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RESEARCH REPORT

Comparison of ascorbate metabolism in fruits of two jujube species with differences in ascorbic acid content

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Abstract

Jujube fruits contain high levels of ascorbic acid (AsA). However, the AsA contents of fruits differ significantly in various jujube germplasm resources, and the factors responsible for these differences remain unknown. To explore the metabolic mechanism of AsA accumulation, we investigated the AsA content in fruits at different developmental stages of two jujube species, *Ziziphus acidojujuba* cv. Yuanxingxiaosuanzao (SZ) and *Ziziphus jujuba* Mill. cv. Luojiangtiaoyuanzao (LZ). The AsA accumulation pattern in the fruits of the two jujube species was similar during fruit development, and AsA content was negatively correlated with titratable acid. We also analysed the expression patterns of AsA metabolism genes. The results showed that AsA biosynthesis in jujube fruits was mainly related to the L-galactose pathway, as well as the inositol pathway. Furthermore, higher expression levels of genes involved in AsA biosynthesis (*GME1*, *GMP1*, *GPP*, *GGP*, and *MIOX1*) and regeneration (*MDHAR1* and *MDHAR2*) were associated with higher AsA concentrations in SZ fruit compared with LZ fruit. In summary, we concluded that more efficient AsA biosynthesis and regeneration was responsible for the higher AsA accumulation in SZ fruit.

Keywords Jujube · Ascorbic acid · Gene expression · L-galactose pathway · Transcriptional regulation

Abbreviations

AsA	Ascorbic acid
GME	GDP-D-mannose 3′,5’-epimerase
GMP	GDP-D-mannose pyrophosphorylase
GPP	L-galactose-1-P phosphatase
GGP	GDP-L-galactosephosphorylase
MIOX	Inositol oxygenase
MDHAR	Monodehydroascorbate reductase
GME	GDP-D-mannose-30,50-epimerase
GalDH	L-galactose dehydrogenase
GalLDH	L-galactono-1,4-lactone dehydrogenase
GalUR	D-galacturonate reductase
AO	Ascorbate oxidase
APX	Ascorbate peroxidase
DHAR	Dehydroascorbate reductase
DHA	Dehydroascorbic acid
TA	Titratable acid

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1 Introduction

Ascorbic acid (AsA), also known as vitamin C (VC), is an essential metabolite in plants and animals (Magwaza et al. 2017). AsA participates in cell wall synthesis, cell division and growth, photosynthesis, senescence hormone synthesis and other metabolic processes in plants, and also plays an important role in the antioxidant protection under biotic and

abiotic stresses (Akram et al. 2017; Chen et al. 2021; Wang et al. 2010). Furthermore, AsA is crucial to maintain human health, such as reducing the risk of cardiovascular diseases, cancer, cataracts, senescence, and other diseases related to oxidative stress (Davey et al. 2000). Humans cannot synthesize AsA owing to a lack of L-gulonolactone oxidase (GulLO) (Huang et al. 2014; Fitzpatrick et al. 2012), therefore fruits and vegetables are the best contributor of AsA in human nutrition.

Four AsA biosynthesis pathways have been identified in plants, namely, L-galactose, D-galacturonate, L-gulose, and myo-inositol pathways (Fenech et al. 2019). Among these, the L-galactose pathway is generally considered the major AsA synthesis pathway in plants (Wheeler et al. 1998), in which AsA is synthesized from D-mannose-1-phosphate through a series of enzymatic reactions. In addition, the regeneration and degradation of AsA is essential for maintaining AsA accumulation in plants (Cruz-Rus et al. 2011). AsA concentration is controlled by a balance between biosynthesis, regeneration and degradation (Ishikawa et al. 2006; Yang et al. 2011) (Fig. 1).

