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METABOLIC MECHANISM OF ASCORBIC ACID IN *ACTINIDIA CHINENSIS* VAR. *CHINENSIS*

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Abstract

Ascorbic acid (AsA) concentration was determined in *Actinidia chinensis* var. *chinensis* 'Jinyang' and 'Hort16A' during fruit development. AsAconcentration was higher at early stage of fruit development, and then declined with fruit development. The expressions of L-Galactose pathway genes were detected and the results showed *GDP-D-mannose pyrophosphorylase 1*(*GMP1*), *GDP-D-mannose-3, 5-epimerase 1*(*GME1*) and *L-galactose dehydrogenase 1*(*GDH1*) genes transcripts were declined with fruit development. Statistically significant correlations analysis results showed that there were significant positive correlation for *GMP1*, *GME1* and *GDH1* expressions with AsA concentration, and the expression level relations between each pair of *GMP1*, *GME1*, *GDH1* are significant positive correlationin these two species, suggesting that *GMP1*, *GME1* and *GDH1* in L-Galactose pathway play important roles in AsA accumulation in *A. chinensis* var. *chinensis*. The trend of *L-ascorbate oxidase 1* (*AO1*) gene transcript was corresponding with the AsA concentration, and there was a significant positive correlation between *AO1* expression and AsAconcentrationin these two species. The expression of four *Monodehydroascorbate reductase* (*MDHAR*) genes and one *Dehydroascorbate reductase* (*DHAR*) gene were different between two *A. chinensis* var. *chinensis* varieties, predicting that *DHAR* or *MDHAR* genes might not key genes for AsA biosynthesis in *A. chinensis* var. *chinensis*.

Key words: *Actinidia chinensis* var. *chinensis*; Ascorbic acid; L-Galactose pathway; Recycling pathway; Gene expression.

Introduction

Ascorbic acid (AsA) also known as vitamin C, is synthesized in plant. Fruit and vegetables, which contain relatively high AsA, are the main dietary sources of AsA for humans. AsA is an enzyme cofactor in photosynthesis, and is vital for cleaning the free radicals (Bulley *et al.*, 2009). Previous reporter showed that AsA controls cell division and affects cell expansion (Smirnoff & Wheeler, 2000).AsAcontents not only act to regulate defense and survival but also act via phytohormones to modulate plant growth under optimal conditions (Pastori, 2003). Furthermore, AsA is not only essential for fruit ripening in climacteric fruit (Green & Prof, 2005; Moori & Eisvand, 2017), but also play a key role in plant fight against various biotic and abiotic stresses (Venkatesh & Park, 2014).

The AsA biosynthetic pathways include L-galactose, D-galacturonate, L-glucose, and *myo*-inositol pathway in plants (Bulley *et al.*, 2009).The L-galactose pathway has been suggested to be the chief AsA biosynthetic route in many plant species (Valpuesta & Botella, 2004). L-galactose-1-phosphate-phosphatase (GPP), GDP mannose-3, 5-epimerase (GME) and GDP-L-galactosephosphorylase (GGP) in the L-galactose pathway are key regulators of AsA accumulation in fruits (Bulley *et al.*, 2009; Gilbert *et al.*, 2009; Ioannidi *et al.*, 2009; Mellidou *et al.*, 2012). Furthermore, the AsA recycling pathway also plays an important role in the regulation of AsA accumulation in plants (Chen *et al.*, 2003). Kiwifruit contain high concentration of AsA (Bulley & Laing, 2016). However, little research about AsA biosynthetic was done in kiwifruit, and the AsA biosynthetic mechanism was not yet clear.

Actinidia chinensis var. *chinensis*'Hort16A' and 'Jinyan' are the major cultivated varieties in the world. 'Hort16A', introduced into New Zealand from China by the Department of Scientific and Industrial Research, was bred from germplasm (Huang, 2016). 'Jinyang' is a superior, yellowed-fleshed kiwifruit cultivar selected from F1 seedlings resulting from interspecific hybridization between *A. eriantha* and *A. chinensis* var. *chinensis*. In this study, the AsA concentration was determined with fruit development in 'Hort16A' and 'Jinyang'. The expressions of corresponding L-Galactose pathway and recycling pathway genes were performed to investigate the AsA biosynthetic mechanism in *A. chinensis* var. *chinensis*.

Materials and Methods

Plant material and harvest dates: *A. chinensis* var. *chinensis* 'Jinyang' and 'Hort16A' vines were grown at Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (32° 18' N 118°52' E). Fruits were collected starting from 19 May 2016 (20 Days after anthesis for 'Jinyang' and 30 DAA for 'Hort16A') with three fruits from each of ten vines sampled about biweekly intervals during 2016. Fruits were matured and harvested at 14 Sep. (138DAA) for 'Jinyang' and 7 Sep. (141 DAA) for 'Hort16A'. For postharvest treatments, fruits from 10 vines were stored at a container with the temperature of 23 ± 2ºC. Samples were collected 7 days when fruit was softened and edible. Kiwifruit fruit flesh of each sample was separated from ten fruits, snap frozen in liquid nitrogen and stored -80°C for later experiments.

Fruit firmness, soluble solids content and AsA concentration measurement: Fruit firmness was assessed on a 1-mm thick slice of skin and on the outer

pericarp (OP) at two locations, 90° to the fruit equator, using a Fruit Texture Analyser (GY-4, China), with a 7.9-mm probe, operating at 20 mm s^{-1}. A refractometer (WYT-4, China) was used to determine the soluble solids content in juice taken from both ends of the fruit.

