Scientia Horticulturae 197 (2015) 637–643

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

The influence of growth media pH on ascorbic acid accumulation and biosynthetic gene expression in tomato

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ARTICLE INFO

Article history:
Received 31 July 2015
Received in revised form 18 October 2015
Accepted 19 October 2015
Available online 4 November 2015

Keywords:
Ascorbic acid
pH
Gene expression

ABSTRACT

The ascorbic acid (AsA) accumulation in plants is affected by various growth, development and environmental factors. The pH of growth media has been reported to affect the accumulation of AsA in plants. In this study, the impact of the pH on the AsA accumulation as well as its biosynthetic gene expression was analyzed in tomato (Solanum lycopersicum 'Ailsa Craig'). The results indicated that with the increase in pH, total AsA content in tomato leaves decreased and redox status (AsA/DHA) was augmented. Similarly, most of the biosynthetic gene expression level decreased with the increment of pH and showed the negative correlation; only a few genes, *e.g.*, genes encoding GDP-Mannose 3′,5′-epimerase 1 (GME1), GDP-L-galactose-1-phosphate phosphorylase 1 (GGP1) and GGP2 showed positive correlation. Moreover, in situ H_2O_2 was detected which accretion was escalated as the pH value surged from 5.0 to 7.0, in agreement to the decreased AsA level. The present study suggests that mild acidity may contribute to the boosted accumulation of AsA in tomato.

1. Introduction

Ascorbic acid (AsA, vitamin C) is one of the most abundant antioxidants in plants. AsA acts as a scavenger of the reactive oxygen species (ROS) generated by a variety of physiological processes such as photosynthesis, oxidative metabolism and abiotic stress (Ye et al., 2015). AsA has additionally been described to be an important cofactor of many enzymes participating in defense, cellular growth and fruit ripening (Arrigoni and De Tullio, 2002; Noctor and Foyer, 1998; Pastori et al., 2003). In plants, biosynthesis of AsA undergoes four different pathways, including the D-mannose/L-galactose (D-Man/L-Gal) pathway, D-galacturonate pathway, gulose pathway and myo-inositol pathway. D-Man/L-Gal or Smirnoff/Wheeler pathway is the major AsA biosynthetic route, involving intermediates such as GDP-D-mannose, GDP-L-galactose, L-galactose and L-galactono-1, 4-lactone (Wheeler et al., 1998).

It is well known that the content of AsA in fruits and vegetables can be affected by various factors such as genotypic differences, preharvest climatic conditions, cultivation practices, and postharvest handling (Zhang et al., 2014). The concentration of AsA in plants is modulated by environmental factors like light (Johkan et al., 2013; Wang et al., 2013), temperature (Tsaniklidis et al., 2014), water deficit (Hu et al., 2005), waterlogging (Alves et al., 2012) and fertilizer (Donno et al., 2013). AsA concentration and stability is also affected by pH (Yahia et al., 2001). AsA stability is pH dependent and irreversibly degraded into 2.3-diketogulonate at physiological pH. During storage of samples containing dehydroascorbate (the oxidized form of ascorbate, DHA), DHA seems to be more stable in acidic solutions having pH 2.0 to 3.0 (Wechtersbach et al., 2011). It was observed during pepper fruit development that the concentration of AsA in apoplast were decreased while the pH of apoplast was increased, contrarily when apoplastic pH was decreased then the concentration of AsA was increased. Moreover the apoplastic AsA content was decreased in blossom end rot affected pepper as the pH got more alkaline (Aloni et al., 2008). When pH increased from 3.0 to 8.0, oxidation of AsA was escalated due to rising of APX activity in Zn treated seedlings of *Helianthus annuus* L.(Mishra et al., 2013). However the mechanism, under which the pH affects the AsA accumulation, remains largely unknown.

Although AsA biosynthesis pathways have been illustrated in plants, a certain plant species showed the different AsA concentration under different growth conditions, indicating that the environmental factors may modulate AsA biosynthesis. However, the mechanism under which the environmental factors like growth medium pH affect AsA accumulation remains elusive. In this study,

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http://dx.doi.org/10.1016/j.scienta.2015.10.031
0304-4238/

we investigated the effect of pH on AsA accumulation in tomato at both the metabolic and transcriptional levels to understand the molecular mechanism involved in the pH dependent AsA accumulation.