Chinese jujube is the most important species in the large cosmopolitan family Rhamnaceae in terms of its economic, ecological, and social importance (Liu et al. 2020). Jujube fruit is reputed as a "natural vitamin pill" due to its high AsA concentration (Liu et al. 2013). There are abundant germplasm resources of jujube in the world, but their fruits differ significantly in AsA content (Wojdyło et al. 2016; Huang et al. 2017). Although AsA levels in jujube fruit have been preliminarily researched, there is very little and contradictory information about the regulation mechanism. The presence of two AsA biosynthetic pathways has been confirmed in cultivar 'Dongzao', L-galactose and myo-inositol pathways, using de novo genome sequencing (Liu et al. 2014). Moreover, *GMP1*, *GMP2*, *GME1*, *GME2*, *GGP* and *GalDH* were involved in AsA accumulation during fruit development and in different genotypes in cultivar 'Junzao' (Zhang et al. 2016). GalLDH, APX and MDHAR were closely associated with high AsA content in 'Jinsixiaozao' and 'Qingjiansuanzao' cultivars (Chen et al. 2016; Huang et al. 2021). Hence, the genes involved in AsA accumulation among jujube germplasm resources remains unclear.

In this study, we systematically investigated the AsA content as well as the expression profiles of genes involved in AsA metabolism and compared the fruit development of 'Yuanxingxiaosuanzao' (wild jujube accession) and 'Luojiangtiaoyuanzao' (cultivated jujube cultivar). The results could provide a theoretical basis for further studies into the quality and molecular regulation of AsA in jujube fruit.

2 Materials and methods

2.1 Materials

We collected *Ziziphus acidojujuba* cv. Yuanxingxiaosuanzao (SZ) and *Ziziphus jujuba* Mill. cv. Luojiangtiaoyuanzao (LZ) in the village of Luojiang County, Sichuan Province, China (31°26′34″ N, 104°42′18″ E). The growth conditions were hilly areas with purple soil, and belong to the subtropical humid monsoon climate, with an annual average temperature of 16.5 °C, average precipitation of 910 mm, a frost-free period of 278 d, and average sunshine duration of 1260 h.

Nine trees of seven-year-old of LZ and SZ with similar growth and field management were selected and randomly divided into three groups of three trees, respectively. At least

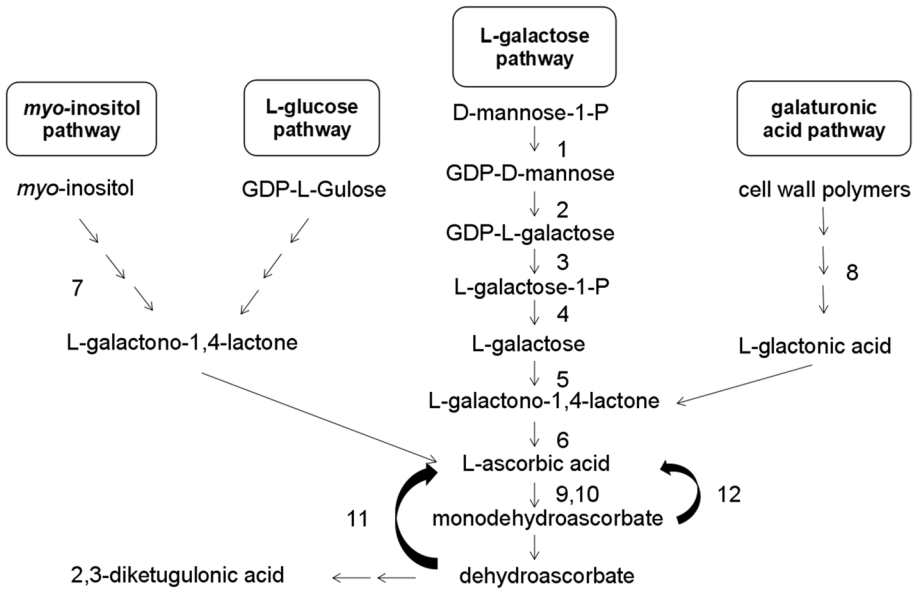

Fig. 1 The pathway of AsA metabolism in plants. Note: 1, GDP-D-mannose pyrophosphorylase (GMP); 2, GDP-D-mannose-30,50-epimerase (GME); 3, GDP-L- galactose phosphorylase (GGP); 4, L-galactose-1-phosphate phosphatase (GPP); 5, L-galactose dehydrogenase (GalDH); 6, L-galactono-1,4-lactone dehydrogenase (GalLDH); 7, Inositol oxygenase (MIOX); 8, D-galacturonate reductase (GalUR); 9, ascorbate oxidase (AO); 10, ascorbate peroxidase (APX); 11, Dehydroascorbate reductase (DHAR); 12, Monodehydroascorbate reductase (MDHAR)

