

Article

Multi-Omics Analysis of High-, Medium-, and Low-Ascorbic-Acid Jujube Cultivars Identifies Potential Key Regulatory Genes in Ascorbic Acid Metabolism

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Abstract

Ascorbic acid (vitamin C) is a critical antioxidant influencing the nutritional quality of fruits. This study integrates metabolomic and transcriptomic analyses to investigate high-ascorbic-acid (HAA), medium-ascorbic-acid (MAA), and low-ascorbic-acid (LAA) jujube cultivars. Metabolomic profiling revealed significant enrichment of ascorbic acid and its precursors in HAA cultivars, whereas MAA and LAA cultivars showed lower levels. Transcriptomic analysis identified 107 differentially expressed genes (DEGs) across the three groups. Compared with LAA, 139 differentially accumulated metabolites (DAMs) were identified in HAA, with more down-accumulated (77) than up-accumulated (62); compared with MAA, 235 DAMs were found in HAA, with significantly more up-accumulated (180) than down-accumulated (55); 250 DAMs with different accumulation patterns between LAA and MAA included 199 up-accumulated and 51 down-accumulated; and 16 DAMs were common in all three comparison combinations. Potential key regulatory genes such as GDP-mannose-3',5'-epimerase (GME) and L-galactose-1-phosphate phosphatase (GPP) were highlighted. Weighted Gene Co-expression Network Analysis (WGCNA) detected possible core genes participating in ascorbic acid biosynthesis pathways. This study provides novel insights into the genetic and metabolic basis of ascorbic acid accumulation, potentially providing valuable targets for molecular breeding.

Keywords: jujube; metabolomic; transcriptomic; ascorbic acid metabolism

1. Introduction

Jujube (*Ziziphus jujuba* Mill.), a globally esteemed fruit species, boasts a rich nutritional composition and extensive medicinal properties [1]. The fruit is notably characterized by its high ascorbic acid (AsA) content [2,3]. AsA, a crucial antioxidant, plays a pivotal role in human health by countering oxidative stress and augmenting immune function [4,5]. The biosynthesis and metabolism of AsA are influenced by genetic factors, environmental conditions, and developmental stages [4,6–8]. Previous research employed the jujube variety “Mazao” as experimental material. Through an integrated analysis of the transcriptome and metabolome, the study investigated the changes in differential genes and metabolites during jujube fruit development and screened potential key genes regulating AsA. The findings indicated that AsA was primarily synthesized during the young fruit stage and the expansion stage, and the ZjMDHAR gene played a significant role in the regeneration cycle of AsA. A total of three gene networks/modules highly correlated with



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AsA were obtained, from which three candidate genes regulating AsA were identified, namely ZjERF17 (LOC107404975), ZjbZIP9 (LOC107406320), and ZjGBF4 (LOC107421670). These results offer a theoretical foundation for the in-depth exploration of key transcription factors regulating AsA anabolism in jujube [9].

The genetic and biochemical foundation of ascorbic acid (AsA) accumulation in jujube continues to be a subject of significant research interest. As is evident in other crops, such as tea (*Camellia sinensis*), where the chemical composition of the leaves is predominantly determined by genetic constitution, environmental factors, and manufacturing procedures [10,11], it is hypothesized that analogous principles govern AsA accumulation in jujube fruits. In jujube, diverse metabolites, including flavonoids, amino acids, lipids, and terpenoids, display differential accumulation among cultivars [12–14]. Studies that integrate widely targeted metabolomics and transcriptomics have offered insights into the metabolic and transcriptional regulation of these bioactive compounds [15–18], providing a framework for comparable research in jujube.

Utilizing advanced multi-omics methods, encompassing metabolomics and transcriptomics, presents an unparalleled opportunity to clarify the molecular mechanisms underpinning AsA biosynthesis and regulation in jujube. High-throughput technologies, such as liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) in conjunction with RNA sequencing, facilitate a comprehensive profiling of metabolic and gene expression alterations [19,20]. Weighted gene co-expression network analysis (WGCNA) has also been demonstrated to be effective in dissecting regulatory networks and identifying key candidate genes associated with specific traits, as evidenced in other horticultural crops, such as tea [21–23].

In contrast to prior research on the ASA anabolic pathway of a single variety across different periods, this study centers on presenting [9]. Through a literature review and preliminary physiological index detection, the jujubes to be tested were classified into three grades: the three varieties with the highest VC content were designated as HAA, those with medium VC content as MAA, and those with the lowest VC content as LAA.

