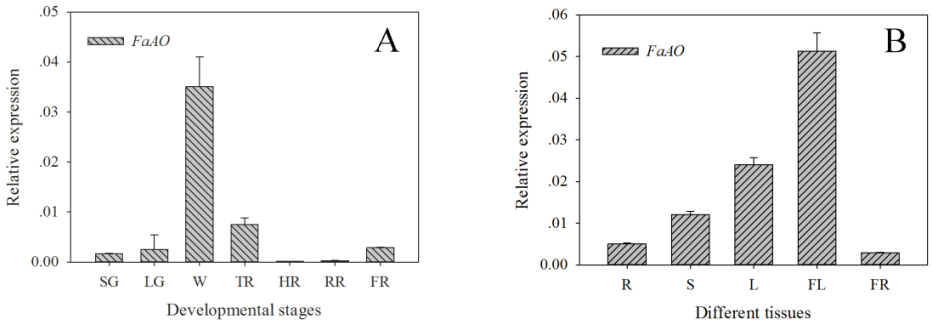

Figure 5. Changes in AO expression during strawberry development and in different tissues

(A) Expression result of FaAO during strawberry fruits development and ripening. The fruits were divided artificially into seven developmental stages. SG: small green, seven days after fruit set. LG: large green, eight days after small green. W: white fruits. TR: turning red, 1/4 area turning to red. HR: half red, 1/2 area turning to red. RR: red ripe, >1/2 area was red. FR: full red. (B) Expression result of FaAO in different tissues. R: roots. S: stems. L: leaves. FL: flowers, fully expanded petals. FR: full red fruits. Standard errors of the analysis are indicated by the vertical bars.

4. Discussion

The cDNA we obtained in this study belongs to multi-copper oxidases family, sharing relativly high similarity at nucleotide and polypeptide level with *AO* genes from other plant species. What we have cloned is *FaAO* gene according to the resonable sequence analysis above. The results of comparison at polypeptide level suggests that *AO* gene from strawberry is relativly conserved since they share above 70% similarity even from different species plants.

As mentioned in introduction, AO catalyzes the oxidative reaction of AA and plays an important role on regulation of the redox state and content of AA pool (Sanmartin et al., 2003a; Zhang et al., 2011). With increasing research on the function of AO, an opinion prevailed that AO controls cell expansion during plant development and growth, although the actual mechanism of its action remains unknown (Kato et al., 2000; Lin et al., 1991; Mertz, 1961). The expression analysis of *CmAO1* exhibited that the transcript abundance declined in young green fruit, while the expression increased during the fruit development and growth, reaching the peak at the outer mesocarp, and declined in over-ripe fruit (Sanmartin et al., 2007). On contrast, the results in our research shows relative expression gradually increased in former stages, while declined dramatically once the fruits are mature and almost cannot be detected at half red and ripe red stages, then it increased again in full red fruits. This Indicats *FaAO* expressed differently with the *CmAO1* in melo. Diallinas et al. (1997) observed high transcription level of the *AO* gene in preclimacteric melon fruits and subsequent repression in the climacteric stage. However, in strawberry the relative expression at turning red stage is higher than the expression at small green stage and large green stage. This possibly results from the higher content of AA in turning red stage than that in small and large green stages. AA is the substrate of the oxidation reaction catalyzed by the enzyme AO, provoking a up-regulation of *AO* expression at turning red stage.

In different tissues, *CmAO1* expressed higher in petals, but the expression was so low in other tissues, while in roots and stems, the expression was scarcely detectable (Sanmartin et al., 2007). In strawberry, the transcript level of *AO* gene is highest in floral tissue, which is the same as *CmAO1*. But the difference is that, in strawberry, a certain amount of expression in roots, stems and leaves can also be detected, the lowest expression was detected in mature fruit, which might because mature fruits hold intense respiration and high ethylene synthesis level, needing more AA content and leading the repression of *FaAO*.

Several researches have been already done to explain AO functions. Gene expression analysis showed *AO* gene is induced by light (De Tullio et al., 2007; Pignocchi, 2003). Overexpression of *AO* gene in the apoplast of transgenic tabacco changed the redox state of AA and increased sensitivity to ozone and salt stress (Balestrini et al., 2012). In current research, expression of *FaAO* in leaves was also detected under 4 ℃ low temperature. The results suggested the expression under low temperature was not higher than normal, on contrast, the expression is slightly lower than normal (data not shown). So we can infer that, *FaAO* is not induced by low temperature, and may depressed under this low temperature stress. Pignocchi found that Increasing the activities of apoplastic AO enzyme in transgenic tobacco plants along with increasing growth (Pignocchi, 2003). And frequently,