2. Materials and methods

2.1. Plant material and growth conditions

Aseptic seeds of tomato *Solanum lycopersicum* 'Ailsa Craig' were germinated in 1/2 MS medium with different pH value (pH 5.0, pH 5.8 and pH 7.0). The pH of growth media was adjusted with HCl and NaOH. Plants were cultivated under controlled environmental conditions (light/dark regimen 16/8 h at 25/20 °C, relative humidity 65–75%). To evaluate the relationship between AsA levels and expression of metabolism genes under different pH treatments, the second to the seventh true leaves were harvested from 1 month-old seedlings for RNA isolation and AsA determination. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C. The experiment was repeated with three replications.

2.2. Ascorbic acid assay

AsA content was assayed according to Rizzolo et al. (1984). Samples of leaves were ground into powder using liquid nitrogen, and powder (0.2–1 g) was homogenized in 5 mL of ice-cold 0.1% (w/v) HPO_3, followed by extraction for about half an hour and centrifugated at 12,000 × *g* for 10 min at 4 °C. The supernatant was filtered by micro porous membrane (0.22 μm) to the new centrifugal tube, shaded and was placed on ice. Then 50 mM DTT (DL-Dithiothreitol) was added to the 300 μL extracts at a 1:1 ratio, followed by reaction for 15 min in the dark at room temperature. Then the total ascorbate was assayed from the extracts by using HPLC. The reduced state of ascorbate was measured by adding equal volume of 0.1% (w/v) HPO_3 to 300 μL supernatant. The difference between the total ascorbate and reduced state of ascorbate was the content of DHA. The chromatographic column SB-aq (Agilent) was eluted with acetate buffer (0.2 M, pH 4.5) at a flow rate of 1.0 mL/min. The reaction solution was detected at 254 nm. AsA concentration was quantified by a standard curve derived from 2 to 40 μg/mL AsA.

2.3. In situ *detection of H_2O_2*

The detection of H_2O_2 in tissues was performed using the method described by Thordal-Christensen et al. (1997). Detached leaves were rinsed with distilled water, incubated in 1 mg/mL 3,3′-diaminobenzidine (DAB)-HCl (pH 3.8) for 10–12 h in dark at room temperature (25 °C). Then chlorophyll was removed by boiling in 96% ethanol for 10 min. After cooling, leaves were inoculated in new 96% ethanol at room temperature for 4 h or longer until chlorophyll completely faded. Loss of electrons in the presence of H_2O_2 leads to formation of shallow brown insoluble product and color change. The results were presented by brown precipitates, formed due to the reaction of 3,3′-diaminobenzidine (DAB) with H_2O_2. At least five leaves from five independent plants were used per treatment.

2.4. qPCR analysis

Total RNA from samples were isolated using TRIzol® 117 reagent (Invitrogen, USA), and DNase was used to remove the genomic DNA before reverse-transcription. Reverse transcription was performed with 3 μg of total RNA with ReverTra Ace® reverse transcriptase (ToYoBo, Osaka, Japan), according to the manufacturer's instructions. Tomato β-*actin* gene was used as internal control for quantitative real-time PCR (qPCR) analysis. Expression of the genes involved in AsA biosynthesis, oxidation and recycling was analyzed by qPCR (Table 1). Gene-specific primers were designed from these sequences of tomato genes by using Primer5 software. qPCR was performed using the Roche Light Cycler 480 qPCR Detection System and the SYBR Green I Master Kit (Roche, Switzerland) according to the supplier's protocols. PCR amplification included a 5 min pre-incubation step at 95 °C, followed by 40 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 20 s. The relative expression of genes was calculated with the $2^{-\Delta\Delta CT}$ method using the *actin* genes as internal control, and presented as the ratio to pH 5.0.