20 fruits were randomly collected as a biological replicate, and three replicates were prepared. Fruits at five developmental stages, i.e., young [10 days after anthesis (DAA)], enlarging (30 DAA), white mature (50 DAA), beginning-red (70 DAA), and half-red (90 DAA) were harvested, labeled as stages I, II, III, IV, and V, respectively. All fruit samples were cut into small pieces, immediately frozen in liquid nitrogen, and stored at − 80 °C.

2.2 Soluble sugar and titratable acid content analysis

The soluble sugar content was determined by anthrone colorimetry (Weng et al. 2013) and the titratable acid (TA) content was determined by acid–base titration (Chutichudet et al. 2008).

2.3 AsA content analysis

The AsA content determination method was essentially the same as that used for chestnut rose (Huang et al. 2014) by high-performance liquid chromatography (HPLC). Frozen tissue (0.5 g) was added to 0.2% metaphosphoric acid (5 mL) and ground using a precooled mortar. The resulting homogenate was centrifuged at 12,000 rpm for 15 min (4 °C), and the supernatant was diluted to 10 mL with 0.2% metaphosphoric acid and filtered through a 0.45 μm nylon filter for determination. The AsA content was determined using a HPLC system (Agilent 1260) with a photodiode array detector, and a reversed-phase C18 column (4.6 mm × 250 mm, 5 μm). The mobile phase was composed of 15% methanol and 85% metaphosphoric acid aqueous solution at pH 2.5, using an injection volume of 10 μL, a flow rate of 0.5 mL·min^{-1}, and a column temperature of 35 °C. AsA quantification was performed at 243 nm.

2.4 RNA extraction, cDNA synthesis, and gene expression analysis using real-time PCR

Total RNA was extracted using the modified cetyl trimethylammonium bromide (CTAB) method (Chang et al. 1993). First-strand cDNA was synthesized using a PrimeScript First Strand cDNA Synthesis Kit (Takara, Japan). The genes involved in AsA metabolism were identified from the report of Zhang et al. (2016). 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 and primers were designed according to Zhang et al. (2016) and Chen et al. (2016) (Supplementary Table S1). 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 by qRT-PCR and melting-curve analysis. The 25 μL reaction system contained 2 × SYBR Premix ExTaq (12.5 μL), 10 μmol·L^{-1} primers (0.5 μL of each), H_2O (10.5 μL), and diluted cDNA (1 μL). Gene expression levels were calculated using the formula $2^{-\Delta\Delta CT}$.

2.5 Statistical analysis

Statistical analyses were conducted using the SPSS 20.0 statistical software (IBM, Chicago, IL, USA). Data were analyzed by one-way analysis of variance, with significant differences (Duncan′s multiple range test) assessed at the 5% confidence level. Pearson correlation analysis was carried out between AsA content and sugar and TA contents during fruit development.

3 Results

3.1 Fruit growth analysis

SZ and LZ had similar harvest seasons, with the fruits harvested in batches and sorted into five developmental stages by size and color (Fig. 2). During fruit development, the growth curves of the two jujube species were in the shape of a single ‘S’ variation, which showed that the expansion of fruit cells and volume mainly occurred at stages II and III (Fig. 2B and 2C). From stage I to II, both fruit peels were green, with the fruit peels of SZ and LZ becoming white at stages III and IV, respectively. Both fruit peels became half-red at stage V. The results showed that LZ fruit developed faster than SZ fruit in the early stage of fruit development.

3.2 Changes in soluble sugar and TA contents during fruit development

The contents of soluble sugar and TA were assessed during fruit development (Fig. 3A and 3B). The soluble sugar content had the same ‘rise-fall-rise’ trend as in the development of both SZ and LZ fruits, with low soluble sugar contents during early fruit development that continuously increased after stage III. The TA content was high at stage I, but decreased at stage II and then increased as the SZ and LZ fruits ripened. At stage V, the soluble sugar contents were 19.64% and 16.19%, while the TA contents were 0.36% and 0.95% in LZ and SZ fruits, respectively. Notably, SZ fruit had significantly lower soluble sugar content, but significantly higher TA content compared with LZ fruit.