researches on AO reported in plant development and growth is correlated with auxin. The analysis of promoter in *AO* gene from pumpkin, indicated there was a cis-acting region upstream that involved in response to auxin (Kisu et al., 1997). And there are already many experiments were done to investigate and explain that AO is closely correlative to auxin (Pignocchi, 2003, 2006). Ethylene, well known as phytohormone, involved in important signal transduction pathways, and AA is specifically required for ethylene synthesis. In our research, *FaAO* expressed lower in mature fruits tissue in which the high ethylene synthesis level occurs, indicates that in full red fruit, high ethylene content is from the high content of AA, which requires the depression of *AO* gene causing the lower expression in mature fruits tissue. All the data may show that *AO* expression is not only correlated with auxin, but also has relationship with other plant hormone like ethylene. However, the details are still unclear. Cloning of *AO* gene from strawberry would be helpful to make the structure forecast of AO protein. In addition, the expression of *FaAO* gene in different tissues and during fruits development stages lie a foundation for further proclaiming the role of AO, if it is correlated with phytohormone and how it works.

5. Conclusion

We have cloned the *AO* gene from strawberry successfully by RT-PCR and Analyzed the expression of this gene during fruits ripening in different tissues. Further, we conducted the analysis on AO functions according to our results and former researches.

Acknowledgements

We sincerely thank for the real-time PCR technical support provided by the College of Animal Science in Sichuan Agricultural University.

References

Balestrini, R., Ott, T., Guther, M., Bonfante, P., Udvardi, M. K., & De Tullio, M. C. (2012). Ascorbate oxidase: The unexpected involvement of a 'wasteful enzyme' in the symbioses with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi. *Plant Physiological Biochemistry, 59*, 71-79. http://dx.doi.org/10.1016/j.plaphy.2012.07.006

Barnes, J., Zheng, Y., & Lyons, T. (2002). Plant resistance to ozone: the role of ascorbate. *Air Pollution and Plant Biotechnology. Springer-Verlag, Tokyo*, 235-254. http://dx.doi.org/10.1007/978-4-431-68388-9_12

De Tullio, M., Liso, R., & Arrigoni, O. (2004). Ascorbic acid oxidase: an enzyme in search of a role. *Biologia Plantarum, 48*(2), 161-166. http://dx.doi.org/10.1023/B:BIOP.0000033439.34635.a6

De Tullio, M. C., Ciraci, S., Liso, R., & Arrigoni, O. (2007). Ascorbic acid oxidase is dynamically regulated by light and oxygen. A tool for oxygen management in plants? *Journal of Plant Physiology, 164*(1), 39-46. http://dx.doi.org/10.1016/j.jplph.2005.09.016

Diallinas, G., Pateraki, I., Sanmartin, M., Scossa, A., Stilianou, E., Panopoulos, N. J., & Kanellis, A. K. (1997). Melon ascorbate oxidase: cloning of a multigene family, induction during fruit development and repression by wounding. *Plant Molecule Biological, 34*(5), 759-770. http://dx.doi.org/10.1023/A:1005851527227

Esaka, M., Hattori, T., Fujisawa, K., Sakajo, S., & Asahi, T. (1990). Molecular cloning and nucleotide sequence of full‐length cDNA for ascorbate oxidase from cultured pumpkin cells. *European Journal of Biochemistry, 191*(3), 537-541. http://dx.doi.org/10.1111/j.1432-1033.1990.tb19154.x

Foyer, C., Rowell, J., & Walker, D. (1983). Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. *Planta, 157*(3), 239-244. http://dx.doi.org/10.1007/BF00405188

Foyer, C. H., & Noctor, G. (2005). Oxidant and antioxidant signalling in plants: a re‐evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell and Environment, 28*(8), 1056-1071. http://dx.doi.org/10.1111/j.1365-3040.2005.01327.x

Jian, R. X., & Hua, Z. Y. (1998). Ascorbic oxidase with wheat resistance to Fusarium head blight relations. *Journal of Sichuan Agricultural Universit, 16*(3), 319-321.