AsA concentration was detected using HPLC technique according to Krupa *et al*., (2011). 10 g sample of fruits was used to extract AsA with the mixture of 3% (w/v) meta-phosphoric (20 mL). A sample achieved from the extraction was purified with the Schoot's filter. AsA was determined by PerkinElmer series 200 HPLC with Diode Array Detector (UV-DAD), and the mobile phase was a 0.1% meta-phosphoric acid. AsA was of HPLC grade and purchased at Sigma. The AsAwas identified on the basis of a standard and expressed in mg/100 g FW. The values of AsA content were calculated using the data from three independent measurements.

Quantitative real time PCR (qRT-PCR): Total RNA was isolated from kiwifruit samples using the cetyltrimethylammonium ammonium bromide (CTAB) method (Tong *et al.*, 2012). The cDNA was synthesized with 1 µg total RNA using PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Code No. RR047A, Daliang, China) according to the manufacturer's instructions.

The AsA biosynthesis genes sequences were download from Kiwifruit Genome Database (http://bioinfo.bti.cornell.edu/cgi-bin/kiwi/home.cgi) according to the report by Huang *et al*., (2013). Gene primers were designed for each gene using the Beacon Designer (Table 1). A single PCR fragment of the expected size was amplified, suggesting that the primers were suitable for qRT-PCR analyses. The resulting PCR product was cloned and sequenced to confirm the expected fragment of the target gene. All samples were harvested, and three biological replicates were run independently. The qRT-PCR was carried out on an Applied Biosystems 7300 Real Time PCR System with SYBRPremix Ex Taq (Perfect Real Time) (TaKaRa Code: DRR041A) according to the method descripted by Zhang *et al*., (2012). Kiwifruit *actin* was used as the housekeeping gene to monitor cDNA abundance (Yin *et al.*, 2012). All samples were examined in triplicate. The relative levels of genes to control *Actin* mRNAs were analysed using the 7300 system software and the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Statistical analysis: Experimental datas were evaluated using analysis of variance (ANOVA) and significant differences among the means of three replicates ($p<0.05$) were determined by Duncan's multiple range tests using the "SPSS 16.0 for Windows" (Chicago, IL, USA). A Pearson's correlation coefficient, r, test was carried out on all the qRT-PCR data to find statistically significant correlations between gene expression and total chlorophyll, carotenoid or AsA content using the "SPSS 16.0 for Windows".

Table 1. Primers used for the paper.

Gene name	Kiwifruit ID	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
PGI1	Achn087691	AACCTGTTGAACCATTGACACTTG	TTGATGCTACGAGGCGAACC
PGI2	Achn197361	CTCTTATCTGTGACACGGAGCAATG	GTGAGTAATCCAATAGCATCCCATCG
PMI1	Achn330131	TTCACCGAACTCATGTCTGCTAG	CTTATCCGTCAACTGCCTCACC
PMM1	Achn302501	TCACAGGCAGGTCCAGTCTC	AAGTGTAGGCAGCAGCAATCTC
GMP1	Achn055281	GGTGGATGAGACCGCAACAATC	GGTTGAGTGCCAGCCGATAATG
GME1	Achn030021	TGGAAAGGTGGAAGGGAGAAAGC	ATGAAGGTGAAAGATCGGGTTTGC
GGP1	Achn155031	GAGGGTGAAAGAGGTTGTTGGTG	CGCAAGCAGTGACATCGTAGC
GGP2	Achn339231	AACAGAGCAACGATAGCAAATCCC	GAGGCAAGCAGTCAAGAACACG
GPP1	Achn262331	CTCAGAGTTCCTCGCCATTGC	GCCCTTATGCTCCACATGCTTG
GPP2	Achn341581	ACTGAACCTTTGTGGGATTGC	CGCTGATGTCAAATTCTTTACCG
GDH1	Achn334011	GCTTTGATTTCAGTGCCGAGAGAG	GGGAGTCCTGTAATACCAATAAACCG
GalLDH1	Achn136491	TTAGGCTGGAGTGATGAGATTCTGG	TCATACTGGGCTTTGTTAAGGTTCC
AO1	Achn228031	ACGACTTCTGGGTGTTGGGATAC	AGGCTCTATGTGGCAGTGGAATG
AO2	Achn230561	AATGCCAACACAATGAATCCCAAC	CTCATAGCAGTCCAGCCGTAGG
APX1	Achn315041	CTCCGCTTATGCTCCGTCTC	ACCTCCAGTCTTTGTCGTCAC
APX2	Achn289741	GCTCTCATCTCCACCAAGAATTGC	TGACCTCAACTGCCACAACACC
APX3	Achn207061	GAACTTCTGAATGAGTCGGAGGAG	ACAAGAGGACGATGGAGTGAACC
DHAR1	Achn224231	ACCTTTGGTAACACCGCCTGAG	ATGCTTGCTCTGTTCCATTGCTG
MDHAR1	Achn005611	GTGGTTGGTGGTGGTTACATTGG	TCGGCGAGGGAAGGAGTAAAC
MDHAR2	Achn132811	AGTGGTGGTGGTTGGTGGTG	GGCGAGGGAAGGAGTAAACAATC
MDHAR3	Achn075231	GGAGGAGGATACATCGGTCTTGAG	GCGTTAAACCCAACAGCCACAG
MDHAR4	Achn297231	AGTCAGGAACCAGAACCAGAACC	CCGATGCTGCCACAATAACACC
Actin		TGCATGAGCGATCAAGTTTCAAG	TGTCCCATGTCTGGTTGATGACT