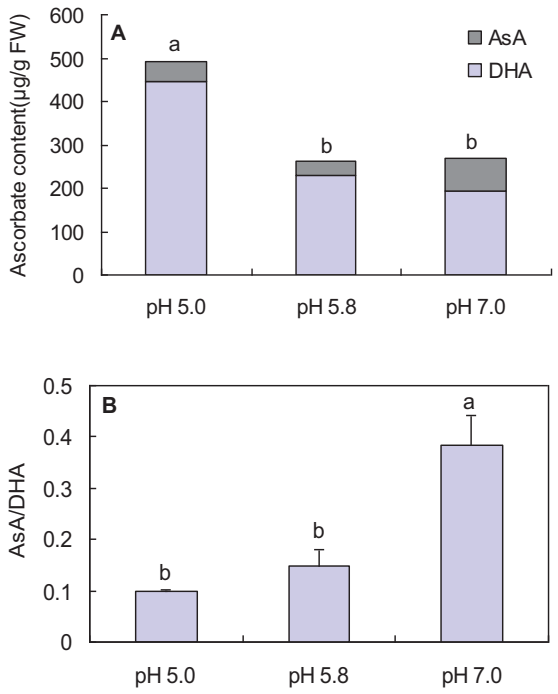

Fig. 1. Levels of total ascorbate and DHA were measured and expressed in micrograms of total ascorbate (black bars) or DHA (grey bars) per g of fresh weight of leaves of plants under different pH treatments (A). AsA/DHA ratios were calculated for each treatment (B). Data is presented as mean ± SD. Statistical analysis was performed using the Duncan's test.

2.5. Statistical analysis

Every treatment was performed with three replications by measuring each sample twice. Results were represented as the means ± standard deviation (SD). Significant differences were calculated using the Duncan's means test at the $P < 0.05$ level. The significance of correlation coefficient was calculated by Data Processing System (http://www.chinadps.net/dps_eng/).

3. Results

3.1. Changes in AsA levels under different pH treatment

The amount of AsA present in tomato leaves under different pH treatments was measured by HPLC. As shown in Fig. 1A, total AsA (T-AsA) and dehydroascorbate (the oxidized form of ascorbate, DHA) were the highest in pH 5.0 leaves with T-AsA being about 1.87- and 1.83-fold higher than that of pH 5.8 and pH 7.0 leaves and DHA were 1.94- and 2.29-fold higher than that of pH 5.8 and pH 7.0 leaves, respectively. But AsA/DHA ratios showed the opposite trend, where pH 7.0 showed the highest AsA/DHA ratio (Fig. 1B). These results indicated that the pH could alter AsA content as well as its redox status in tomato leaves.

Table 1
Primers used for qPCR.

Gene	Full name	Accession No.	Forward primer (5′–3′)	Reverse primer (5′–3′)
GPI	Glucose-6-phosphate isomerase	Solyc04g076090	TGCTCTTCAAAAGCGTGTCC	CGGCAATAAGTGCTCTGTCA
PMI	Phosphomannose isomerase	Solyc02g086090	TACATTGTGGTGGAACGAGGA	ACCCCATTTGGCAAGAACAG
PMM	Phosphomannomutase	Solyc05g048760	TTTACCCTCCATTACATTGCTGA	TCTTCTTGACTACAGTTTCTCCCA
GMP	GDP-D-mannose pyrophosphorylase	Solyc03g096730	AAACCTGAAATCGTGATGTGAGA	TGAAGAAGAGGAGAACTGGAAAC
GME1	GDP-mannose 3′,5′-epimerase 1	Solyc01g097340	AATCCGACTTCCGTGAGCC	CTGAGTTGCGACCACGGAC
GME2	GDP-mannose 3′,5′-epimerase 2	Solyc04g077020	CCATCACATTCCAGGACCAGA	CGTAATCCTCAACCCATCCTT
GGP1	GDP-L-galactose-1-phosphate phosphorylase 1	Solyc06g073320	GAAATCTGGTCTGTTCCTCTGTGA	TTCACACACCAACTCCACATTACA
GGP2	GDP-L-galactose-1-phosphate phosphorylase 2	Solyc02g091510	CTGTTGTCTTGGTTGGAGGTTGT	AGCACAGTCAAAACACCAACAAA
GP1	L-Galactose-1-phosphate phosphatase 1	Solyc04g014800	AGCCGCTACAAACCCTCATCT	TGTCCGCTTTCCATCTCCTAT
GP2	L-Galactose-1-phosphate phosphatase 2	Solyc11g012410	GGTTAGGTCCCTTCGTATGTG	TTTCACAATCACAGCACCACC
GalDH	L-Galactose dehydrogenase	Solyc01g106450	CTTCTTACTGAGGCTGGTGGTC	AACCTCTTTAACAGACTTCATCCC
GLDH	L-Galactono-1,4-lactone dehydrogenase	Solyc10g079470	ATTGAGGTTCCCAAGGACATAG	ATGTTATTAGATAGGATGCGGTTT
MIOX	myo-Inositol oxygenase	Solyc12g008650	ACTACTCTTCCTTCTGCTGCTTTA	AATGTTGAGCCACTTCATGTTCT
MDHAR1	Monodehydroascorbate reductase 1	Solyc09g009390	GGTGATGTTGCCACTTTTCCTTT	CGACAGACTTCCCTTGCTCACT
DHAR1	Dehydroascorbate reductase 1	Solyc05g054760	CCTACCTTCGTCTCATTTCCG	TGAACAAACATTCTGCCCATT
AOBP	Ascorbate oxidase promoter-binding protein	Solyc06g69760	GCTTTCTCCCATTGAACTCCAG	GCCAGCCACTTGCTCTTATTGT
AO	L-ascorbate oxidase	Solyc04g054690	AGGATGGCTCAGAGTGTT	ATCAGGTAAGGCGTATGG
cAPX	Cytoplasm ascorbate peroxidase	Solyc06g005150	TGGAGCCCATTAGGGAGCA	GCCAGGGTGAAAGGGAACAT
tAPX	Thylakoidal ascorbate peroxidase	Solyc11g018550	CTTTCTTCAATGGCTTCTCTCACCG	CAACCTGGTAGCGAAACACATGGG
APX1	Ascorbate peroxidase 1	Solyc06g005160	TGGAGCCCATTAGGGAGCA	GCCAGGGTGAAAGGGAACAT
APX2	Ascorbate peroxidase 2	Solyc06g005150	TGGGAGGGTGGTGACATATTTT	TTGAAGTGCATAACTTCCCATCTTT
APX3	Ascorbate peroxidase 3	Solyc09g007270	TTCAACAGCAACTACTCCAGCC	GGAACAGTTCCCAATCCTATCC
APX4	Ascorbate peroxidase 4	Solyc01g111510	GGAACAGTTCCCAATCCTATCC	CATAGGTTCCTGCATCATGCCACC
APX5	Ascorbate peroxidase 5	Solyc02g083620	AGTAGATGCAGAGTATCTGAAGGA	CATAGGTTCCTGCATCATGCCACC
APX7	Stromal ascorbate peroxidase 7	Solyc06g060260	CTTTCTTCAATGGCTTCTCTCACCG	CAACCTGGTAGCGAAACACATGGG
Actin	*Actin*	Solyc11g005330	GTCCTCTTCCAGCCATCCA	ACCACTGAGCACAATGTTACCG

