

Comparison of ascorbic acid metabolism during the development of two jujube varieties

Yong Huang¹, Guoying Song², Yang Wang³, Kun Zhang¹,
Jiayun Zhou¹, Zhenchao Yuan¹, Qunxian Deng^{1,*}

¹ College of Horticulture, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

² The Bureau of Agriculture and Rural Affairs of Shizhong District, Leshan, Sichuan 614000, China

³ Chengdu Agricultural College, Faculty of Agriculture and Horticulture, Chengdu, Sichuan 611130, China

ABSTRACT

Jujube (*Ziziphus jujuba* Mill.) is a fruit crop containing a high level of ascorbic acid (AsA). However, the regulation of AsA accumulation differs significantly in various jujube germplasm resources and tissues, and the factors contributing to this discrepancy remain unknown. To explore the mechanisms underlying AsA metabolism, we investigated the dynamics of AsA content in the fruits, leaves and petals during the development of two jujube varieties, *Z. jujuba* Mill. cv. 'Zhanshanmizao 1' (ZM1) and *Z. jujuba* Mill. cv. 'Zhanshanpingguozao' (ZP). The diversity of AsA content in different jujube tissues demonstrated that the fruits had the highest levels of AsA, whereas the petals had the lowest. The pattern of AsA accumulation in the fruits and leaves was similar during the development of both jujube varieties. The activity of GalDH was correlated with the AsA levels in the ZM1 fruits. The activity of GalLDH was positively correlated with the AsA levels in ZP leaves. In contrast, the activities of GalDH and GalLDH were not correlated with AsA content during leaf growth in ZM1. We also analysed the expression patterns of genes involved in AsA metabolism. The results showed that AsA biosynthesis in jujube fruits was mainly related to the L-galactose pathway. Moreover, expression of genes involved in AsA biosynthesis (*GME1*, *GMPI*, *GGP* and *GalLDH*) was associated with the AsA concentrations in ZM1 and ZP fruits. *GME*, *GMP* and *GalDH* may be the key genes for AsA synthesis in ZM1 and ZP leaves.

Keywords: ascorbic acid, dynamic development, gene expression, jujube, L-galactose pathway

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GMP, GDP-D-mannose pyrophosphorylase; GGP, GDP-L-galactose phosphorylase; GME, GDP-D-mannose 3',5'-epimerase; GPP, L-galactose-1-P phosphatase; MDHAR, monodehydroascorbate reductase; T-AsA, total ascorbic acid.

INTRODUCTION

Chinese jujube (*Ziziphus jujuba* Mill.) is the most important member of the family Rhamnaceae due to its important nutritional and medicinal properties. Jujube

fruit is rich in various vitamins, with the reputation of being a 'natural vitamin pill'. ZM1 (*Z. jujuba* Mill. cv. 'Zhanshanmizao 1') originated in Mianyang City,

*Corresponding author.

e-mail: dengqx@sicau.edu.cn (Qunxian Deng).

Sichuan Province, and has been cultivated for nearly 300 years, with a cultivated area of nearly 26.67 km². Its fruit is ovoid to oblong in shape, with a thin skin, thin flesh, fine crisp slag, juicy and sweet, small kernel, sweet and a good quality fresh food. ZP (*Z. jujuba* Mill. cv. 'Zhanshanpingguozao') is a mutant variety of ZM1, and its fruit is rounded orbicular to oblate spherical, shaped like a small apple, having a thin skin, juicy, small kernel, fruity sour flavour, pulpy, fine crisp and flavourful and with a strong flavour. It is also considered to be an excellent quality of fresh food.

L-ascorbic acid (AsA), also called vitamin C, is an important antioxidant that plays a key role in several plant biological processes, including defence mechanisms, cell division and photosynthesis (Zhang et al., 2016). Fruits and vegetables contain relatively high levels of AsA; however, the AsA content varies markedly among plant tissues and varieties. As early as the 1950s, the synthetic pathway of AsA in mammals was initially explored (Lehninger and Ul Hassan, 1956). Four biosynthetic pathways have been identified for AsA biosynthesis in plants (Figure 1): the L-galactose

pathway, galacturonate pathway, L-glucose pathway and myo-inositol pathway (Dowdle et al., 2007; Imai et al., 2009; Li et al., 2010a; Bulley et al., 2012). The L-galactose pathway is thought to be the dominant route for AsA biosynthesis in many plants (Linster et al., 2007; Linster and Clarke, 2008; Imai et al., 2009). It undergoes a total of nine enzymatic reactions, converting D-glucose to AsA via GDP-D-mannose, and the last five enzymes (GDP-D-mannose 3',5'-epimerase [GME], L-galactose-1-P phosphatase [GPP], L-galactose dehydrogenase [GalDH] and L-galactono-1,4-lactone dehydrogenase [GalLDH]), which catalyse the conversion of GDP-D-mannose to AsA, are specific to AsA biosynthesis (An et al., 2007; Laing et al., 2007). In AsA recycling, AsA is produced by the reduction of oxidised forms of AsA, including MDHAR and DHAR, which also play a role in regulating the AsA content (Alos et al., 2013). Whole genome sequencing of 'Dongzao' and 'Junzao' identified the presence of two AsA synthesis pathways in jujube, namely the L-galactose pathway and the myo-inositol pathway (Liu et al., 2014; Zhang et al., 2016). Moreover, *ZjMDHAR* plays a key role in AsA recycling during

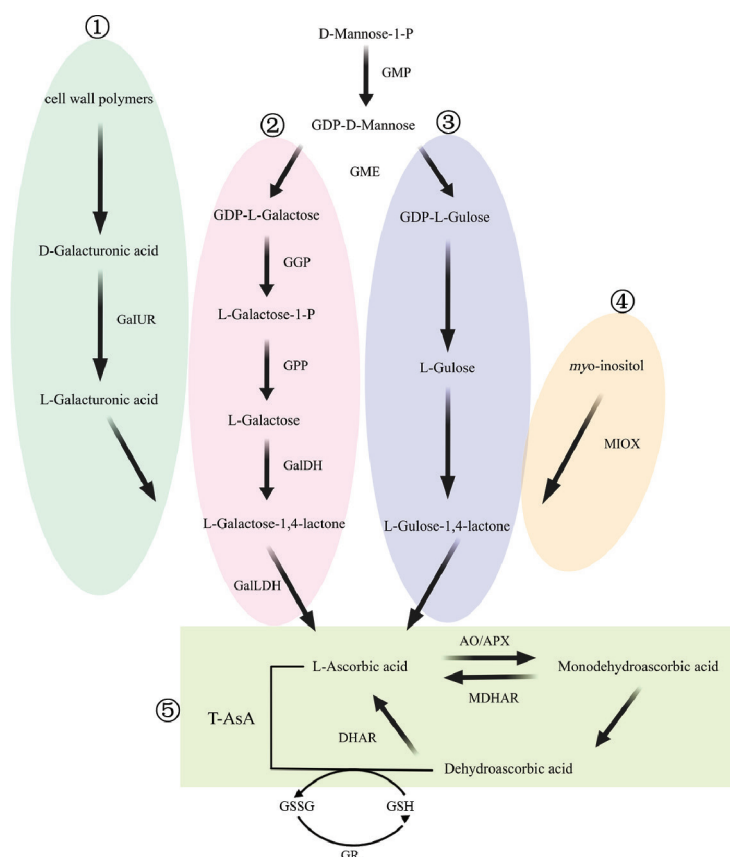


Figure 1. The pathway of AsA metabolism in plants. *Note:* ① galacturonic acid pathway; ② L-galactose pathway; ③ L-glucose pathway; ④ myo-inositol pathway; ⑤ AsA regeneration and degradation pathway. AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GGP, GDP-L-galactose pyrophosphatase; GME, GDP-D-mannose 3',5'-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GPP, L-galactose-1-P phosphatase; GSH, glutathione; GSSG, oxidised glutathione; MDHAR, monodehydroascorbate reductase; MIOX, myo-inositol oxygenase; T-AsA, total ascorbic acid.

fruit development in cultivar Mazao (Lu et al., 2022). *GMP1*, *GMP2*, *GME1*, *GME2*, *GGP* and *GalDH* are involved in AsA accumulation during fruit development and different genotypes in cultivar Junzao (Zhang et al., 2016). Although AsA levels in jujube fruit have been preliminarily researched, there is very little information about the synthesis and regulation mechanism.