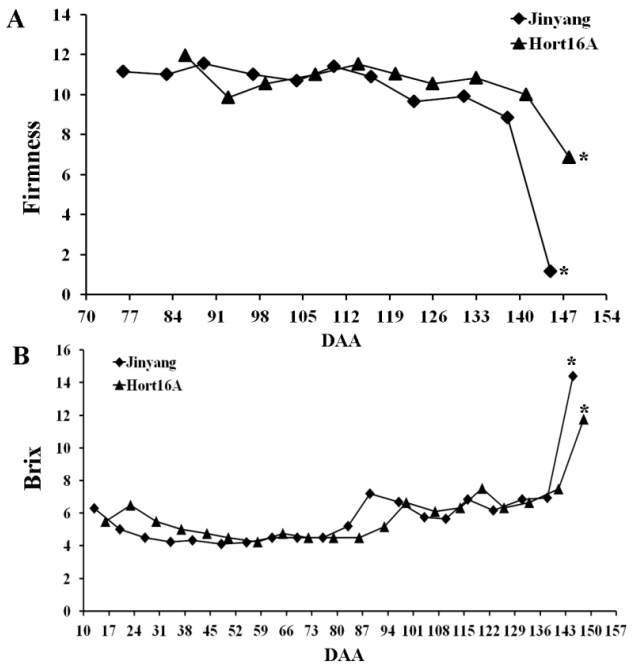

Fig. 1. Changes of firmness (A) and soluble solids content (B) in *A. chinensis* var. *chinensis* 'Jinyang' and 'Hort16A' during fruit development. Each value is presented as the mean ± standard deviation (n=10). Experimental data were evaluated using analysis of variance (ANOVA) and significant differences among the means of three replicates (p<0.05) were determined by Duncan's multiple range tests, using the "SPSS 16.0 for Windows". The * indicate the significant difference at 0. 05 level.

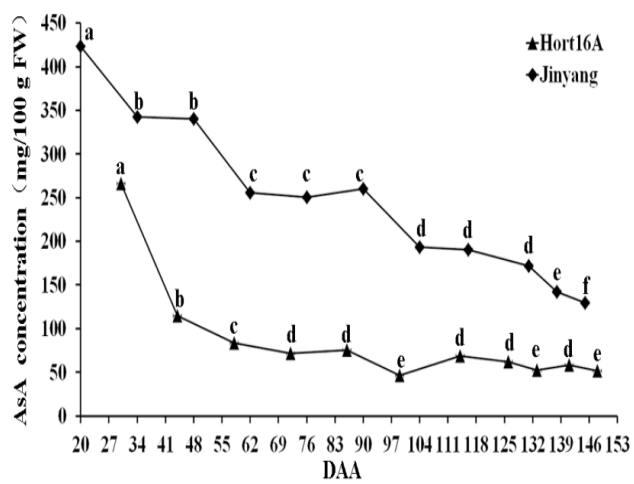

Fig. 2. Change of ascorbic acid (AsA) concentration during *A. chinensis* var. *chinensis* 'Jinyang' and 'Hort16A' fruit development. Each values is presented as mean ± standard deviation (n=10). Experimental data were evaluated using analysis of variance (ANOVA) and significant differences among the means of three replicates (p<0.05) were determined by Duncan's multiple range tests, using the "SPSS 16.0 for Windows".The different small letters in the same variety indicate the significant difference at 0. 05 level.

Results

The changes of fruit firmness, soluble solids content and AsA concentration in 'Jinyang' and 'Hort16A' during fruit development: Fruit flesh firmness measurements were started at 76 DAA for 'Jinyang' and 86 DAA for 'Hort16A'. No change in flesh firmness was observed before fruit harvest, and firmness decreased rapidly at 7 d after harvest for these two species (Fig. 1A). There was a significant increase in the soluble solids content in the fruit as the fruit ripening (Fig. 1B).

AsAconcentrations were higher at early stage of fruit development, 424.80 mg/100 g FW at 20 DAA in 'Jinyang' (Fig. 2) and 265.94 mg/100 g FW at 30 DAA in 'Hort16A' (Fig. 2). AsAconcentration was declined gradually until fruit ripening (138DAA, 142.10 mg/100 g FW) and softening (145 DAA, 129.26.10 mg/100 g FW) in 'Jinyang' (Fig. 2). However, AsA concentrationwas declined rapidly until 58 DAA in 'Hort16A' (83.91 mg/100 g FW), and then stable. AsA concentration was 68.87 mg/100 g FW at the edible period (153 DAA). Although the concentrations of AsA were declined with fruit development, the declined patterns were different betweenthese two varieties (Fig. 2).

The expressions of L-Galactose pathway gene members in *A. chinensis* var. *chinensis*: To study the AsA biosynthesis mechanism, the highest point, middle point, fruit mature point and fruit edible (soften) point were selected according to the content of AsA with fruit development. Twelve genes involving in L-Galactose pathway of AsA biosynthesis were analyzed using qRT-PCR in 'Jinyang' (Fig. 3) and Hort16A (Fig. 4). The expressions of the *GDP-D-mannose pyrophosphorylase 1* (*GMP1*), *GME1*, *L-galactose dehydrogenase 1* (*GDH1*), and *L-galactono-1*, *4-lactone dehydrogenase1* (*GalLDH1*) showed high similar patterns with high expression at early stage of fruit development (20 DAA) but decreasing rapidly in 'Jinyang' fruit (Fig. 3). The expression of *glucose-6-phosphate isomerase 1* (*PGI1*) did not change obviously during fruit development. The expression of *PGI2, pectinesterase 1* (*PMI1*) and *GGP2* were upregulated at 76 DAA, and decreased up to fruit softening. *GPP1* and *GPP2* transcripts were upregulated at 76 DAA, and decreased at fruit mature, then not obvious change with fruit softening. *phosphomannomutase 1* (*PMM1*) and *GGP1* were decreased at 76 DAA, but increased at fruit mature, then decreased with fruit softening (Fig. 3).