Fig. 2. *In situ* detection of H_2O_2 in tomato leaves under different pH treatments. Loss of electrons in the presence of H_2O_2 leads to formation of shallow brown insoluble product and color change in leaves. The ROS level is elevated as pH increases.

3.2. Histochemical localization of H_2O_2

AsA varied in response to pH and the ROS might also be affected under different pH treatment. Comparing the plants grown under pH 5.8 and pH 7.0 with plants under pH 5.0, plants cultivated under pH 5.0 showed less concentration of H_2O_2 (Fig. 2) and the leaves of the plants under pH 5.8 and pH 7.0 accumulated more ROS so we assumed that the higher level of AsA under pH 5.0 might help to scavenge the excessive ROS.

3.3. Changes in mRNA level of genes involved in AsA metabolism

To determine the relationship between AsA content and expression of the ascorbate biosynthesis related genes, the gene transcription level was determined by qPCR. Among these biosynthetic genes: genes encoding glucose-6-phosphate isomerase (*GPI*), phosphomannomutase (*PMM*), GDP-D-mannose pyrophosphorylase (*GMP*), GDP-Mannose 3′,5′-epimerase (*GME*), GDP-L-galactose-1-phosphate phosphorylase (*GP*), L-galactose-1-phosphate phosphatase (*GPP*), L-galactose dehydrogenase (*GalDH*) and L-galactono-1,4-lactone dehydrogenase (*GLDH*) are involved in the D-mannose/L-galactose pathway for AsA biosynthesis. myo-inositol oxygenase (*MIOX*) is involved in the myo-inositol pathway. As shown in Fig. 3, expression of *GPI*, *PMM*, *GMP*, *GP1*, *GalDH*, *GLDH* and *MIOX* was more copious in pH 5.0 leaves than in pH 5.8 and pH 7.0 leaves. monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) are two key enzymes for recycling AsA. Here, relative expression of *MDHAR1* and *DHAR1* showed a higher level in the pH 5.0 leaves (Fig. 4).