In this study, a multi-omics approach is employed to explore the differential metabolic and transcriptional profiles of HAA, MAA, and LAA jujube cultivars. Through the integration of metabolomic and transcriptomic data, key metabolites and potential regulatory genes associated with AsA biosynthesis have been identified. Although genes involved in the ASA biosynthetic pathway in Chinese jujube have been reported in relevant studies, there are few reports on the roles of these genes in regulating fruit ripening and stress resistance, especially disease resistance. Our findings offer a more profound comprehension of the metabolic and genetic factors influencing AsA accumulation in jujube, which may lay the foundation for the development of high-AsA jujube cultivars via molecular breeding and genetic engineering.

2. Materials and Methods

2.1. Jujube Plant Materials and Chemicals

Fresh fruit samples of jujube cultivars with high (HZ), medium (MZ), and low ascorbic acid (LZ) contents were procured from the National Jujube Germplasm Repository (29°59' N, 118°49' E) in Shandong Province, China. The fruits were harvested at physiological maturity under standardized agronomic practices. Subsequently, they were promptly flash-frozen using liquid nitrogen and stored at −80 °C for subsequent analysis. Each cultivar was sampled in three independent biological replicates.

2.2. Metabolite Extraction and Detection

The extraction of freeze-dried samples was carried out in accordance with the procedure described by Chen et al. [24]. The samples were ground using a grinder and then dissolved in a 70% methanol solution, followed by storage at 4 °C overnight. During this time, the mixture was vortexed six times. After incubation, the supernatant was collected, filtered, and stored in sample vials for subsequent analysis. To evaluate repeatability, a quality control mix was incorporated into both test samples.

Internal standard information is as follows: name: 2-Chlorophenylalanine; purity: 98%; manufacturer: J&K Scientific; batch Number: LBCOR15; CAS number: 14091-11-3.

Metabolite profiling was performed using a UPLC–ESI–MS/MS system (SHIMADZU Nexera X2 UPLC (Shimadzu Corporation, Kyoto, Japan)) and Applied Biosystems 4500 QTRAP mass spectrometer (SCIEX, Boston, MA, USA). Chromatographic separation employed an Agilent SB–C18 column (1.8 µm, 2.1 mm × 100 mm), with mobile phase A being ultrapure water (0.1% formic acid) and mobile phase B being acetonitrile (0.1% formic acid). The gradient conditions adhered to those detailed in Lin et al. [25], with a flow rate of 0.35 mL min^{−1}, a column temperature of 40 °C, and an injection volume of 2 µL. Mass spectrometric analysis was conducted under electrospray ionization (ESI) conditions with the following settings: an ion source temperature of 550 °C, a positive ion spray voltage of 5500 V, a negative ion spray voltage of 4500 V, ion source gas I at 55 psi, gas II at 60 psi, and a curtain gas pressure of 25 psi. The collision-induced ionization was set at a high level.

Metabolites were identified based on their fragmentation patterns, retention times, and accurate m/z values through comparison with the standards in the MetWare Database (MWDB). Data processing was accomplished using Analyst 1.6.3 Software.

The widely targeted metabolomics analysis for plants uses Metware Database (MWDB), a plant-exclusive metabolite database independently developed by Metware. This database is constructed from multi-dimensional information, including secondary mass spectrometry (MS²), retention time (RT), accurate mass (m/z), collision energy (CE) and other parameters collected from a large number of plant samples via the UPLC–MS/MS system (SCIEX, Boston, MA, USA).

Plant widely targeted metabolomics uses the high-resolution mass spectrometer AB Sciex TripleTOF 6600 (SCIEX, Boston, MA, USA) for qualitative detection of pooled samples, and then uses AB Sciex 4500 QTRAP (SCIEX, Boston, MA, USA) for quantification. This approach combines the advantages of untargeted and targeted metabolomics: high-resolution mass spectrometry is used for accurate qualitative identification, and triple quadrupole mass spectrometry with high sensitivity, high specificity and excellent quantitative capability is used as a complementary tool. The parameters matched in metabolite identification include accurate Q1 molecular mass, secondary fragments, retention time, and isotopes. The qualitative and quantitative mass spectrometry analysis of metabolites in project samples is based on the MetWare Database (MWDB) and multiple reaction monitoring (MRM). Metabolite identification is based on the accurate mass of metabolites, MS2 fragments, isotope distribution of MS2 fragments, and retention time (RT). Through the intelligent secondary spectrum matching method independently developed by our company, the secondary spectra and RT of metabolites in project samples are intelligently matched one by one with the secondary spectra and RT in our company's database. The mass tolerance and MS2 tolerance are set to 20 ppm, and the RT tolerance is set to 0.2 min [26–28].