There are large climatic differences between the north and south of China, which may affect the differences in metabolic mechanisms and thus lead to differences in the intrinsic quality of jujube fruits. Although the mechanism of AsA accumulation in northern fresh jujube has been reported, the AsA accumulation pattern among different varieties and tissues of southern fresh jujube has been less reported. In this study, we systematically investigated the contents of AsA, enzyme activities and expression profiles of genes related to AsA metabolism during the development of ZM1 and ZP fruits and leaves, with a view to finding out the main synthesis pathway of AsA in southern jujube and the key genes for AsA accumulation. These results can provide a theoretical basis for further research on the quality and molecular regulation of AsA in southern jujube fruits.

MATERIALS AND METHODS

Plant materials and sample preparation

Seven-year-old trees *Z. jujuba* Mill. cv. 'Zhanshanmizao 1' (ZM1) and 7-year-old grafted trees *Z. jujuba* Mill. cv. 'Zhanshanpingguozao' (ZP) (the grafting rootstock is ZM1) with similar vigour and load (based on trunk cross-sectional area), which were grown in Zhanshan Village, Santai County, Mianyang City, Sichuan Province, were used for this study. The orchard and growing conditions are hilly terraces with yellowish reddish purple clay soil. The climate is subtropical monsoon, with an average annual temperature of 16.8°C, an average annual precipitation of 828 mm and a frost-free period of 291 days. Petals were collected during the first bloom period. Initial leaf sampling was conducted on 5-day-old foliage (from the fifth to seventh nodes of fruit-bearing shoots). Subsequent collections were performed at 20-day intervals, covering foliage ages of 25, 45, 65 and 85 days. Fruits were collected at 10, 30, 50, 70 and

90 days after flowering (DAF). In total, 50 leaves and 30 fruits of uniform size were collected from different orientations at 10 a.m. Three replicates were included for each sampling. Samples were packed with ice and were transported to the laboratory immediately. First, the packed samples were counted and weighed. Next, they were frozen in liquid nitrogen. Then, they were stored at −80°C for further use. Three technical replicates were included for each analysis.

AsA content measurement

The AsA content was determined using HPLC method according to Huang et al. (2014), with some modifications. Exactly 0.5 g of sample was ground in 5 mL of 0.2% metaphosphoric acid solution. The mixture was then centrifuged at $8000 \times g$ for 20 min at 4°C. The supernatant was diluted to a final volume of 10 mL, filtered through a 0.22 µm membrane and used for AsA detection. However, unlike AsA quantification, total ascorbic acid (T-AsA) quantification required the addition of 10 µL of fresh DTT (200 mM) solution to the supernatant. After being kept at room temperature for 4 h in the dark, the mixture was used for T-AsA quantification; a 15% methanol solution was used as the mobile phase. The separation was achieved by an Agilent 1260 MP-C18 column (250 mm × 4.6 mm, 5 µm). The flow rate was set to 0.5 mL · min^{−1}, and the column temperature was maintained at 25°C. The AsA and T-AsA contents were quantified based on the detected peak areas at 243 nm by comparing with external standards. Dehydroascorbic acid (DHA) content was calculated by subtracting the AsA content from the T-AsA content.

GalDH enzyme activity assays

The GalDH enzyme activity was assayed according to the method of Gatzek et al. (2002). Two grams of flesh was homogenised with 5 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 mM phenylmethanesulfonyl fluoride, 0.5% (v/v) Triton X-100, 0.2% (v/v) 2-mercaptoethanol and 2% (w/v) PVP, and then centrifuged at $12000 \times g$ at 2°C for 20 min. The supernatants were collected to assay GalDH. One unit of GalDH activity was defined as the amount of enzyme causing an increase of 0.01 in the absorbance per min at 340 nm at 25°C.



Figure 2. Fruit developmental stages of two jujube varieties. DAF, days after flowering.

GalLDH enzyme activity assays

The GalLDH enzyme activity was assayed according to the method of Gatzek et al. (2002), with some modifications. Exactly 0.5 g sample was ground in 8 mL of 0.1 mM phosphate buffer (containing 0.4M sucrose, 10% glycerin, 50 mM β -mercaptoethanol, 1 mM EDTA and 2% PVP, pH 7.4). The mixture was then centrifuged at $5000 \times g$ at 4°C. After centrifugation, the supernatant was collected and further centrifuged at $12000 \times g$ at 4°C to obtain the pellet. The pellet was subsequently suspended in Tris-HCl buffer (containing 10% glycerin and 5 mM GSH). The reaction system contained 2 mL of 50 mM phosphate buffer (containing 1 mg/mL cytochrome *c*, pH 8.0), 0.4 mL of galactono-1,4-lactone and 0.1 mL of enzyme solution. The reaction mixture was pre-incubated at 27°C for 1 min. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 per min at 550 nm.

AO and APX enzyme activity assays

The ascorbate oxidase (AO) enzyme activity was assayed according to the method of Esaka et al. (1990). The ascorbate peroxidase (APX) enzyme activity was assayed according to the method of De Pinto et al. (1999). One unit of AO and APX activities was defined as the amount of enzyme causing an increase of 0.01 in the absorbance per min at 265 nm and 290 nm at 25°C, respectively.

Gene expression analysis

Total RNA was isolated from the petals, leaves and fruits of ZM1 and ZP at different developmental stages using the improved CTAB method. RNA quality was assessed by electrophoresis on a 1% agarose gel, and RNA concentration was determined. First-strand cDNA was synthesised using a PrimeScript strand synthesis Kit (Takara RR047A, China). The expression of genes related to AsA metabolism in ZM1 and ZP was analysed using qPCR at different developmental stages of petals, leaves and fruits. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Premix Ex Taq Kit on a Bio-Rad CFX96 instrument. *ZjH3* was the reference gene. The PCR protocol comprised initial heating at 95°C for 3 min, followed by 39 cycles at 95°C for 5 s, 55°C for 30 s and 72°C for 10 s. Primer specificity was determined using qRT-PCR and melting-curve analysis. The 25 μ L reaction system contained $2 \times$ SYBR Premix ExTaq (12.5 μ L), 10 μ mol/L primers (0.5 μ L of each), H_2O (10.5 μ L) and diluted cDNA (1 μ L). Gene expression levels were calculated using the formula $2^{-\Delta\Delta CT}$. The primers used for qPCR analysis are listed in Table S1 in Supplementary Materials.

Statistical analysis

The correlation analysis was conducted using Origin 2022 software. All results are expressed as mean $\pm$ standard deviation ($n = 3$), with differences considered significant at $p < 0.05$ and highly significant

at $p < 0.01$, as determined by Student's *t*-tests. At the same time, a one-way analysis of variance was performed on the fruits and leaves of the two varieties at different stages of development, with significant differences (Duncan's multiple range test) assessed at the 5% confidence level.