The transcription level of several genes involved in the AsA oxidation was measured in leaves by qPCR and the results exhibited that the transcription levels of ascorbate oxidase gene (*AO*), *APX4*, *APX5* and *APX7* were highest in the pH 7.0 leaf tissues, which might accelerate the oxidation of AsA leading to a lower level of T-AsA at pH 7.0. However, the relative expression level of *APX1*, *APX2* and *APX3* and genes encoding cytoplasm ascorbate peroxidase (*cAPX*), ascorbate oxidase promoter-binding protein (*AOBP*) was the highest in the pH 5.0 leaf tissues (Fig. 5). We speculate that the several genes in AsA oxidation may play a complementary role in the regulation of AsA accumulation.

3.4. The correlation between pH and gene expression and AsA accumulation

In order to explore the relationship between gene expression and AsA accumulation, we calculated the correlation coefficient between them. Among the AsA biosynthetic genes, there is a strong positive correlation between the relative expression of *GLDH* and the amount of total AsA. Moreover *GPI*, *PMM*, *GMP*, *GaLDH* and *MIOX* showed the general positive correlation with total AsA. Nonetheless, the expression level of *GME*, *GGP*, *GP2* and a gene encoding phosphomannose isomerase (*PMI*) depicted weak correlation or even negative correlation with the content of AsA in the treated