3.3 Changes in AsA content during fruit development

The AsA accumulation in LZ and SZ fruits was similar during fruit development (Fig. 3C). The AsA concentration

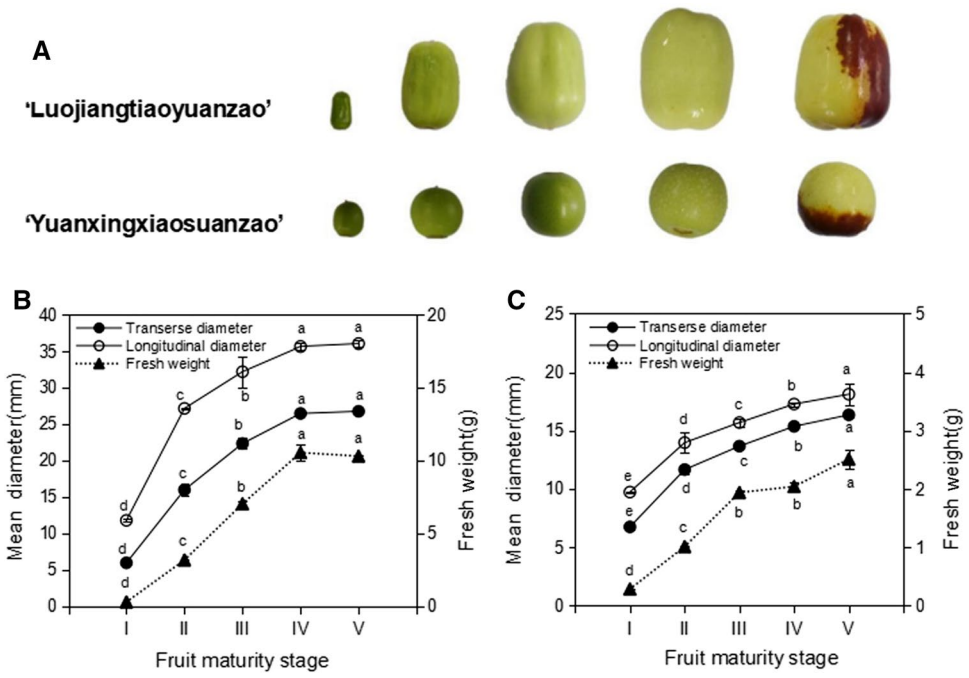

Fig. 2 Fruit developmental stages of two jujube species. **A** Whole fruit separated into five stages. **B** Dynamics of growth and development of LZ fruit. **C** Dynamics of growth and development of SZ fruit. Values are means of three replicates ± SD and different letters indicate significant differences (Duncan′s multiple range test, $p < 0.05$)