RESULTS

Dynamic change of AsA content during fruit development

The growth dynamics of ZM1 and ZP are shown in Figure 2. Overall, the results showed that the change patterns of AsA and T-AsA content (Figure 3A) during the development of ZM1 and ZP fruits were consistent, showing an initial increase followed by a decrease. A sharp increase was observed from 10 DAF to 30 DAF, with AsA and T-AsA content in ZP being higher than that in ZM1. Both reached their maximum values at 50 DAF. However, the maximum content in ZM1 was significantly higher than that of ZP by 18.35% and 16.96%, and thereafter, a sharp decrease was observed in both from 50 DAF to 70 DAF. Although the DHA content (Figure 3A) generally increased during fruit development, the pattern of increase differed between ZM1 and ZP. Both ZM1 and ZP showed the same increasing trend from 10 DAF to 50 DAF. Nevertheless, the turning point occurred at 50 DAF, with a slight increase observed in ZP from 50 DAF to 90 DAF, increasing by 22.20%. In ZM1, the trend showed a sharp decrease followed by an increase. AsA/DHA (Figure 3A) reflects the degree of redox of AsA; the higher the ratio, the more favourable the accumulation of AsA. At the 10 DAF, the ratio in ZM1 was higher than that in ZP by 196.77%. However, at 30 DAF, the ratio was exactly the opposite. After 50 DAF, the ratio in ZM1 was higher than that in ZP by 13.73%.

Changes in AsA content during leaf growth

As shown in Figure 3B, the DHA and T-AsA contents in ZM1 were significantly higher than those in ZP from 5 days to 65 days, and the AsA contents in ZM1 were significantly higher than those in ZP by 560.77%. At 85 days, the AsA, DHA and T-AsA contents in ZP were higher than those in ZM1. Although there was an overall increasing trend in AsA content during leaf development, the pattern of increase differed between ZM1 and ZP. From 5 days to 45 days, a gradual increase occurred in ZM1 and ZP. However, a sharp increase was observed from 45 days to 85 days, followed by a sharp decrease in ZM1. On the contrary, the AsA content in ZP decreased from 45 days to 85 days, followed by a sharp decrease thereafter.

AsA content of petal

According to the results (Figure 3C), petals also contain AsA, but the content was significantly lower than that in the leaves and fruits. The AsA content in ZP was higher

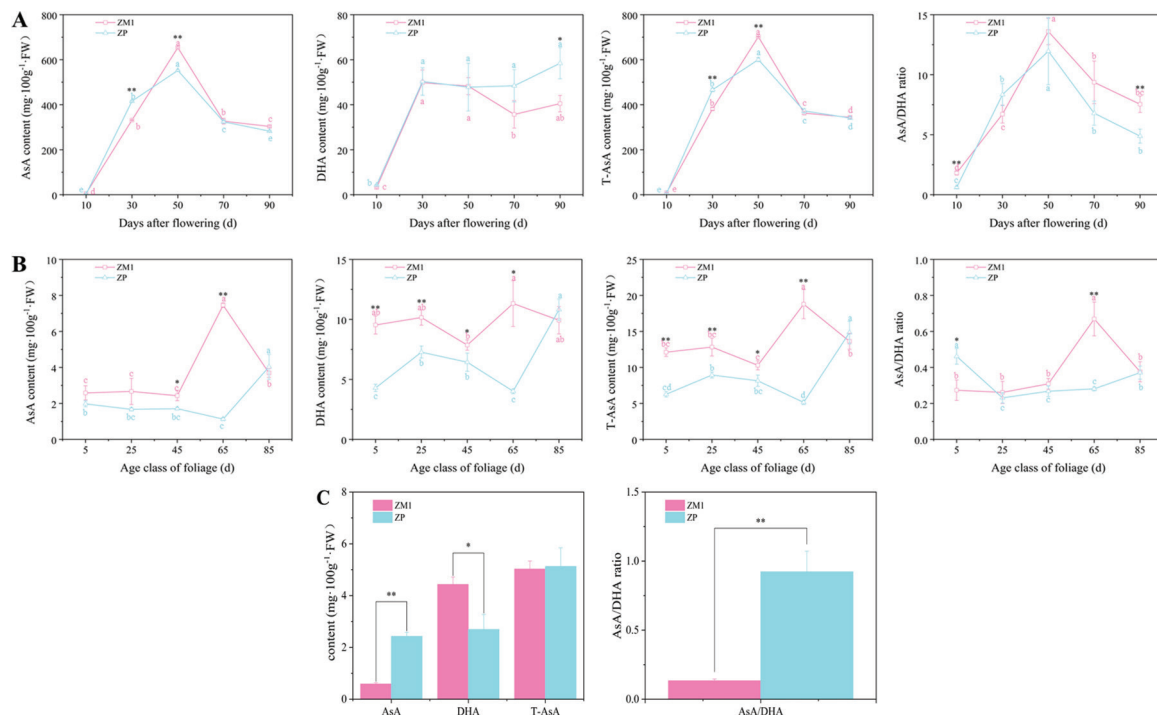


Figure 3. Changes in AsA, DHA, T-AsA contents and AsA/DHA ratio during the development of different tissues in two jujube varieties. (A) Changes in AsA, DHA, T-AsA contents and AsA/DHA ratio during fruit development of two jujube varieties. (B) Changes in AsA, DHA, T-AsA contents and AsA/DHA ratio during leaf development of two jujube varieties. (C) Comparison of AsA, DHA, T-AsA contents and AsA/DHA ratio in the petals of two jujube varieties. Note: * and ** indicate independent samples *t*-test, which analyse the differences between ZM1 and ZP in direct quantitative data at the same developmental stage. * indicates a significant difference ($0.01 < p < 0.05$), ** indicates a highly significant difference ($**p < 0.01$); a, b, c, etc. represent one-way analysis of variance between different developmental stages of two varieties, with significant differences (Duncan's multiple range test) assessed at the 5% confidence level. AsA, ascorbic acid; DHA, dehydroascorbic acid; T-AsA, total ascorbic acid; ZM1, 'Zhanshanmizao 1'; ZP, 'Zhanshanpingguozao'.

than that in ZM1 by 311.86%. The DHA content in ZM1 was higher than that in ZP by 64.40%, but there was no significant difference between the two in T-AsA content. The noteworthy point was that the AsA/DHA ratio in ZP was much higher than that in ZM1 by 588.95%.

AsA biosynthesis and recycling during the fruit development

Changes in the expression of critical AsA biosynthetic genes involved in the L-galactose pathway, as well as the activities of corresponding enzymes during fruit development, were analysed in two varieties with significantly different AsA content (Figure 4A). In this pathway, an overall increase in GalLDH activity was observed, but a decrease occurred at 70 days in both species, decreasing by 244.97% and 45.94% respectively, compared with 50 DAF. The AsA content in the fruits of both varieties decreased sharply from 50 days to 70 days, and the changes in AsA content corresponded to the changes in enzyme activity. GalDH activity exhibited different changes in different varieties. In ZM1, it increased from 10 DAF to 50 DAF, representing a significant increase of 1056.59%, and then decreased from 50 DAF to 90 DAF, while in ZP, it increased from

10 DAF to 70 DAF, showing a sharp increase from 50 DAF to 70 DAF, followed by a relatively stable level in the later stage. The expression levels of *GME* and *GME1* demonstrated similar trends in both varieties (Figure 5). The expression levels of *GMP* and *GMP1* displayed similar trends (Figure 5). In both varieties, the expression levels of *GPP*, *GalDH* and *GalLDH* showed a significant increase during fruit development (Figure 5). The expression levels of the ZM1 gene increased by 15768.26%, 16214.28% and 46688.17%, respectively, while the expression levels of the ZP gene increased by 14350.74%, 75644.64% and 137379.42%, respectively. *GPP* showed a relatively stable level from 10 DAF to 70 DAF, followed by a sharp increase and subsequent decrease in the later stages in ZM1. By contrast, the expression levels of *GPP* demonstrated a gradual increase from 10 DAF to 50 DAF and then gradually decreased from 50 DAF to 90 DAF in ZM1.