In 'Hort16A', the expressions of the *PMI1*, *PMM1*, *GMP1*, *GME1* and *GDH1* showed high similar patterns with high expression at early stage of fruit development (30 DAA) but decreased rapidly up to fruit ripening (148 DAA) and then stable (Fig. 4). *GGP1* and *GalLDH1* transcripts were declined until fruit mature and then increased with fruit softening. *PGI1* and *GGP2* transcripts were not obviously change during fruit maturing, but then increased with fruit softening. The expression of *PGI1* and *GPP2* were not obviously change during fruit development (Fig. 4).

Statistically significant correlations between gene expression and total AsA concentration with fruit development in 'Jinyang' (Table 2) and 'Hort16A' (Table 3) were analyzed, and the results showed that there were significant positive correlation for *GMP1*, *GME1* and *GDH1* expression with AsAconcentration, and the expression level relations between each pair of *GMP1*, *GME1*, *GDH1* were significant positive correlationin these two species (Table 2).

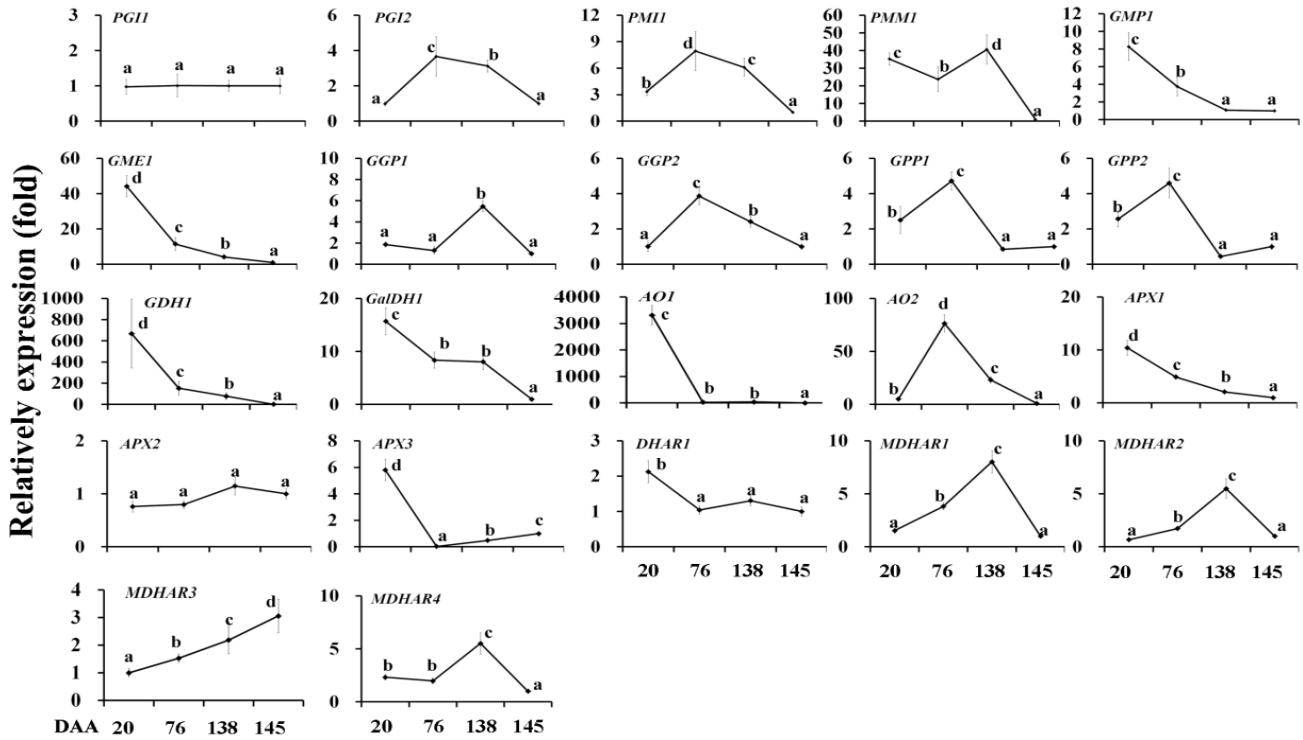

Fig. 3. The expression of AsA biosynthesis and recycling pathway genes during fruit development in 'Jinyang'. *AO*, *L-ascorbate oxidase*; *APX*, *L-ascorbate peroxidase*; DAA, Days after anthesis; *DHAR*, *dehydroascorbatereductase*;*GalLDH*, *L-galactono-1,4-lactone dehydrogenase*; *GDH*, *L-galactose dehydrogenase*; GGP, *GDP-L-galactosephosphorylase*; *GME*, *GDP-D-mannose-3,5-epimerase*; *GMP*, *GDP-D-mannose pyrophosphorylase*; *GPP*, *L-galactose-1-phosphate phosphatase*; *MDHAR*, *monodehydroascorbatereductase*; *PGI*, *glucose-6-phosphate isomerase*; *PME*, *pectinesterase*; *PMI*, *mannose-6-phosphate isomerase;PMM*, *phosphomannomutase*. Error bars indicate standard error (n = 3). Experimental data were evaluated using analysis of variance (ANOVA) and significant differences among the means of three replicates (p<0.05) were determined by Duncan's multiple range tests, using the "SPSS 16.0 for Windows". The different small letters indicate the significant difference at 0. 05 level.