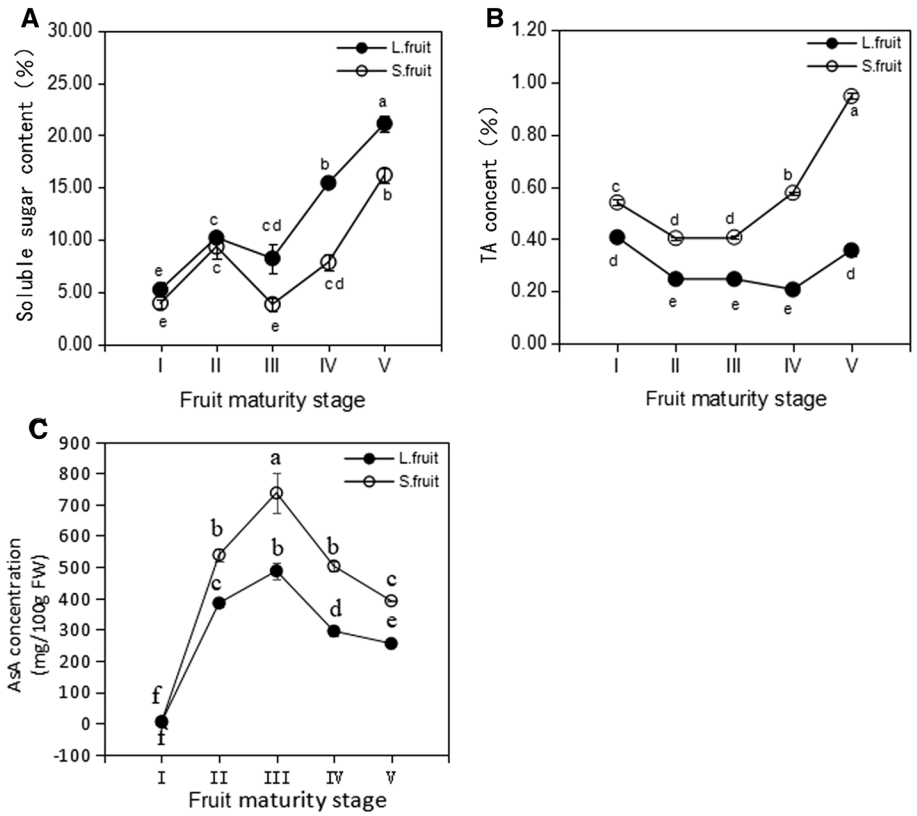

Fig. 3 Changes of AsA, soluble sugar and TA contents during fruit development of two jujube species. **A** Changes of soluble sugar content during fruit development. **B** Changes of TA content during fruit development. **C** Changes of AsA content during fruit development. "L" indicates 'Luojiangtiaoyuanzao' (LZ) and "S" indicates 'Yuanxingxiaosuanzao' (SZ). Values are means of three replicates ± SD and different letters indicate significant differences (Duncan′s multiple range test, $p < 0.05$)

in both LZ and SZ fruits was low at the young fruit stage, peaked at stage III, and then declined with fruit ripening. Notably, the AsA content in SZ was higher than that in LZ during fruit development. At stage V, the AsA contents

of SZ and LZ fruits were 391.27 and 254.80 mg/100 g^{-1} FW, respectively.

3.4 Correlation of AsA content with soluble sugar content and TA content during fruit development

The correlation analysis showed that the positive correlation between AsA content and soluble sugar content was not significant in the two jujube species (Table 1). The AsA content was negatively correlated with TA content, but positively correlated with the sugar to acid ratio during LZ fruit development. Furthermore, there was no obvious correlation between AsA content and TA content and the sugar to acid ratio in SZ fruit.

3.5 Expression profiles of genes involved in AsA biosynthesis during fruit development

We examined the expression patterns of seven AsA biosynthetic genes during fruit development of LZ and SZ (Fig. 4A). The expression patterns of *GME1*, *GMP1*, *GalDH*, and *GalLDH* were similar in LZ and SZ fruits. The expression levels of *GME1* and *GMP1* were upregulated and then downregulated, peaking at stage III, and the expression levels of *GME1* and *GMP1* were 1.5-fold higher in SZ than in LZ at stage III. Furthermore, *GalDH* and *GalLDH* maintained high expression levels at stages III and V, with higher expression levels observed in LZ. *GGP* transcripts accumulated rapidly during early fruit development until stage III in both SZ and LZ fruits, and the *GGP* expression level at stage III was 6.9-fold higher in SZ fruit than in LZ fruit. *GPP* maintained the highest expression level at stage V in the fruits of SZ and LZ. For *MIOX1*, the highest expression level was observed at stage III in SZ fruit, but an increase in expression was observed throughout LZ fruit development. *GME1*, *GMP1*, *GGP*, *GPP*, and *MIOX1* had high expression levels, in agreement with the change in AsA content, which indicates these genes might be the key limiting factors involved in AsA biosynthesis in jujube.