APX and AO are important degrading enzymes of plant AsA. As shown in Figure 4, AO activity displayed similar changes in ZM1 and ZP. It was highest in the first stage, sharply decreased at 30 DAF and then decreased significantly by 147.64% and 762.01%, respectively. Then it gradually maintained

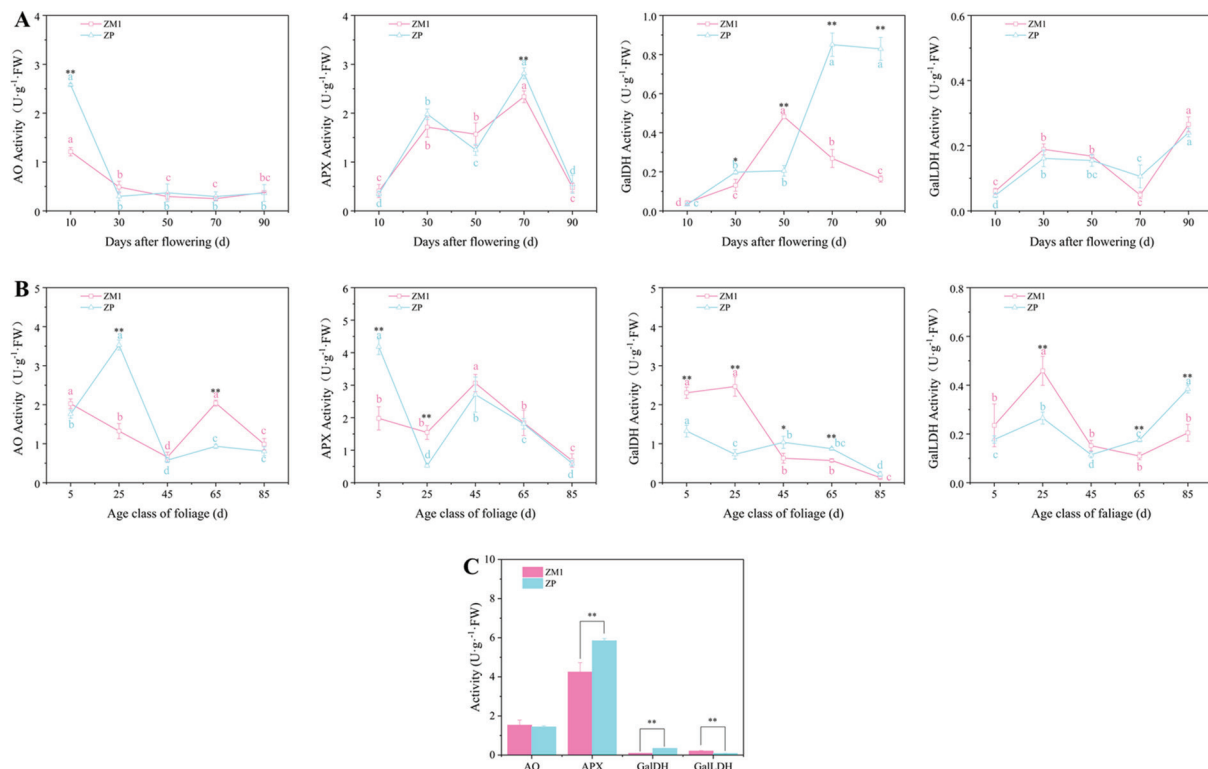


Figure 4. Changes in the activities of AsA-related enzymes during the development of different tissues in two jujube varieties. (A) Changes in the activities of AsA-related enzymes during fruit development of two jujube varieties. (B) Changes in the activities of AsA-related enzymes during leaf development of two jujube varieties. (C) Comparison of AsA-related enzyme activities in the petals of two jujube varieties. *Note:* * and ** indicate independent samples *t*-test, which analyse the differences between ZM1 and ZP in direct quantitative data at the same developmental stage. * indicates a significant difference ($0.01 < p < 0.05$), ** indicates a highly significant difference ($**p < 0.01$); a, b, c, etc. represent one-way analysis of variance between different developmental stages of two varieties, with significant differences (Duncan's multiple range test) assessed at the 5% confidence level. AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; ZM1, 'Zhanshanmizao 1'; ZP, 'Zhanshanpingguozao'.

during the latter stage. In addition, APX activity exhibited similar changes in different varieties. It increased in the first stage, increased from 30 DAF to 70 DAF and then decreased significantly during the latter stage by 389.63% and 415.47%. AO showed the same trend in ZM1 and ZP, respectively (Figure 5). It reached the lowest level at 10 DAF, gradually increased from 10 DAF to 50 DAF, significantly increased by 857.98% and 2342.01%, respectively, and then sharply decreased. Eventually, it increased sharply from 70 DAF to 90 DAF and increased significantly by 287.72% and 1863.02%, respectively. The expression of APX showed a pattern of continuous increase during the ZM1 fruit development, significantly increased by 78044.47%, while it showed a different trend in ZP (Figure 5). In ZP, APX expression increased during the former stage, with a significant increase of 15658.65%, decreased from 50 DAF to 70 DAF and then sharply increased during the latter stage by 909.10%, peaking at 90 DAF. The expression of MDHAR in ZM1 and ZP showed a gradual increase and increased significantly by 8061.03% and 7983.09%, respectively. But the

expression of DHAR exhibited different changes in ZM1 and ZP (Figure 5). In ZM1, the expression of DHAR generally increased from 10 DAF to 30 DAF, while a decrease was observed at 50 DAF, followed by an increase during the latter stage. In addition, the expression of DHAR generally increased from 10 DAF to 70 DAF, and a sharp increase occurred from 70 DAF to 90 DAF by 1608.90% in ZP.

AsA biosynthesis and recycling during the leaf growth

As shown in Figure 4B, the activity of GalLDH showed less variability during the leaf growth in ZM1 and ZP, but GalDH activity exhibited different changes in different varieties. In ZM1, it peaked at 25 days and decreased continuously, while in ZP, it decreased sharply, then increased and finally decreased during the latter stage. GalDH activity decreased by 1660.31% at 85 days compared with 10 days. GalDH activity was not consistent with changes in ZM1 and ZP AsA content, respectively. The expression levels of GME, GME1, GMP, GMP1, GGP, GPP, GalDH and GalLDH