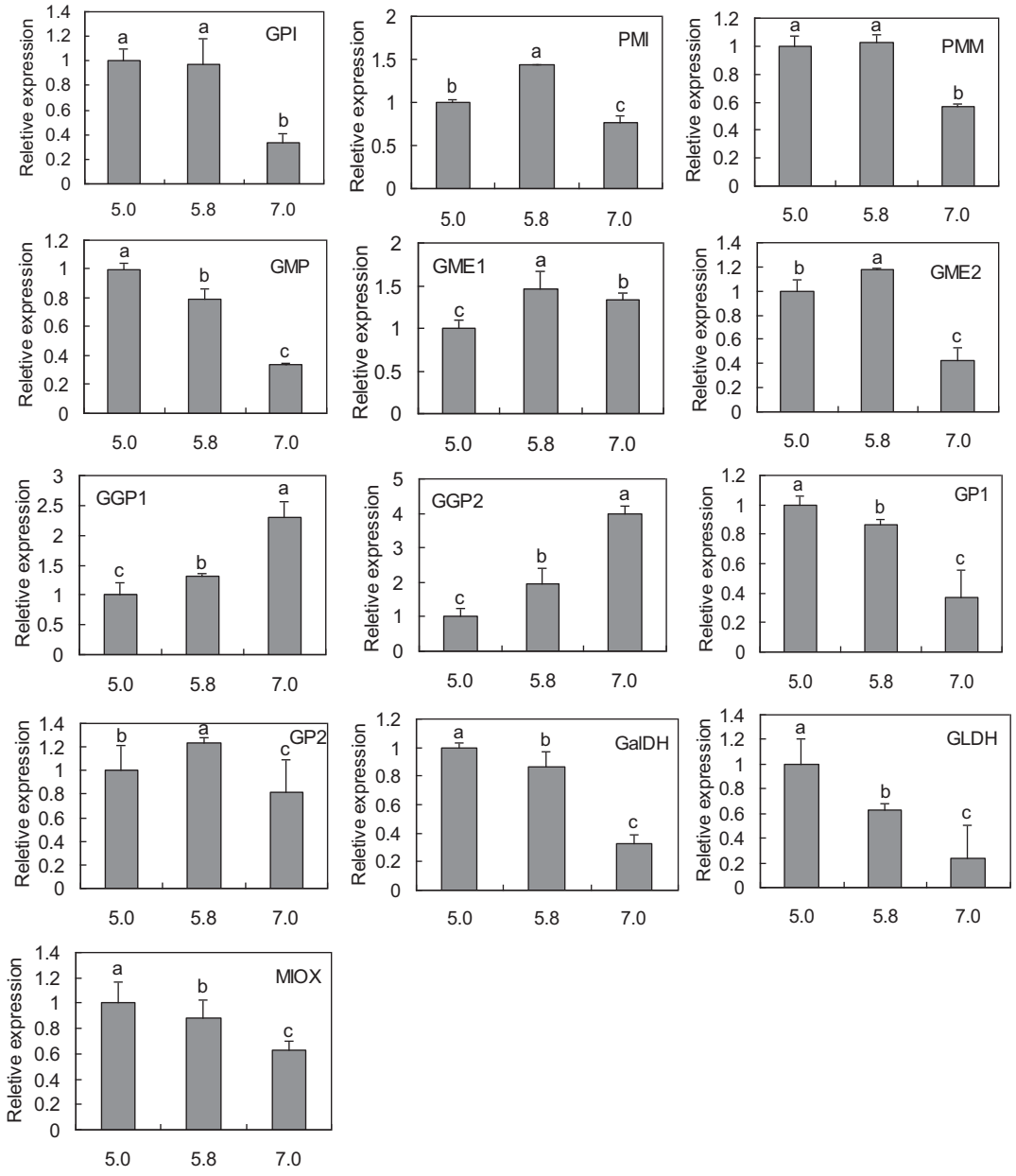

Fig. 3. The relative mRNA transcription of AsA biosynthetic genes performing qPCR with specific primers designed for coding sequences of AsA biosynthesis-related genes. Data is presented as mean values ± SD ($n = 3$).

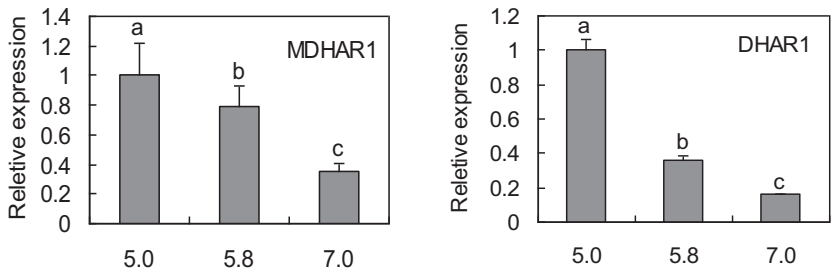

Fig. 4. The relative mRNA transcription of AsA recycling genes, using specific primers designed from coding sequences of *MDHAR1* and *DHAR1*, was assayed in 30-day old seedlings under different pH treatments. Data is presented as mean values ± SD ($n = 3$).