Table 1 Correlation of AsA content with soluble sugar content and TA acid content during fruit development of two jujube species

Index	Soluble sugar content	TA content	Sugar to acid ratio
AsA content in LZ fruit	0.204	−0.797**	0.603*
AsA content in SZ fruit	0.042	−0.287	0.314

*Denotes significant difference at 0.05,

**Denotes significant difference at 0.01

3.6 Expression profiles of genes involved in AsA degradation and recycling during fruit development

We also analyzed the expression profiles of AsA degradation and recycling genes in the jujube fruits (Fig. 4B). As SZ fruit ripened, the expression of *AO1*, *AO2*, *AO3*, and *AO4* significantly increased and then decreased, while the expression of *AO5* showed no significant change. In contrast, the expression of *AO1*, *AO3*, and *AO4* showed little change in LZ fruit, while the expression level of *AO5* was low at the early stages and then rose sharply with fruit ripening. The expression of *APX3* increased continuously during LZ fruit development, while *APX1*, *APX2*, and *APX4* showed a rise–fall–rise expression pattern similar to that in SZ. Regarding AsA recycling genes, the expression of *MDHAR1* and *MDHAR2* showed no significant change during LZ fruit development, but significantly increased during SZ fruit development at stages III and V. For *DHAR*, LZ and SZ showed similar trends, with two peaks observed at stages III and V, but a higher expression level was maintained in LZ fruit.

4 Discussion

4.1 The difference of AsA content in fruits of two jujube species is determined by multiple factors

AsA accumulation can vary between species or cultivars, as observed in oranges (Martí et al. 2009), blueberries (Liu et al. 2015) and tomatoes (Mellidou et al. 2012b). In the present study, the AsA content in SZ fruit was significantly higher than that in LZ fruit. As the growing environment and harvesting times of LZ and SZ were similar, the differences in AsA content were speculated to be mainly genetically determined.

Sugar is the precursor for AsA and could regulate the expression of enzymes involved in AsA metabolism as a signaling molecule (Sawake et al. 2015). In this study, soluble sugar and AsA contents were not necessarily related (Table 1), which was similar to previous results (Massot et al. 2010; Yang et al. 2013). Although the total soluble solids contents were clearly higher in 'Newhall' than in 'Dream', the AsA contents in pulps of the two cultivars were similar (Yang et al. 2011). Furthermore, the AsA content showed a very significant negative and positive correlation with the TA content and sugar to acid ratio during LZ fruit development, respectively (Table 1). Together, these differences in AsA content in LZ and SZ appeared not to be linked to primary metabolism of sugars during maturation, while low acid concentration may be considered an advantage for AsA accumulation in LZ fruit. Similar results have been reported in apple and tomato (Fang et al. 2017;