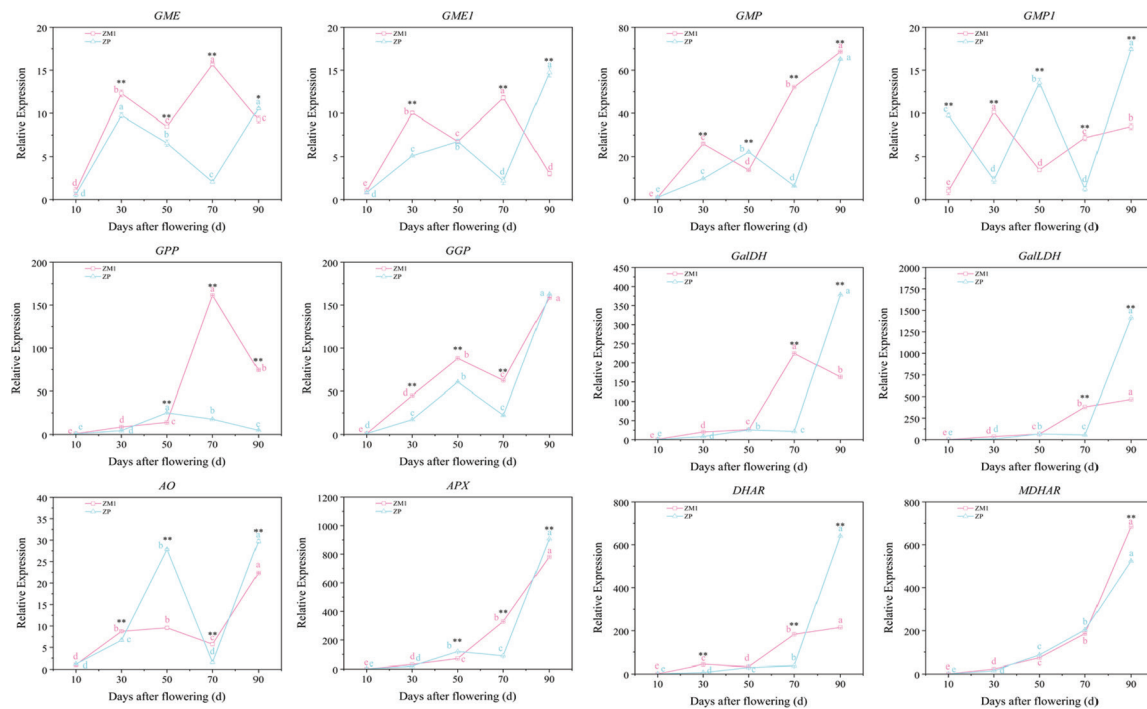


Figure 5. Relative expression levels of genes involved in AsA metabolism during fruit development of two jujube varieties. *Note:* * and ** indicate independent samples *t*-test, which analyse the differences between ZM1 and ZP in direct quantitative data at the same developmental stage. * indicates a significant difference ($0.01 < p^* < 0.05$), ** indicates a highly significant difference ($**p < 0.01$); a, b, c, etc. represent one-way analysis of variance between different developmental stages of two varieties, with significant differences (Duncan's multiple range test) assessed at the 5% confidence level. AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GGP, GDP-L-galactose phosphorylase; GME, GDP-D-mannose 3',5'-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GPP, L-galactose-1-P phosphatase; MDHAR, monodehydroascorbate reductase; ZM1, 'Zhanshanmizao 1'; ZP, 'Zhanshanpingguozao'.

demonstrated similar changes in ZP (Figure 6). They showed low expression levels during the former stage, increased sharply at 45 days, then decreased and finally increased sharply at 85 days by 1842.71%, 334.69%, 3839.66%, 2428.13%, 1456.25%, 408.65%, 2297.29% and 8286.52%. The expression levels were not in agreement with the changes in AsA content. *GME*, *GME1*, *GMP*, and *GGP* showed low expression levels during ZM1 leaf growth. The expression of *GGP* peaked at 25 days, then decreased sharply. They were almost not expressed at 85 days. The expression of *GMP1* and *GalLDH* peaked at 65 days, in contrast to the changes in AsA content during leaf growth, suggesting that these genes might be the key limiting factors involved in AsA biosynthesis in jujube.

APX activity sharply decreased from 5 days to 25 days by 28.17% and 694.55%, then increased at 45 days and decreased during the later stages in ZM1 and ZP, with similar trends (Figure 4B). It peaked at 45 days in ZM1, while in ZP, it peaked at 5 days. Both AO and APX reached their lowest levels at 85 days (Figure 4B). This result indicated that jujube leaf senescence might be associated with APX activity.

The expression of *AO* showed different changes in ZM1 and ZP (Figure 6). In ZP, it increased sharply, with a significant increase of 1865.29% and peaked at 45 days, then decreased sharply at 65 days, and increased again during the later stage. By contrast, in ZM1, *AO* expression increased in the first stage, then decreased at 45 days, gradually increased afterwards and finally peaked at 85 days. A similar expression pattern of *APX* was observed during ZP leaf growth, while it was rarely detected in ZM1. In ZP, the expression of *DHAR* was higher at 45 days and 85 days, which was consistent with the AsA content. In ZM1, the expression of *DHAR* was higher at 65 days, also in agreement with AsA content. These results indicated that *DHAR* might be the key gene involved in the regeneration of AsA in both ZM1 and ZP. The expression of *MDHAR* was rarely detected in ZM1. However, in ZP, *MDHAR* expression was lower in the early stages until 25 days, then increased sharply and peaked at 70 days.

AsA biosynthesis and recycling in petals

As shown in Figure 4C, the comparison of AsA enzyme activities in the petals of different varieties revealed that both GalDH and GalLDH activities were lower, which may explain the low AsA content in the petals.

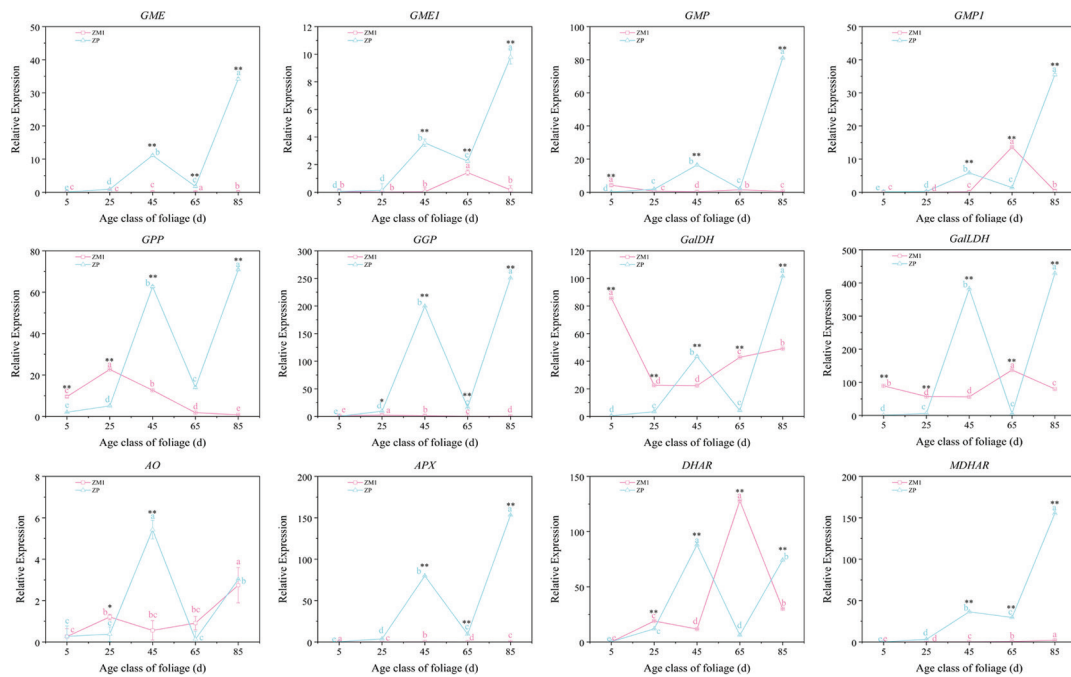


Figure 6. Relative expression levels of genes involved in AsA metabolism during leaf development of two jujube varieties. *Note:* * and ** indicate independent samples *t*-test, which analyse the differences between ZM1 and ZP in direct quantitative data at the same developmental stage. * indicates a significant difference ($0.01 < p < 0.05$), ** indicates a highly significant difference ($**p < 0.01$); a, b, c, etc. represent one-way analysis of variance between different developmental stages of two varieties, with significant differences (Duncan's multiple range test) assessed at the 5% confidence level. AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GGP, GDP-L-galactose phosphorylase; GME, GDP-D-mannose 3',5'-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GPP, L-galactose-1-P phosphatase; MDHAR, monodehydroascorbate reductase; ZM1, 'Zhanshanmizao 1'; ZP, 'Zhanshanpingguozao'.