Missing values were imputed with one fifth of the minimum value of each row (each metabolite), then the CV value of QC samples was calculated, and metabolites with CV value less than 0.5 were retained to obtain the final data file [29]. Principal component analysis (PCA) was conducted using the R package (4.1.2) (<https://www.r-project.org/>)

project.org/) accessed on 31 July 2026. Significantly altered metabolites (SCMs) were defined as those with a variable importance in projection (VIP) score of ≥ 1.0 and a fold change (FC) of ≥ 1.5 or ≤ 0.67 , as determined by the OPLS-DA model.

2.3. Transcriptomic Analysis

Total RNA was extracted from the samples using an RNAprep Pure Plant Kit (DP441, TIANGEN Biotech, Waldshut-Tiengen, Germany). The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide. Sequencing was carried out on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA). The sequenced reads were trimmed by Fastp v0.20.1 and then mapped to the jujube genome using HISAT2 v2.2.1 [30]. Gene expression was estimated using StringTie v2.2.1 [31] with the -G option. The expression level was quantified using the number of reads per kilobase of exon sequence in a gene per million mapped reads (FPKM). DESeq2 software (Michael Love professor, North Carolina State University, the United States of America, github.com/mikelove/DESeq2) was utilized to conduct analyses of differentially expressed genes (DEGs) [32]. The *p*-value was corrected by multiple hypothesis tests, and the range of the *p*-value was determined by controlling the false discovery rate (FDR) [33]. In this study, $|\log_2(\text{fold-change})| \geq 1$ and $p\text{-adj} < 0.05$ were employed as the threshold for screening DEGs.

2.4. Weighted Gene Co-Expression Network Analysis (WGCNA)

WGCNA was employed to identify gene-metabolite associations critical for AsA regulation. Genes with TPM > 1 and metabolites from high-throughput LC-MS were integrated into a co-expression network constructed with soft-thresholding powers optimized at $\beta = 12$. Cytoscape software (v3.9.1) was used for visualizing modules enriched for candidate AsA pathways [34].

2.5. The Method of Identifying Potential Hub Genes

Candidate hub genes were pinpointed based on their intramodular connectivity within individual modules via the WGCNA package. Genes with module membership (MM) > 0.8 and gene significance (GS) > 0.5 were regarded as highly connected module-associated genes. The top genes ranked by intramodular connectivity (kWithin) were selected as representative potential hub genes for network visualization and candidate gene analysis.

2.6. Real-Time Quantitative (RT-PCR)

Quantification was performed through a two-step reaction process: reverse transcription (RT) and PCR. Each RT reaction consisted of 0.5 μg RNA, 2 μL of 5 $\times$ TransScript All-in-one SuperMix for qPCR, and 0.5 μL of gDNA Remover, with a total volume of 10 μL . Reactions were carried out in a GeneAmp[®] PCR System 9700 (Applied Biosystems, The United States of America) for 15 min at 42 °C and 5 s at 85 °C. The 10 μL RT reaction mix was then diluted 10-fold in nuclease-free water and stored at −20 °C. Real-time PCR was conducted using a LightCycler[®] 480 II Real-time PCR Instrument (Roche Diagnostics, Basle, Switzerland) with a 10 μL PCR reaction mixture that included 1 μL of cDNA, 5 μL of 2 $\times$ ChamQ SYBR qPCR Master Mix, 0.2 μL of forward primer, 0.2 μL of reverse primer, and 3.6 μL of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche, Swiss) at 94 °C for 30 s, followed by 45 cycles of 94 °C for 5 s and 60 °C for 30 s. Each sample was run in triplicate for analysis. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. The primer sequences were designed in the laboratory and synthesized by TsingKe Biotech (Beijing, China) based on the mRNA sequences obtained from the NCBI database as follows:

the sequence of primers is shown in Supplemental Table S12. The expression levels of mRNAs were normalized to *ZjUBQ* and were calculated using the $2^{-\Delta\Delta C_t}$ method [35].