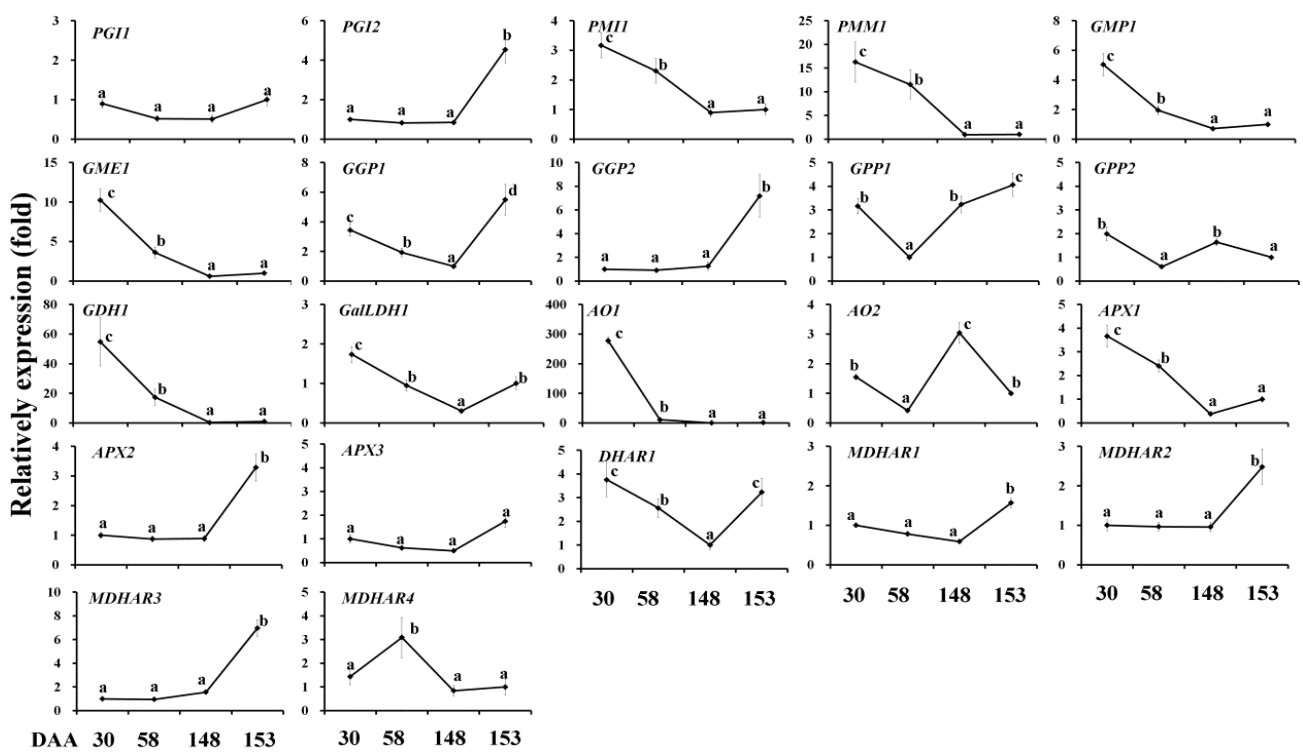

Fig. 4. The expression of AsA biosynthesis and recycling pathway genes during fruit development in 'Hort16A'. Error bars indicate standard error (n = 3). Experimental data were evaluated using analysis of variance (ANOVA) and significant differences among the means of three replicates (p<0.05) were determined by Duncan's multiple range tests, using the "SPSS 16.0 for Windows". The different small letters indicate the significant difference at 0. 05 level.

Table 2. Pearson's correlation (r) comparing relative gene expression over fruit development to AsA concentration in 'Jinyang'.

	PGI1	***PGI2***	***PMI1***	***PMM1***	***GMP1***	***GME1***	***GGP1***	***GGP2***	***GPP1***	***GPP2***	***GDH1***	***GalLDH1***	***AO1***	***AO2***	***APX1***	***APX2***	***APX3***	***DHAR1***	***MDHAR1***	***MDHAR2***	***MDHAR3***	***MDHAR4***
AsA concentration	-0.824	-0.289	0.046	0.419	**0.999****	**0.981****	-0.302	-0.163	0.474	0.521	**0.972***	0.894	**0.918***	-0.018	**0.997****	-0.834	0.855	0.855	-0.401	-0.523	-0.859	-0.205
	0.088	0.355	0.477	0.291	**0.000**	**0.009**	0.349	0.418	0.263	0.239	**0.014**	0.053	**0.041**	0.491	**0.001**	0.083	0.073	0.073	0.299	0.239	0.070	0.398
PGI2	0.648																					
	0.176																					
PMI1	0.378	**0.943***																				
	0.311	**0.029**																				
PMM1	-0.389	0.391	0.576																			
	0.306	0.305	0.212																			
GMP1	-0.836	-0.323	0.010	0.395																		
	0.082	0.338	0.495	0.303																		
GME1	**-.0917***	-0.405	-0.077	0.451	**0.984****																	
	0.041	0.298	0.462	0.275	**0.008**																	
GGP1	0.061	0.409	0.350	0.708	-0.319	-0.208																
	0.470	0.296	0.325	0.146	0.340	0.396																
GGP2	0.638	**0.958***	**.0934***	0.249	-0.195	-0.319	0.136															
	0.181	**0.021**	**0.033**	0.376	0.402	0.341	0.432															
GPP1	0.107	0.464	0.629	0.081	0.455	0.295	-0.483	0.682														
	0.446	0.268	0.185	0.459	0.272	0.352	0.259	0.159														
GPP2	0.047	0.367	0.543	0.023	0.506	0.346	-0.565	0.602	0.994**													
	0.476	0.316	0.228	0.489	0.247	0.327	0.218	0.199	0.003													
GDH1	**-0.929***	-0.403	-0.077	0.485	**0.974***	**0.999****	-0.158	-0.332	0.258	0.306												
	0.035	0.298	0.462	0.258	**0.013**	**0.001**	0.421	0.334	0.371	0.347												

Table 2. (Cont'd.).