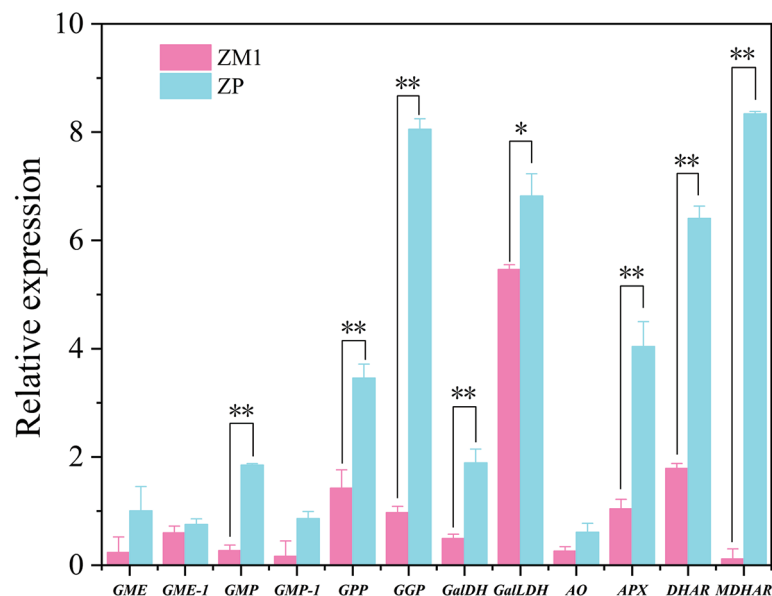


Figure 7. Comparison of the expression of AsA metabolic genes in petals of two jujube varieties. *Note:* * and ** indicate independent samples *t*-test, which analyse the differences between ZM1 and ZP in direct quantitative data at the same developmental stage. * indicates a significant difference ($0.01 < p < 0.05$), ** indicates a highly significant difference ($**p < 0.01$). AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GGP, GDP-L-galactose phosphorylase; GME, GDP-D-mannose 3',5'-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GPP, L-galactose-1-P phosphatase; MDHAR, monodehydroascorbate reductase; ZM1, 'Zhanshanmizao 1'; ZP, 'Zhanshanpingguozao'.

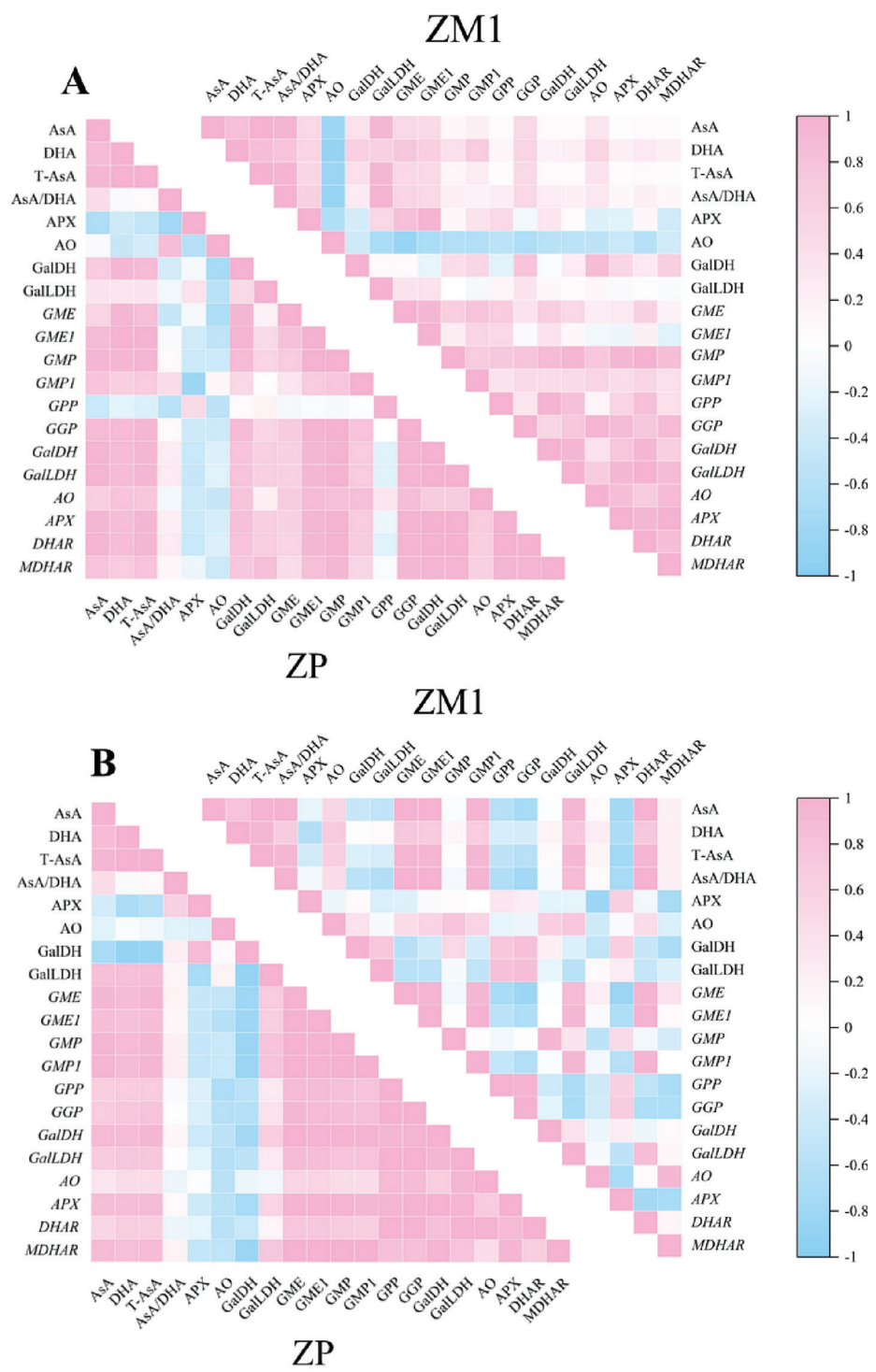


Figure 8. Correlation analysis of AsA accumulation with metabolic enzyme activities and gene expression in two jujube varieties. (A) Correlation analysis of AsA accumulation with metabolic enzyme activities and gene expression during fruit development in two jujube varieties. (B) Correlation analysis of AsA accumulation with metabolic enzyme activities and gene expression during leaf development in two jujube varieties. AO, Ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid; AsA, ascorbic acid; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; GalDH, L-galactose dehydrogenase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GGP, GDP-L-galactose phosphorylase; GME, GDP-D-mannose 3',5'-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GPP, L-galactose-1-P phosphatase; MDHAR, monodehydroascorbate reductase; T-AsA, total ascorbic acid; ZM1, 'Zhanshanmizao 1'; ZP, 'Zhanshanpingguozao'.

Compared with ZM1, the petals of ZP exhibited higher GalDH activity but lower GalLDH activity. *GME*, *GME1*, *GMP* and *GMPI* showed lower expression levels in ZM1 and ZP, respectively (Figure 7), which corresponded to the lowest levels of their AsA. Notably, *GalLDH* had higher expression in both ZM1 and ZP (Figure 7). Additionally, *GGP* expression was detected at a high level in ZP but was rarely detected in ZM1.

APX and AO showed higher activity in ZM1 and ZP, respectively (Figure 4C). Their activities were consistent with the highest T-AsA content. The expression level of *AO* was lower in jujube petals. Compared with ZM1, the petals of ZP exhibited higher expression of *DHAR* and *MDHAR*.