2.7. Statistical Analysis

All statistical analyses were performed using R software (4.1.2) unless otherwise specified. Data are presented as mean $\pm$ standard deviation (SD) from three independent biological replicates. Statistical differences among different experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for multiple comparisons. A p value < 0.05 was considered statistically significant. Pearson correlation analysis was performed to evaluate the associations between gene expression levels and metabolite abundances. The significance of correlations was determined based on the correlation coefficient (r) and corresponding p values, with $|r| \geq 0.8$ and adjusted $p < 0.05$ considered significant.

3. Result

3.1. Observation and Characterization of Different Jujube Cultivars

We conducted an extensive study on three widely cultivated elite jujube tree lines from the same cross combinations ('Dongzao' $\times$ 'Jinsisihao'), namely the Dongyou series (Dongyou No. 1, Dongyou No. 2, Dongyou No. 3), the Jindong series (Jindong No. 1, Jindong No. 2, Jindong No. 3), and the Jinyou series (Jinyou No. 1, Jinyou No. 2, Jinyou No. 3), as detailed in Supplemental Table S1. All cultivars were harvested at the half-red stage, a commonly recognized maturity point for jujube fruit (Figure 1a). To gain insights into the metabolic variations among jujube cultivars with differing ascorbic acid (AsA) levels, we classified the cultivars into three distinct groups based on their AsA content: High-Ascorbic-Acid Group (HAA), Medium-Ascorbic-Acid Group (MAA), and Low-Ascorbic-Acid Group (LAA) (Figure 1b). The results revealed clear distinctions in AsA content across these groups, with the LAA group showing the lowest AsA content, followed by the MAA group, and the HAA group exhibiting the highest AsA levels.

Notably, fruit sizes also varied between the groups, with the LAA group possessing the largest horizontal diameter (average 28.60 mm) (Figure 1c) and vertical diameter (average 30.24 mm) (Figure 1d, Supplemental Table S1). We further evaluated other key physiological parameters such as total acid content (TA) and sugar content in the different jujube cultivars. However, a significant increase in sugar content was observed in the MAA group compared to both the HAA and LAA groups (Figure 1e). Additionally, we identified that the total acid content in Jindong No. 2 was significantly higher than in the other cultivars, indicating potential variations in fruit composition within this series (Figure 1f), as detailed in Supplemental Table S2.

3.2. Metabolic Variations Across Different Jujube Cultivars

To explore the metabolic differences between jujube cultivars with high, medium, and low ascorbic acid (vitamin C) contents, we performed metabolomic analysis across the three groups: High-Ascorbic-Acid Group (HAA), Medium-Ascorbic-Acid Group (MAA), and Low-Ascorbic-Acid Group (LAA). Principal component analysis (PCA) revealed clear distinctions in metabolite profiles between different groups (Figure 2a), and hierarchical clustering analysis showed that different jujube cultivars had different metabolic patterns (Supplemental Figure S1a). Thus, the PCA and cluster analysis together indicated that the cultivars have distinct metabolite profiles. Data reliability analysis revealed that metabolomic data were highly replicable [Pearson correlation (r_P) ≥ 0.9] and qualified for further analyses (Supplemental Figure S1b). Furthermore, a total of 1121 non-volatile metabolites were detected in all jujube cultivars

(Supplemental Figure S1c, Supplemental Table S3), including 167 alkaloids, 181 amino acids and derivatives, 152 flavonoids, 57 lignans and coumarins, 94 lipids, 45 nucleotides and derivatives, 58 organic acids, 117 phenolic acids, 10 quinones, 10 tannins, 93 terpenoids and 137 other metabolites. Supplemental Table S4 has been added to this study, which provides a summary of comprehensive annotation information for all identified metabolites detected in the current research. This information covers metabolite names, molecular formulas, theoretical m/z values, observed m/z values, mass errors (in ppm), retention times (RT), and ion modes. The table also includes supporting evidence for metabolite annotation, specifically covering MS/MS fragmentation patterns and database matching information obtained from the MetWare Database (MWDB). Specifically, the HAA group showed a significantly higher abundance of metabolites associated with antioxidant defense, such as phenolic acids, terpenoids and flavonoids, compared to the MAA and LAA groups (Figure 2b).