	PGI1	*PGI2*	*PMI1*	*PMM1*	*GMP1*	*GME1*	*GGP1*	*GGP2*	*GPP1*	*GPP2*	*GDH1*	*GalLDH1*	*AO1*	*AO2*	*APX1*	*APX2*	*APX3*	*DHAR1*	*MDHAR1*	*MDHAR2*	*MDHAR3*	*MDHAR4*
GalLDH1	-0.765	-0.015	0.307	0.782	0.882	0.899	0.146	0.000	0.355	0.359	0.909*											
	0.118	0.493	0.347	0.109	0.059	0.051	0.427	0.500	0.322	0.320	0.045											
AO1	**-0.981***	-0.567	-0.265	0.392	**0.927***	**0.976***	-0.169	-0.512	0.090	0.150	0.980**	0.829										
	0.010	0.217	0.368	0.304	**0.036**	**0.012**	0.416	0.244	0.455	0.425	0.010	0.085										
AO2	0.562	0.866	0.883	0.152	-0.047	-0.195	-0.083	**0.973***	0.826	0.766	-0.219	0.051	-0.404									
	0.219	0.067	0.058	0.424	0.477	0.402	0.458	**0.014**	0.087	0.117	0.391	0.474	0.298									
APX1	**-0.833**	-0.259	0.078	0.481	**0.995****	**0.984****	-0.233	-0.150	0.452	0.494	**0.978***	0.923*	**0.922***	-0.018								
	0.084	0.370	0.461	0.259	**0.002**	**0.008**	0.383	0.425	0.274	0.253	**0.011**	0.039	**0.039**	0.491								
APX2	0.452	0.170	-0.095	0.013	-0.834	-0.728	0.715	-0.078	-0.779	-0.836	-0.692	-0.561	-0.611	-0.291	-0.799							
	0.274	0.415	0.453	0.493	0.083	0.136	0.142	0.461	0.110	0.082	0.154	0.219	0.195	0.354	0.101							
APX3	**-0.991***	-0.684	-0.408	0.296	0.869	**0.933***	-0.190	-0.638	-0.034	0.035	**0.938***	0.740	**0.988****	-0.533	0.855	-0.543						
	0.004	0.158	0.296	0.352	0.065	**0.034**	0.405	0.181	0.483	0.482	**0.031**	0.130	**0.006**	0.233	0.073	0.228						
DHAR1	**-0.974***	-0.460	-0.168	0.583	0.859	0.936*	0.086	-0.473	-0.024	0.016	**0.952***	0.881	**0.967***	-0.420	0.876	-0.439	**0.948***					
	0.013	0.270	0.416	0.208	0.070	0.032	0.457	0.263	0.488	0.492	**0.024**	0.060	**0.016**	0.290	0.062	0.280	**0.026**					
MDHAR1	0.375	0.746	0.659	0.652	-0.428	-0.383	**0.911***	0.525	-0.170	-0.274	-0.347	0.044	-0.423	0.320	-0.339	0.634	-0.485	-0.190				
	0.312	0.127	0.171	0.174	0.286	0.308	**0.045**	0.237	0.415	0.363	0.327	0.478	0.289	0.340	0.330	0.183	0.258	0.405				
MDHAR2	0.369	0.603	0.469	0.554	-0.544	-0.469	**0.950***	0.348	-0.390	-0.486	-0.427	-0.085	-0.458	0.122	-0.462	0.790	-0.486	-0.219	**0.973***			
	0.316	0.199	0.265	0.223	0.228	0.266	**0.025**	0.326	0.305	0.257	0.287	0.458	0.271	0.439	0.269	0.105	0.257	0.391	**0.014**			
MDHAR3	0.586	-0.199	-0.512	-0.763	-0.841	-0.814	-0.084	-0.250	-0.572	-0.563	-0.817	**-0.964***	-0.692	-0.314	-0.885	0.637	-0.573	-0.728	-0.096	0.081		
	0.207	0.401	0.244	0.119	0.080	0.093	0.458	0.375	0.214	0.219	0.092	**0.018**	0.154	0.343	0.058	0.182	0.213	0.136	0.452	0.459		
MDHAR4	0.039	0.491	0.468	0.793	-0.226	-0.131	**0.988****	0.235	-0.350	-0.437	-0.084	0.253	-0.122	0.029	-0.135	0.613	-0.165	0.132	**0.932***	**0.937***	-0.215	
	0.481	0.255	0.266	0.104	0.387	0.435	**0.006**	0.382	0.325	0.282	0.458	0.374	0.439	0.486	0.433	0.193	0.417	0.434	**0.034**	**0.032**	0.392	

**Correlation is significant at the $p<0.01$ level (1-tailed), *correlation is significant at the $p<0.05$ level (1-tailed)

Table 3. Pearson's correlation (r) comparing relative gene expression over fruit development to AsA concentration in 'Hort16A'.

	PGI1	***PGI2***	***PMI1***	***PMM1***	***GMP1***	***GME1***	***GGP1***	***GGP2***	***GPP1***	***GPP2***	***GDH1***	***GalLDH1***	***AO1***	***AO2***	***APX1***	***APX2***	***APX3***	***DHAR 1***	***MDHAR1***	***MDHAR2***	***MDHAR3***	***MDHAR4***
AsA concentration	0.437	-0.286	0.871	0.828	**0.988****	**0.981****	0.191	-0.344	0.063	0.632	**0.978***	0.894	**0.995****	-0.099	0.883	-0.281	0.058	0.692	0.053	-0.303	-0.374	0.018
	0.281	0.357	0.064	0.086	**0.006**	**0.009**	0.404	0.328	0.469	0.184	**0.011**	0.053	**0.003**	0.45	0.058	0.36	0.471	0.154	0.474	0.349	0.313	0.491
PGI2	0.734																					
	0.133																					
PMI1	0.127	-0.487																				
	0.437	0.257																				
PMM1	0.05	-0.532	**0.996****																			
	0.475	0.234	**0.002**																			
GMP1	0.35	-0.36	**0.936***	**0.904***																		
	0.325	0.32	**0.032**	**0.048**																		
GME1	0.312	-0.394	**0.949***	**0.921***	**0.999****																	
	0.344	0.303	**0.026**	**0.04**	**0**																	
GGP1	**0.944***	0.877	-0.017	-0.078	0.129	0.094																
	0.028	0.062	0.492	0.461	0.436	0.453																
GGP2	0.693	**0.998****	-0.547	-0.591	-0.419	-0.453	0.842															
	0.153	**0.001**	0.227	0.205	0.29	0.273	0.079															
GPP1	0.688	0.629	-0.425	-0.5	-0.088	-0.131	0.567	0.638														
	0.156	0.186	0.288	0.25	0.456	0.435	0.216	0.181														
GPP2	0.222	-0.3	0.257	0.204	0.535	0.517	-0.105	-0.307	0.518													
	0.389	0.35	0.372	0.398	0.233	0.241	0.447	0.346	0.241													
GDH1	0.287	-0.418	**0.952***	**0.925***	**0.998****	**1.000****	0.067	-0.477	-0.147	0.52												
	0.357	0.291	**0.024**	**0.037**	**0.001**	**0**	0.466	0.262	0.426	0.24												