Correlation analysis

To deeply explore the mechanism of AsA accumulation in ZM1 and ZP, the correlation between AsA content, biosynthetic enzyme activities and gene expression was analysed in fruits of different varieties with contrasting AsA content. As shown in Figure 8A, in ZM1, the expression levels of AsA-related genes were not significantly correlated with the AsA pool, except for the *GME*, *GME1* and *GGP*, which showed a positive correlation with the AsA pool, respectively. Similarly, *GMPI* displayed a positive correlation with DHA. The activities of APX, GalDH and GalLDH were positively correlated with the AsA pool, whereas AO activity was negatively correlated with the AsA pool. On the contrary, in ZP, the expression levels of AsA-related genes were positively correlated with AsA, DHA and T-AsA, but not significantly correlated with AsA/DHA ratio, except for *GPP*, which was negatively correlated with the AsA pool. APX activity was negatively correlated with the AsA pool, and the activity of AO was negatively correlated with DHA and T-AsA. GalDH activity was significantly correlated with AsA, DHA and T-AsA. However, GalLDH activity was not significantly correlated with the AsA pool.

During the leaf growth (Figure 8A), in ZM1, the expression levels of *GME*, *GME1*, *GMPI*, *GalLDH* and *DHAR* were positively correlated with the AsA pool, whereas the expression levels of *GPP*, *GGP* and *APX* were negatively correlated with the AsA pool. The expression levels of *GMP*, *GalDH* and *MDHAR* were not significantly correlated with the AsA pool. The activity of APX was negatively correlated with the AsA pool, whereas the activity of AO was positively correlated with the AsA pool. GalDH and GalLDH were not correlated with the AsA pool. Meanwhile, in ZP, the expression levels of all the genes were correlated with AsA, DHA and T-AsA, but none of them were significantly correlated with the AsA/DHA ratio. The activity of AO was not significantly correlated with the AsA pool. The activities of APX and GalDH were negatively correlated with AsA, DHA and T-AsA, whereas the activity of GalLDH was positively correlated with AsA, DHA and T-AsA. Apart from APX and AO, the activities of GalDH and GalLDH were not significantly correlated with the AsA/DHA ratio.

DISCUSSION

Characteristics of AsA accumulation in jujube fruit, leaf and petals

AsA content can vary significantly among species or cultivars, such as peaches (Imai et al., 2009), strawberries (Araguez et al., 2013) and blackcurrants (Ioannidi et al., 2009). In this study, the AsA content in ZP fruit was higher than that in ZM1 fruit from 10 DAF to 30 DAF, while ZM1 fruit had higher AsA content than ZP fruit from 50 DAF to 90 DAF. Since the growing environment and harvesting times of ZM1 and ZP were similar, the differences in AsA content were likely genetically determined. The patterns of AsA accumulation vary among varieties. For example, during fruit development, the total AsA content remained unchanged or increased slightly in oranges (Alos et al., 2021), decreased in apples (Fang et al., 2017) and sweet cherry (Liang et al., 2017), and increased in grapes (Cruz-Rus et al., 2010) and chestnut rose (Huang et al., 2014). In this study, AsA content increased during the early stage of fruit development, peaked at the white mature stage and then gradually decreased with fruit ripening. This pattern suggests that rapid AsA accumulation during the early stage may promote fruit growth and that AsA accumulation mainly occurs during the fruit expansion stage. Our results were consistent with the findings for kiwifruit (Lin et al., 2022) and blueberry (Liu et al., 2015), but differed from those for northern fresh jujube (Chen, 2015).

The T-AsA content in the green photosynthetic tissues of species or cultivars is generally higher than that in fruits, such as apple (Li et al., 2010a), kiwifruit (Li et al., 2010b) and strawberry (Bulley et al., 2012). Nevertheless, in this study, the T-AsA content in the leaves of ZM1 and ZP was much lower than that in the fruit. The results were consistent with the findings for acerola (Badejo et al., 2007), Chinese jujube (Zhang et al., 2016) and *Rosa roxburghii* fruit (An et al., 2007; Yan et al., 2015). This result may be attributed to the transfer of AsA content from the leaves to the fruits. DHA content was higher than AsA content during the leaf growth, a finding consistent with studies in apple (Li et al., 2010a) and *R. roxburghii* fruit (Li et al., 2017). This result suggests that jujube leaves may contain high levels of oxidised substances. In *R. roxburghii*, AsA content remained at a high level with no significant change during the early stage of leaf development (Li et al., 2017). However, in this study, AsA content increased as the leaves matured and decreased when the leaves began to senesce, which is similar to the study of apple leaves (Li et al., 2010a). It suggests that the rate of AsA synthesis exceeds the rate of oxidation loss in young leaves, whereas oxidation loss exceeds biosynthesis in senescent leaves. Moreover, the change in AsA content in the leaves was more pronounced but significantly different from that in the fruits. The analysis of AsA and DHA content revealed that petals had the lowest of T-AsA levels, which were 120 times lower than the maximum levels detected in fruits. Interestingly, the AsA content in

orange leaves differed from this study, with orange leaves showing the highest levels of T-AsA (Alos et al., 2021). The physiological reason for the accumulation of AsA in petals is not well understood. These findings suggest that each organ or tissue exhibits a distinct pattern of AsA accumulation during development.

AsA metabolism-related enzymes play crucial roles in AsA accumulation in jujube fruit, leaf and petals

The activities of the relevant metabolic enzymes showed different trends in ZM1 and ZP. In this study, GalDH activity correlated significantly with AsA content in ZM1 during fruit development, while in ZP, it was continuous throughout fruit development. The result indicated that GalDH plays a significant role in the AsA synthesis process. A study on 'Jinsixiaozao' (Wang et al., 2019) showed that GalLDH activity correlated with changes in AsA content. In this study, GalLDH activity was low during the early stage of fruit development and increased significantly with ripening in ZM1 and ZP, suggesting that GalLDH may play a role in synthesis at maturity. In the most widely accepted L-galactose pathway, GalDH and GalLDH are the key enzymes (Wheeler et al., 1998). In apple (Li et al., 2010a), changes in GalDH activity showed a strong correlation with AsA content in leaves of different ages. In this study, GalDH activity was negatively correlated with AsA content in ZP leaves, and AsA content remained low during leaf growth. Further investigation is necessary on the role of GalDH in controlling AsA synthesis via the L-galactose pathway. GalLDH activity was positively correlated with AsA in ZP leaves. As suggested by Ishikawa et al. (2007), GalLDH activity is strongly correlated with AsA content in developing tissues and cells. In addition, in ZM1, the activities of GalDH and GalLDH were not correlated with AsA content during leaf growth, suggesting that GalDH and GalLDH do not significantly affect AsA synthesis in ZM1 leaves. Young leaves exhibited much lower DHA content and much higher AO and APX activities compared with mature leaves, yet both had low AsA content. The results indicated that much of the AsA in young leaves may be oxidised and broken down, and mature leaves may contain higher levels of oxides. AO and APX activities were particularly low in aged leaves of ZP and ZM1, suggesting that leaf senescence may be associated with a decrease in APX and AO activities. GalDH and GalLDH activities were extremely low, while AO and APX activities were high in petals of ZP and ZM1. Meanwhile, ASA content was low, and DHA content was high. The results implied that AsA in petals is likely oxidised due to the high DHA/AO activity of ZP and ZM1.