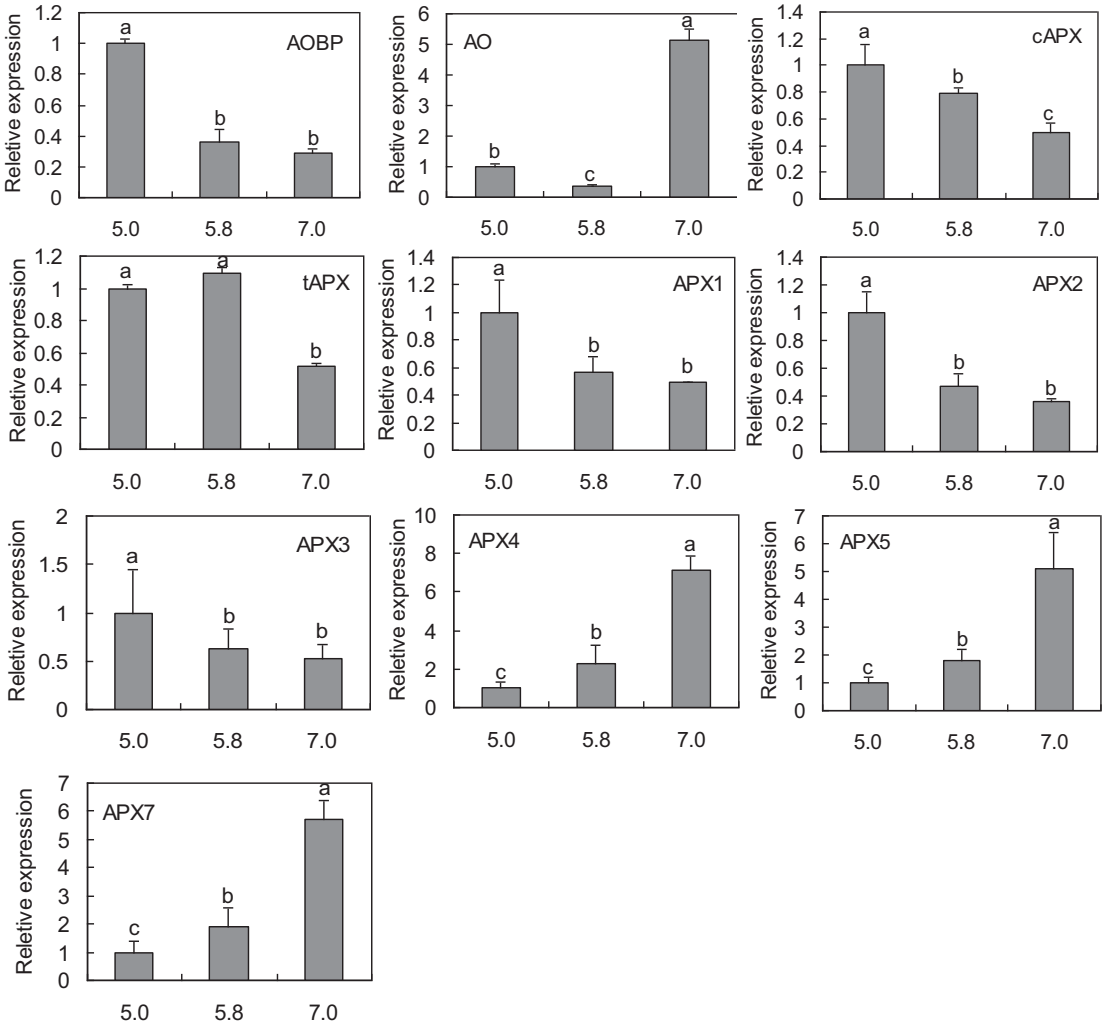

Fig. 5. The relative mRNA transcription of AsA metabolism genes was assayed by performing qPCR with specific primers designed for coding sequences of AsA metabolism-related genes. Data is depicted as mean values ± SD ($n = 3$).

Table 2
Correlation coefficient between AsA biosynthetic gene expression and AsA accumulation as well as growth medium pH.

Gene	GPI	PMI	PMM	GMP	GME1	GME2	GGP1	GGP2	GP1	GP2	GalDH	GLDH	MIOX	MDHAR1	DHAR1
r_{DHA}	0.64	−0.05	0.57	0.82	−0.93	0.41	−0.77	−0.82	0.75	0.06	0.74	0.91	0.82	0.82	0.99**
r_{AsA}	−0.95	−0.91	−0.97*	−0.66	0.002	−0.99**	0.87	0.83	−0.66	−0.95	−0.89	−0.71	−0.83	−0.83	−0.46
$r_{T\text{-}AsA}$	0.52	−0.19	0.44	0.73	−0.97*	0.27	−0.67	−0.73	0.65	−0.09	0.63	0.84	0.73	0.73	0.97*
r_{pH}	−0.93	−0.45	−0.90	−1.00**	0.63	−0.80	0.98*	0.99**	−0.98*	−0.54	−0.97*	−0.99**	−0.99**	−0.99**	−0.91

Statistical analysis was performed using the DPS.
* Indicated significant correlation at $P < 0.05$.
** Indicated significant correlation at $P < 0.01$.

Table 3
Correlation coefficient between AsA oxidative gene expression and AsA accumulation as well as growth medium pH.

Gene	AOBP	AO	cAPX	tAPX	APX1	APX2	APX3	APX4	APX5	APX7
r_{DHA}	0.99**	−0.5	0.88	0.47	0.99**	0.99**	0.99**	−0.75	−0.74	−0.74
r_{AsA}	−0.33	0.99*	−0.76	−0.99**	−0.38	−0.4	−0.44	0.88	0.89	0.89
$r_{T\text{-}AsA}$	0.99**	−0.37	0.80	0.34	0.99*	0.98*	0.97*	−0.65	−0.63	−0.63
r_{pH}	−0.85	0.85	−0.99**	−0.84	−0.88	−0.88	−0.91	0.98*	0.97*	0.97*

Statistical analysis was performed using the DPS.
* Indicated significant correlation at $P < 0.05$.
** Indicated significant correlation at $P < 0.01$.

leaves (Table 2). Similar correlation pattern was observed between DHA and biosynthetic gene expression. For reduced AsA, only *GGP1* and *GGP2* displayed positive correlation (Table 2). The positive correlation coefficient between the T-AsA and relative transcrip-

tion levels of *MDHAR1* and *DHAR1* are 0.73 and 0.97, respectively (Table 2). These revealed that the two genes might play an important role in the process of ascorbate accumulation. For oxidation related genes, *AO*, *APX4*, *APX5* and *APX7* presented negative correlation, while others showed strong positive correlation (Table 3).