Table 3. (Cont'd.).

	PGI1	*PGI2*	*PMI1*	*PMM1*	*GMP1*	*GME1*	*GGP1*	*GGP2*	*GPP1*	*GPP2*	*GDH1*	*GalLDH1*	*AO1*	*AO2*	*APX1*	*APX2*	*APX3*	*DHAR 1*	*MDHA R1*	*MDHA R2*	*MDHA R3*	*MDHA R4*
GalLDH1	0.643	0.04	0.84	0.797	.901*	0.891	0.515	-0.029	0.026	0.278	0.879											
	0.179	0.48	0.08	0.101	0.049	0.055	0.243	0.485	0.487	0.361	0.06											
AO1	0.421	-0.308	0.831	0.786	**0.972***	**0.964***	0.153	-0.361	0.118	0.706	**0.962***	0.85										
	0.29	0.346	0.084	0.107	**0.014**	**0.018**	0.424	0.319	0.441	0.147	**0.019**	0.075										
AO2	-0.277	-0.297	-0.399	-0.415	-0.193	-0.201	-0.477	-0.251	0.453	0.695	-0.19	-0.497	0.001									
	0.362	0.352	0.3	0.292	0.404	0.4	0.262	0.374	0.274	0.152	0.405	0.252	0.499									
APX1	0.25	-0.362	**0.990****	**0.980***	**0.941***	**0.950***	0.12	-0.426	-0.356	0.224	**0.949***	**0.902***	0.837	-0.475								
	0.375	0.319	**0.005**	**0.01**	**0.029**	**0.025**	0.44	0.287	0.322	0.388	**0.025**	**0.049**	0.082	0.263								
APX2	0.738	1.000**	-0.483	-0.53	-0.355	-0.389	0.879	**0.997****	0.632	-0.295	-0.413	0.044	-0.303	-0.296	-0.358							
	0.131	0	0.258	0.235	0.323	0.306	0.06	**0.001**	0.184	0.352	0.293	0.478	0.349	0.352	0.321							
APX3	**0.915***	**0.939***	-0.177	-0.238	-0.017	-0.054	**0.987****	**0.914***	0.642	-0.124	-0.081	0.374	0.028	-0.388	-0.042	**0.941***						
	0.042	**0.03**	0.411	0.381	0.491	0.473	**0.007**	**0.043**	0.179	0.438	0.46	0.313	0.486	0.306	0.479	**0.03**						
DHAR1	0.794	0.362	0.637	0.591	0.692	0.675	0.758	0.296	0.126	0.039	0.656	**0.936***	0.632	-0.664	0.737	0.366	0.643					
	0.103	0.319	0.181	0.205	0.154	0.163	0.121	0.352	0.437	0.48	0.172	**0.032**	0.184	0.168	0.131	0.317	0.179					
MDHAR1	0.897	**0.932***	-0.137	-0.193	-0.008	-0.042	**0.990****	**0.904***	0.562	-0.202	-0.069	0.399	0.014	-0.477	0	**0.933***	**0.995****	0.676				
	0.051	**0.034**	0.431	0.404	0.496	0.479	**0.005**	**0.048**	0.219	0.399	0.466	0.3	0.493	0.261	0.5	**0.033**	**0.002**	0.162				
MDHAR2	0.721	**1.000****	-0.496	-0.54	-0.375	-0.408	0.87	**0.998****	0.617	-0.318	-0.432	0.026	-0.326	-0.303	-0.372	**1.000****	**0.933***	0.352	**0.927***			
	0.14	**0**	0.252	0.23	0.313	0.296	0.065	**0.001**	0.192	0.341	0.284	0.487	0.337	0.349	0.314	**0**	**0.033**	0.324	**0.037**			
MDHAR3	0.67	**0.993****	-0.585	-0.628	-0.453	-0.487	0.818	**0.999****	0.652	-0.298	-0.51	-0.072	-0.388	-0.211	-0.468	**0.993****	0.897	0.252	0.884	**0.994****		
	0.165	**0.003**	0.208	0.186	0.274	0.257	0.091	**0.001**	0.174	0.351	0.245	0.464	0.306	0.395	0.266	**0.004**	0.051	0.374	0.058	**0.003**		
MDHAR4	-0.427	-0.396	0.504	0.568	0.169	0.206	-0.269	-0.422	**-0.946***	-0.636	0.215	0.186	-0.062	-0.717	0.477	-0.399	-0.363	0.161	-0.269	-0.385	-0.448	
	0.286	0.302	0.248	0.216	0.415	0.397	0.366	0.289	**0.027**	0.182	0.393	0.407	0.469	0.142	0.261	0.301	0.318	0.42	0.366	0.307	0.276	