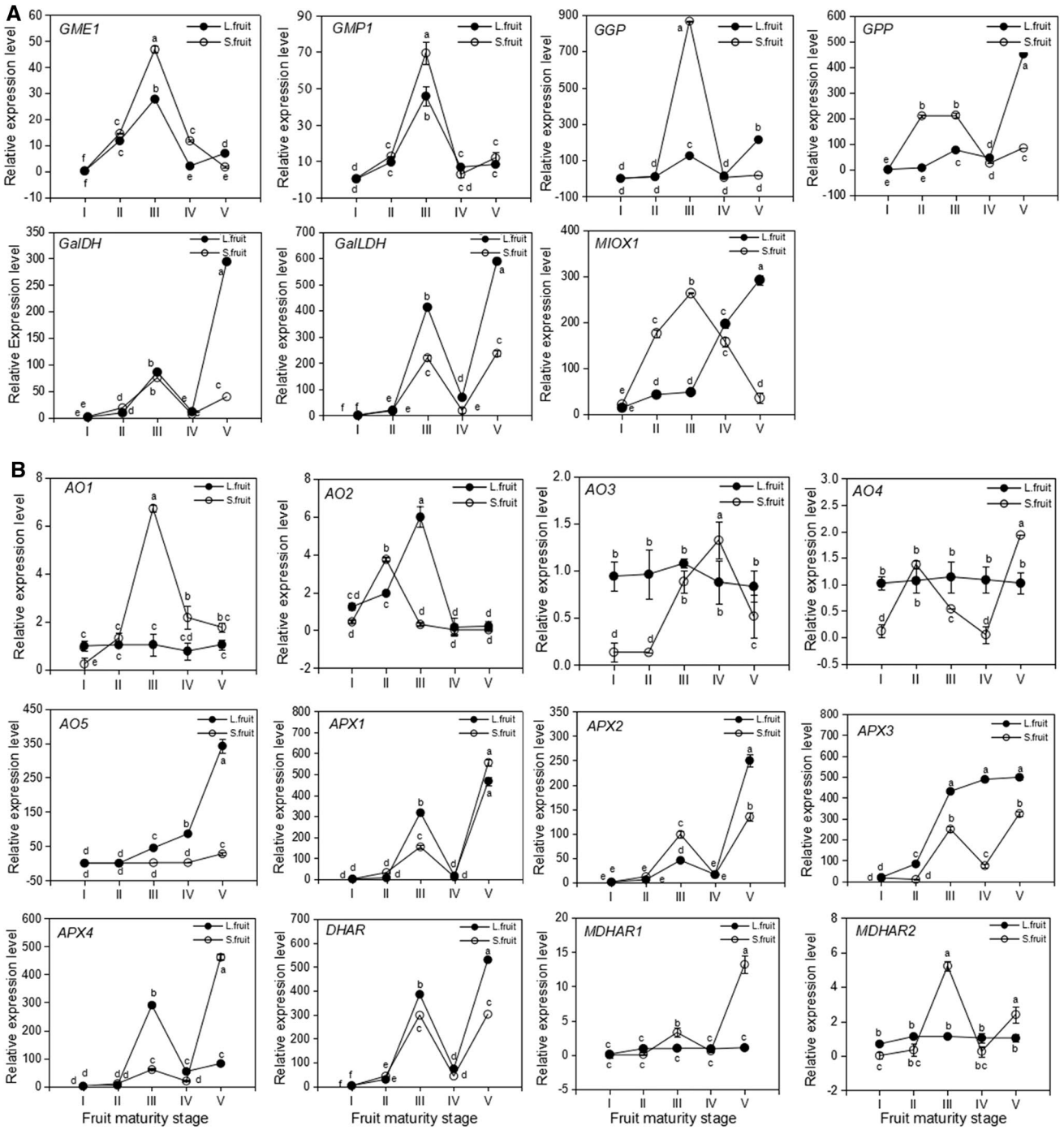

Fig. 4 Relative expression levels of genes involved in AsA biosynthesis and recycling during fruit development. **A** Relative expression levels of genes involved in AsA biosynthesis. **B** Relative expression levels of genes involved in AsA recycling. Values are means of three replicates ± SD and different letters indicate significant differences (Duncan's multiple range test, $p < 0.05$)

Mellidou et al. 2012b). The high concentration of AsA in plants improves the resistance to extreme environments, which is the main reason why the AsA content of wild jujube accessions is higher than that of cultivated jujube cultivars (Mellidou et al. 2021). The difference in AsA content of jujube fruits between two genotypes was the indirect result of human selection of large, low-acid and high-sugar fruits (Zhang et al. 2016).

The pattern of AsA accumulation in different species varies. For instance, the total AsA content remained unchanged or increased slightly in strawberry (Agius et al. 2005) and oranges (Yang et al. 2011), decreased in apple (Li et al.

2011) and sweet cherry (Liang et al. 2017), and increased in grape (Cruz-Rus et al. 2010) and chestnut rose (Huang et al. 2014) during fruit development. In this study, AsA content increased gradually at the early stage of fruit development, peaked at the white mature stage, and then decreased gradually with fruit ripening, which implied that the final content in jujube fruit was subject to developmental regulation. Our results were similar to the findings for kiwifruit (Li et al. 2010) and blueberry (Liu et al. 2015).

4.2 AsA biosynthesis plays crucial roles in AsA accumulation in jujube species

The L-galactose pathway is generally regarded as the main route of AsA biosynthesis (Fenech et al. 2019). GMP converts D-mannose-1-P into GDP-D-mannose (Badejo et al. 2009), and GME converts GDP-D-mannose into GDP-L-galactose (Huang et al. 2014; Liu et al. 2015). GGP catalyzes GDP-L-galactose to release L-galactose-1-P (Bulley et al. 2012; Mellidou et al. 2012a). In this study, the expression patterns of *GMP1*, *GME1* and *GGP* showed the best correlation with the change in AsA content in both jujube species during fruit development. *MIOX* is the key gene involved in the myo-inositol pathway (Fenech et al. 2019). We found that *MIOX1* was differentially expressed during LZ and SZ fruit development, indicating that the inositol pathway plays diverse roles in AsA accumulation in various jujube species.