At the same time, we have made a correlation analysis between pH value and gene expression. As shown in Tables 2 and 3, pH exerts obvious effect on AsA biosynthetic gene expression. Most of the biosynthetic genes such as *GPI*, *PMM*, *GMP*, *GME2*, *GP1*, *GP2*, *GaLDH*, *GLDH*, *MIOX*, *MDHAR1* and *DHAR1* indicated the negative correlation with the pH value; however, the oxidation related genes such as *AO*, *APX4*, *APX5* and *APX7* are positively related to the pH value. This is in agreement to decreasing AsA accumulation and increasing levels of ROS along with pH value.

4. Discussion

Numerous findings have been exploring the influence of environmental factors on AsA accumulation (Alves et al., 2012; Johkan et al., 2013; Ye et al., 2015; Zhang et al., 2014), while there were few reports about the relationship between the pH and AsA of plants. Light intensity has been shown as the most important pre-harvest factor to determine the final AsA content of the agricultural product (Johkan et al., 2013). The final step of D-mannose/L-galactose pathway contributing to pool size was controlled by light, as L-galactono-1, 4-lactone oxidation to AsA was promoted in high light acclimated leaves (Smirnoff, 1995). The light signal transduction pathway mediated by high-pigment-1 (hp1) (Zhang et al., 2014) and the CSN5B protein that interacts with GMP (Wang et al., 2013) may contribute in part to the light-dependent AsA accumulation.

In this study, regarding the effect of pH on AsA accumulation, it is revealed that the lower pH leads to higher AsA content. As a major antioxidant, AsA protects plants from oxidative stress through the decomposition of ROS generated by normal oxygenic metabolism and abiotic stress (Noctor and Foyer, 1998; Conklin and Barth, 2004). Extreme low pH will cause damage to cell growth and plant development and plant may activate its antioxidant capacity by enhancing AsA biosynthesis (Koyama et al., 2001). On the other hand, the mild acidic conditions may help to keep stability of ascorbate, as ascorbic acid itself is an organic acid.

The expression of AsA biosynthetic genes is not always correlated to their metabolite accumulation. Most of the genes expressions tend to show a positive correlation for DHA and total AsA but for reduced state of AsA, the genes expressions incline to exhibit a negative correlation (Table 2). Although most of these genes have been demonstrated for their roles in AsA biosynthesis, *e.g., GMP* (Badejo et al., 2007; Wang et al., 2011), *GME* (Zhang et al., 2011), *GLDH* (Tabata et al., 2002) and *DHAR* (Chen and Gallie, 2005; Haroldsen et al., 2011), the final concentration of AsA may result from the collective function of pathway genes rather than a single gene. When the pH value increases to 7.0, expression of most of the genes declined (Table 3, Fig. 3) and this may contribute to the decrease in the accumulation of AsA toward high pH value (Fig. 1). It is worth noting that the expression abundance of AsA biosynthetic genes was obtained from the qPCR with the only internal control of *actin* gene. More housekeeping genes like EFα1 (elongation factor α1), TBP (TATA binding protein), RPL8 (ribosomal protein L8) and TUA (α-tubulin) would provide more accurate quantification of the gene expression (Expósito-Rodríguez et al., 2008)

For the oxidation of AsA, pH also exhibited significant effect on the gene expression. Different gene members of APX family showed opposite correlation (Table 3), suggesting the complementary roles of gene members in metabolite synthesis and stress response. It is worthy to note that AOBP expression was positively correlated with AsA accumulation, whereas AO seem to have a negative correlation with AsA accumulation (Fig. 5), suggesting a negative correlation between AOBP and AO (Table 3). Alongside of tomato fruit development, *AOBP* expression rises to peak level at breaker stage (42 days after pollination), presenting a positive correlation with AsA accumulation (Alba et al., 2005). In pumpkin, the AOBP bound to the promoter of *AO* gene, but did not show a positive correlation with AO (Kisu et al., 1998).

In conclusion, this study revealed differences in the accumulation of AsA under different pH conditions of growth media. Low pH will lead to higher AsA accumulation and less ROS accumulation, and the pH dependent AsA accumulation was possibly lessened due to the systematic down regulation of biosynthetic genes as pH increases. In addition to this study, enzymatic assay may provide further evidences for elucidating the mechanism of pH influence on AsA accumulation.

Acknowledgements

This work was supported by the National Basic research program of China (No. 2011CB10060), the National Natural Science Foundation of China (31171974) and Fundamental Research Funds for the Central Universities (2014PY006, 2662015PY225).

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