**Correlation is significant at the $p<0.01$ level (1-tailed), *correlation is significant at the $p<0.05$ level (1-tailed)

The expression of recycling pathway members in *A. chinensis* var. *chinensis*: The expressions of two *ascorbate oxidase* (*AO*) and three *L- ascorbate peroxidase* (*APX*) genes were studied in 'Jinyang' (Fig. 3) and 'Hort16A' (Fig. 4) with fruit development. In 'Jinyang', transcript of *AO1* decreased significantly at 76 DAA and then stable. But the *AO2* expression levels was increased obviously at 76 DAA and then decreased at 138 DAA and 145 DAA with fruit mature and softening (Fig. 3). The expression of *APX1* was decreased gradually up to fruit softening. There was not obvious change of *APX2* gene expression. The transcript of *PAX3* was declined significantly at 76 DAA and then increased slightly. Transcripts of *monodehydroascorbatereductase 1* (*MDHAR1*), *MDHAR2* and *MDHAR4* peaked at 138 DAA when fruit ripening and then decline with fruit softening. *MDHAR3* expression was increased gradually up to fruit softening. The expression of *dehydroascorbatereductase 1* (*DHAR1*) was decreased significantly at 76 DAA and then stable (Fig. 3).

The expression analyses of recycling pathway members in 'Hort16A' were as Fig. 4. The expression pattern of *AO1* in 'Hort16A' was similar with those of *AO1* in 'Jinyang'. The expression pattern of *AO2* was fluctuant with fruit development. The expression pattern of *APX2*, *MDHAR2* and *MDHAR3* were resemble with not obvious change before fruit mature and then increased with fruit softening. Transcripts of *APX3* and *DHAR1* were decreased significantly with fruit development. *MDHAR4* expression was increased obviously at 58 DAA and then decreased with fruit mature. There were not obviously changes of *APX3* and *MDHAR1* gene expression with fruit development. There was a significant positive correlation for *AO1* expression with AsA concentration in 'Jinyang' (Table 2) and 'Hort16A' (Table 3).

Discussion

Previous reported showed that kiwifruit contains high AsA concentration, five or six times as much as a banana, ten times as much as an apple (Ferguson & Huang, 2007). AsA concentrations among *Actinidia* species are considerable variation, *A. henryi* has low values (4.4 mg/100 g FW), and *A. latifolia* has very high (671–2140 mg/100 g FW) (Huang *et al.*, 2004). AsA concentration also has large variation within *A. chinensis* var. *chinensis* or *A.chinensis* var. *deliciosa* (Ferguson & Huang, 2007). There is range from 50 to 420 mg ascorbate/100 g FW in accessions of *A. chinensis* var. *chinensis* (Huang *et al.*, 2004). The AsA content of 'Jinyang' and 'Hort16A' were 129.26.10 and 68.87 mg/100 g FW at the condition of edible in our study, respectively. The concentration of AsA peaked between 28 DAA and 42 DAA, before decreasing as the fruit progressed toward maturation (Bulley*et al.*, 2009). In our study, the concentrations of AsA were declined with fruit development in our detected range, but declined trends were different between these two species, providing an excellent model to investigate gene factors that regulate AsA.

The *MgGMP* expression data was coinciding with AsA contents of acerola (*Malpighia glabra* L.) during fruit ripening (Badejo *et al.*, 2007), and the AsA content of transgenic tobacco plants overexpressing the *MgGMP* gene including its promoter was about 2-fold higher than that of the wild type (Badejo *et al.*, 2008). The *Solanum lycopersicum* 'Money maker' cultivar overexpression of Yeast-derived *GMP* gene increased AsA levels of up to 70% in leaves, 50% in green fruit, and 35% in red fruit (Cronje *et al.*, 2012). Overexpressing of tomato *GMP* gene in tobacco plants could significantly increase the content of AsA in the leaves (Wang *et al.*, 2011). These results showed that *GMP* plays a major role in the proposed AsA biosynthetic pathway in plants. Transcripts of *GME* and *GGT* were higher in *A. eriantha* than other genotypes (*A. chinensis* and *A. deliciosa*) during the period of highest increase in AsA concentration (Bulley *et al.*, 2009). *GGT* and *GME* gene expression increased at high light intensities where AsA levels were also increased in *Arabidopsis* (Laing *et al.*, 2007). Transient expression experiments showed that tobacco overexpression of *GME* alone has little affect accumulation of AsA in leaf, overexpression of *GGT* led to an approximate 3-fold increase in leaf AsA, but co-expressed *GME* and *GGT* resulted in an 8～12- fold increase in leaf AsA (Laing *et al.*, 2007). The expression patterns of *GMP1*, *GME1* and *GDH1* were corresponding with the tendency of AsA concentration in two *A. chinensis* varieties, and there were significant positive correlation for *GMP1*, *GME1* and *GDH1* expression with AsA concentration, and the expression level relations between each pair of *GMP1*, *GME1*, *GDH1* are significant positive correlation in these two species, suggested that L-Galactose pathway is one of important routes for AsA biosynthesis, and *GMP1*, *GME1*, *GDH1* genes play key roles in L-Galactose pathway forAsA biosynthesis.

Transgenes plants overexpression of *DHAR* or *MDHAR* gene does not affected the concentration of AsA. None of studies were performed in kiwifruit (Bulley & Laing, 2016). There are five *MDHAR* genes and three *DHAR* genes in *Arabidopsis*. Four *MDHAR* genes and one *DHAR* gene expressions were studied in kiwifruit during fruit development. The results showed that the expressions of these genes were different between two *A. chinensis* varieties, predicting that *DHAR* or *MDHAR* genes might not key genes for AsA biosynthesis in kiwifruit.

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