Kato, N., & Esaka, M. (1996). cDNA cloning and gene expression of ascorbate oxidase in tobacco. *Plant Molecule Biological, 30*(4), 833-837. http://dx.doi.org/10.1007/BF00019015

Kato, N., & Esaka, M. (2000). Expansion of transgenic tobacco protoplasts expressing pumpkin ascorbate oxidase is more rapid than that of wild-type protoplasts. *Planta, 210*(6), 1018-1022. http://dx.doi.org/10.1007/s004250050712

Kisu, Y., Harada, Y., Goto, M., & Esaka, M. (1997). Cloning of the pumpkin ascorbate oxidase gene and analysis of a cis-acting region involved in induction by auxin. *Plant and Cell Physiology, 38*(5), 631-637. http://dx.doi.org/10.1093/oxfordjournals.pcp.a029214

Lin, L. S., & Varner, J. E. (1991). Expression of ascorbic acid oxidase in zucchini squash (*Cucurbita pepo* L.). *Plant Physiology, 96*(1), 159-165. http://dx.doi.org/10.1104/pp.96.1.159

Mertz, D. (1961). Distribution and cellular localization of ascorbic acid oxidase in the maize root tip. *American Journal of Botany, 48*(5), 405-413. http://dx.doi.org/10.2307/2439333

Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual review of plant biology, 49*(1), 249-279. http://dx.doi.org/10.1146/annurev.arplant.49.1.249

Ohkawa, J., Okada, N., Shinmyo, A., & Takano, M. (1989). Primary structure of cucumber (Cucumis sativus) ascorbate oxidase deduced from cDNA sequence: homology with blue copper proteins and tissue-specific expression. *Proceedings of the National Academy of Sciences, 86*(4), 1239-1243. http://dx.doi.org/10.1073/pnas.86.4.1239

Pignocchi, C. (2003). The Function of Ascorbate Oxidase in Tobacco. *Plant Physiology, 132*(3), 1631-1641. http://dx.doi.org/10.1104/pp.103.022798

Pignocchi, C., Kiddle, G., Hernandez, I., Foster, S. J., Asensi, A., Taybi, T., … Foyer, C. H. (2006). Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. [Research Support, Non-U.S. Gov't]. *Plant Physiological, 141*(2), 423-435. http://dx.doi.org/10.1104/pp.106.078469

Plöchl, M., Lyons, T., Ollerenshaw, J., & Barnes, J. (2000). Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. *Planta, 210*(3), 454-467. http://dx.doi.org/10.1007/PL00008153

Qing, C., Yu, H., Wang, X., Xie, X., Yue, X., & Tang, H. (2012). An alternative cetyltrimethylammonium bromide-based protocol for RNA isolation from blackberry (*Rubus* L.). *Genet Molecule Research, 11*(2), 1773-1782. http://dx.doi.org/10.4238/2012.June.29.10

Sanmartin, M., Drogoudi, P. A., Lyons, T., Pateraki, I., Barnes, J., & Kanellis, A. K. (2003a). Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. *Planta, 216*(6), 918-928.

Sanmartin, M., Drogoudi, P. D., Lyons, T., Pateraki, I., Barnes, J., & Kanellis, A. K. (2003b). Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. *Planta, 216*(6), 918-928. http://dx.doi.org/10.1007/s00425-006-0399-5

Sanmartin, M., Pateraki, I., Chatzopoulou, F., & Kanellis, A. K. (2007). Differential expression of the ascorbate oxidase multigene family during fruit development and in response to stress. *Planta, 225*(4), 873-885. http://dx.doi.org/10.1007/s00425-006-0399-5

Smirnoff, N. (2000). Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current Opinion in Plant Biology, 3*(3), 229-235. http://dx.doi.org/10.1016/S1369-5266(00)80070-9

Yamamoto, A., Bhuiyan, M. N. H., Waditee, R., Tanaka, Y., Esaka, M., Oba, K., … Takabe, T. (2005). Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and Arabidopsis plants. *Journal of Experimental Botany, 56*(417), 1785-1796. http://dx.doi.org/10.1093/jxb/eri167

Yan, G., Jie, Z., Zicheng, X., & Shuichenga, Z. (2008). Progress of ascorbic acid oxidase in plant science. *Chinese Agricultural Science Bulletin, 24*(3), 196-199.

Yan, X. H., Hao, R. T., Yong, Z., Ya, L., & Qing, C. (2009). Cloning and expression analysis of ascorbate peroxidase gene during fruit development and ripening in *Fragaria* × *ananassa* cv. Toyonaka. *World Journal of Agricultural Sciences, 5*(6), 675-679.

Ze, G. Y., & Li, Y. Z. (2003). A preliminary study of the wheat ascorbic oxidase activity and disease. *Hubei Agricultural Sciences,* (001), 31-33.

Zhang, Y., Li, H., Shu, W., Zhang, C., Zhang, W., & Ye, Z. (2011). Suppressed expression of ascorbate oxidase gene promotes ascorbic acid accumulation in tomato fruit. *Plant Molecular Biology Reporter, 29*(3), 638-645. http://dx.doi.org/10.1007/s11105-010-0271-4