AsA metabolism-related genes play a key role in AsA accumulation in jujube fruits and leaves

AsA biosynthesis is recognised as the primary determinant of AsA accumulation in most plant

varieties, though the predominant metabolic pathways vary among taxa. Current evidence indicates that *in situ* synthesis serves as the principal mechanism for AsA accumulation in numerous varieties, including blackcurrant (Ioannidi et al., 2009), kiwifruit (Li et al., 2010b) and strawberry (Duan et al., 2012). In this study of ZM1 and ZP, we identified eight cDNA sequences associated with the L-galactose pathway. The results of qRT-PCR showed that eight genes were expressed during the development of ZM1 and ZP. The expression patterns of *GMP*, *GGP* and *GalLDH* exhibited strong correlation with AsA accumulation patterns in both cultivars from 10 DAF to 50 DAF. The expression of *GME* and *GME1* in ZM1 fruits showed 'M' type changes during development, but the general trend was first increasing and then decreasing, which was consistent with the changes. These findings align with previous studies demonstrating that *GME1*, *GMP1*, *GGP*, *GPP* and *GalLDH* function as key regulators of AsA biosynthesis during 'Junzao' fruit development, while *GME2* and *GMP2* appear particularly important during the ripening stages (Zhang et al., 2016). The expression of *GMP1* in ZM1 fruits showed a general trend of increasing and then decreasing, but the expression of the gene increased from 50 days to 70 days. The reason for analysing may be that the *GMP* gene regulates the generation of mannose from D-mannose-1-phosphate, which is the substrate of the L-galactose pathway, and the elevated expression of the *GMP1* gene synthesises more substrates of the L-galactose pathway, but the activity of the enzyme GalLDH decreased. Its gene expression is reduced, resulting in lower AsA content. Furthermore, *GalLDH* has been identified as the crucial gene controlling AsA production in 'Dongzao', 'Jinsixiaozao' and 'Zanhuangdazao' fruits (Chen, 2015). Collectively, these results strongly suggest that the L-galactose pathway represents the predominant route for AsA biosynthesis in southern jujube fruits, with *GME1*, *GMP*, *GGP*, *GPP*, *GalDH* and *GalLDH* serving as key regulatory genes. However, it is important to note that final AsA in fruits represents a complex balance between multiple processes, including not only biosynthesis but also catabolism, oxidation, reduction, and transport mechanisms (Ishikawa et al., 2007).

The results of this study showed that the expression of *AO* genes was low at the young fruit stage, whereas the results of the 'Junzao' study showed that the expression of most of the *AO*-type genes exhibited high expression during early fruit development. Similar studies have been documented in sweet orange (Alos et al., 2014), chilli pepper (Alos et al., 2013) and *R. roxburghii* Tratt (Huang et al., 2014). These discrepancies may reflect genotypic differences, environmental influences or the multifaceted roles of AO in physiological processes such as cell division and rapid fruit expansion (Sanmartin et al., 2007). Regarding the AsA regeneration pathway, our results demonstrated coordinated regulation by both *MDHAR* and *DHAR* in ZM1 and ZP fruits. In cherry (Eltelib et al.,

2011), AsA accumulation was not correlated with *DHAR*, but in *R. roxburghii* Tratt (Huang et al., 2014), *DHAR* was the key gene for AsA accumulation, indicating that the key genes of the AsA regeneration pathway were different in different varieties. Genotypes and the strength of transcriptional regulation of key genes for AsA synthesis, degradation and regeneration vary by environment.

The key genes for AsA synthesis in leaves of different varieties differ. *GPP* is the key gene for AsA synthesis in apple leaves, overexpression of the *GPP* gene in kiwifruit led to a dramatic increase in AsA content, and co-expression of *GPP* and *GME* in *Arabidopsis thaliana* led to a seven-fold increase in AsA content. In this study, we found that the expression of *GMPI* and *GalLDH* in ZM1 leaves was similar to the changes in their AsA content, suggesting that the L-galactose pathway plays an important role in the synthesis of AsA in southern fresh jujube leaves, and that *GMPI* and *GalLDH* play a key role in the synthesis of AsA in ZM1 leaves. In ZP leaves, on the other hand, it seems that we could not find a single gene whose expression change was consistent with the change of its AsA content, which was similar to the study of *R. roxburghii* Tratt leaves (Huang et al., 2014), suggesting that some other pathways may be involved in the synthesis of AsA in jujube leaves, or post-transcriptional regulation may have occurred. In *R. roxburghii* Tratt leaves (Huang et al., 2014), *MDHAR* was the key gene of the cycling pathway, and *APX* was the main oxidase, and similar reports were also found in tomato (Stevens et al., 2008). The expression of *AO* in ZM1 leaves increased continuously, and the changes in the expression of *DHAR* were in line with the changes in the content of AsA, but the expression of *APX* and *MDHAR* was almost non-existent, which indicated that *AO* played a key role in the oxidation of AsA, and *DHAR* was the key gene of AsA regeneration in ZP leaves at 45 days and 85 days. These results suggest that the regeneration pathway plays an important role in the accumulation of AsA in fresh jujube leaves, but the key genes differed among genotypes.

CONCLUSIONS

In this study, we compared AsA content, the activities of enzymes and the expression of genes involved in AsA biosynthesis, degradation and regeneration between fruits, leaves and petals of ZM1 and ZP. The pattern of AsA accumulation in the two jujube fruits and leaves was similar, and ZM1 had a higher level of AsA content during fruit and leaf development. The analysis of AsA content revealed that fruits had the highest levels of AsA, while petals had the lowest levels. The activity of *GalDH* was correlated with AsA levels in ZM1 fruits, and the key enzymes involved in AsA synthesis were scarcely detected in the leaves of ZM1 and ZP. The L-galactose pathway is the predominant route for AsA biosynthesis in jujube fruits, with key expression of

synthesis genes (*GME1*, *GMPI*, *GPP* and *GalLDH*). *GME*, *GMP* and *GalDH* contribute to AsA biosynthesis in jujube leaves.

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AUTHOR CONTRIBUTIONS

YH drafted and edited the manuscript. GYS performed the experiments with YH and analysed the data. YW collected the data. KZ edited the manuscript with JYZ. ZCY revised the manuscript. QXD was the project investigator and research supervisor. Yong Huang and Guoying Song contributed equally to this work. The published version of this manuscript was revised and agreed upon by all authors. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIALS

Table S1. Primers for qRT-PCR.

Gene	Primer sequence (5'-3')	Gene	Primer sequence (5'-3')
<i>GME</i>	F:AGAATGAGCACATGACCGAAGA R:TCAGCAGCAAGGTTGAAGACA	<i>APX</i>	F:CCTGAAATCCCATTCCATC R:GCACCTTCCCAGAGTATGAC
<i>GME1</i>	F:GGCACCTACCATGAGATTG R:AGATGACCCATAAATTGACAG	<i>AO</i>	F:GGTGAGTGGACGGAGAA R:GGATTTACAACCCTAACATA
<i>GMP</i>	F:GAAGGCACTTATTCTTGTCG R:GGATCATAGGTTTGTTA	<i>DHAR</i>	F:CCCTGAACCTGCCCTTGT R:CAGCGGTAACCTTCTCCC
<i>GMP1</i>	F:CAATAGCGATGTTATCAGTG R:GATGGCTCGTCAACCTTA	<i>MDHAR1</i>	F:GCAAAACCTGCTGTCAGTCCC R:TCAACTCGTGCCATACGGTC
<i>GGP</i>	F:TGAGCGTTTGCCACAGA R:ACCGTCCAACCTTGATTATTT	<i>MDHAR2</i>	F:GCCATAGCCAAAGCCACAAGG R:GAGTAGATCCACGACTATCGG
<i>GPP</i>	F:GGTGGTGCTGTTATTGTTA R:GGTTTGAAGCTGCTACTCT		
<i>GalDH</i>	F:GGTGTCCAAGAAATGAGTAT R:CTCCTAGTCACTCTATCAGC		
<i>GalLDH</i>	F:AGCAGATTGGAGGCATTA R:CGATTGTTCCCTTAGCAG GCACCTTCCCAGAGTATGAC		

qRT-PCR, quantitative real-time PCR.