More transcripts of *GME1*, *GMP1*, *GGP*, *GPP*, and *MIOX1* were detected in SZ than in LZ before stage IV, particularly at stage III, which was in agreement with the higher AsA content in SZ. These results indicated that the L-galactose and myo-inositol pathways play predominant roles during SZ fruit development, and the higher expression of *GME1*, *GMP1*, *GGP*, *GPP*, and *MIOX1* promoted higher AsA concentrations in SZ fruit. The higher transcript levels of *GME*, *GGP*, *GalDH* and *GalLDH* in orange was the main reason for its higher ascorbate content than in Satsuma mandarin (Alós et al. 2014). Liu et al. (2015) demonstrated that higher expression levels of *GME*, *GGP*, and *GalDH* were associated with higher AsA content in 'Bluecrop' compared with 'Berkeley'. These results suggested that induction of the genes involved in AsA biosynthesis could be vital for the differences in AsA content between genotypes during jujube fruit development.

4.3 The degradation and regeneration of AsA play crucial roles in AsA accumulation in jujube species

AsA consumption and recycling was mainly determined by the oxidation of AO and APX, and reduction of MDHAR and DHAR (Fenech et al. 2019). In this study, expression of the genes involved in the degradation and regeneration of AsA varied during jujube fruit development. *APX1*, *APX2*, *APX3*, and *AO5* showed the highest expression levels in mature LZ and SZ fruit, which was consistent with the decline of AsA content in another jujube cultivar (Zhang et al. 2016). The significant upregulation of *AO* and *APX* might be the main reason for the decreased AsA content in the latter stages of fruit development (Huang et al. 2014; Liu et al. 2015).

In this study, *DHAR* expression was closely related to the high AsA accumulation at stages III and V of SZ and LZ fruits. In addition, the up-regulation of *MDHAR1* at stage III and *MDHAR2* at stage V contributed to the AsA accumulation in SZ fruit, but had no significant effect on LZ fruit. These results indicated that *MDHAR* and *DHAR* are key genes in AsA regeneration of SZ fruits, while *DHAR* is a key factor in LS fruits. We speculated that the higher AsA regeneration efficiency was partially responsible for higher AsA accumulation in SZ fruit, especially regarding *MDHAR* expression. *MDHAR* and *DHAR* coregulated AsA content and had a complementary relationship in maintaining the redox state of AsA during the development of kiwifruit (Li et al. 2010), bilberry (Cocetta et al. 2012) and blueberry (Liu et al. 2015). In another study, the change in AsA content correlated well with *MDHAR* expression and was negatively correlated with *DHAR* expression in strawberry (Cruz-Rus et al. 2011). These results indicated that the AsA regeneration pathway differs in various species.

5 Conclusion

In this study, we compared AsA concentration and expression profiles of genes involved in AsA biosynthesis, degradation, and regeneration between fruits of LZ and SZ. The pattern of AsA accumulation in the two jujube species was similar, and SZ had a higher level of AsA content during fruit development. AsA content was negatively correlated with TA content. The L-galactose and inositol pathways are the predominant routes for AsA biosynthesis in jujube fruits, with higher expression of synthesis genes (*GME1*, *GMP1*, *GGP*, *GPP*, and *MIOX1*) and a recycling gene (*MDHAR*), possibly responsible for the higher AsA concentration in SZ fruits.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13580-022-00431-9.

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Author contributions YW drafted and edited the manuscript, and assisted GYS in completing the experiment. GYS performed the experiments with YW and analyzed the data. DL proposed the re-search, and facilitated the work. HX collected the data. HFZ edited the manuscript with YW. XL revised the manuscript. QXD was the project investigator and research supervisor. 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.

Declarations

Conflict of interest Authors declare no conflict of interest.

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