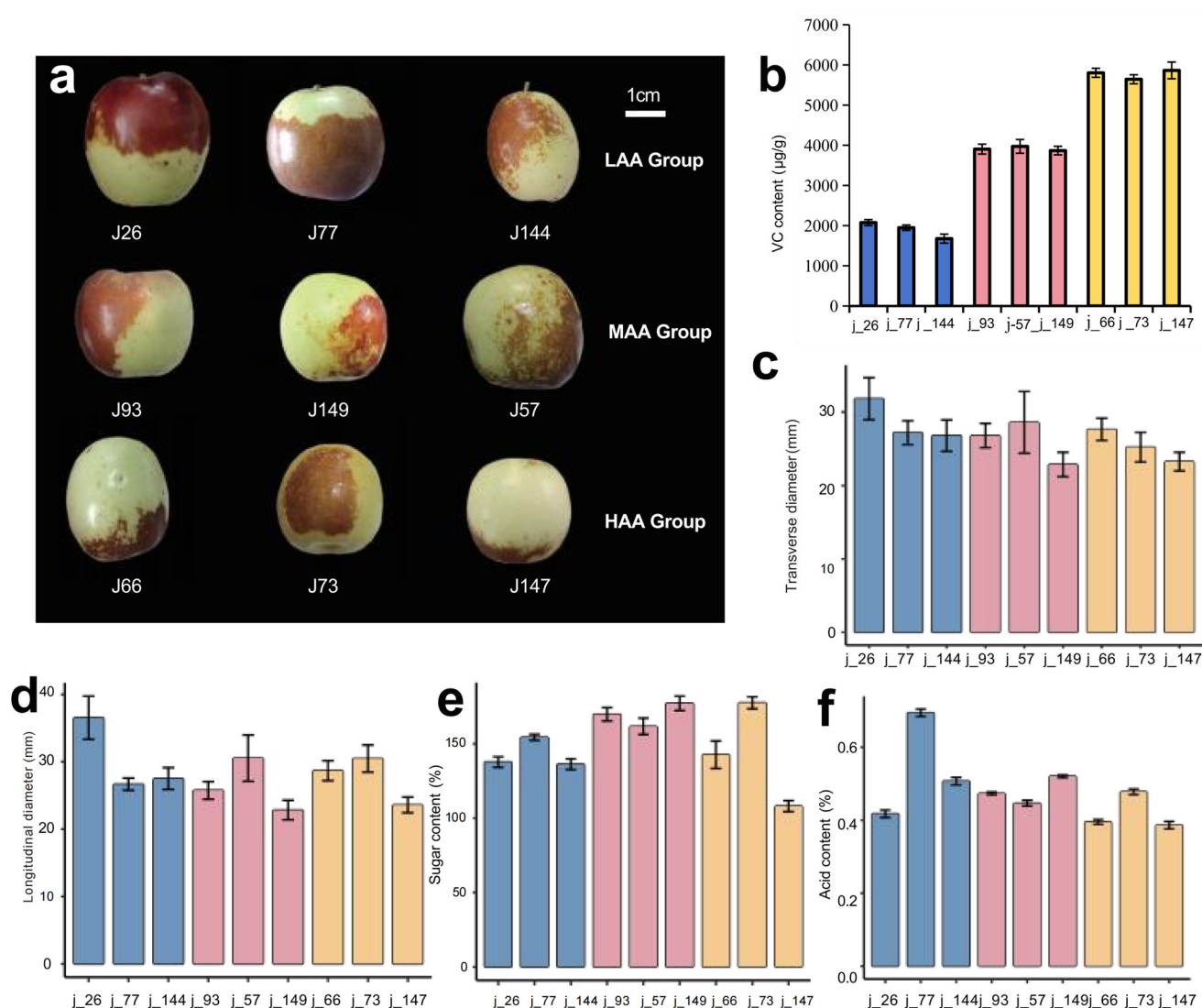


Figure 1. Phenotypic and physiological characteristics of jujube from three ascorbic acid content groups. (a) Representative fruit phenotypes of the low-ascorbic-acid (LAA), medium-ascorbic-acid (MAA), and high-ascorbic-acid (HAA) groups. Scale bar = 1 cm. (b–f) Quantitative comparisons of (b) ascorbic acid (VC) content, (c) transverse diameter (mm), (d) longitudinal diameter (mm), (e) sugar content (%), and (f) acid content (%) among the three groups. Data are presented as mean $\pm$ standard deviation (SD).

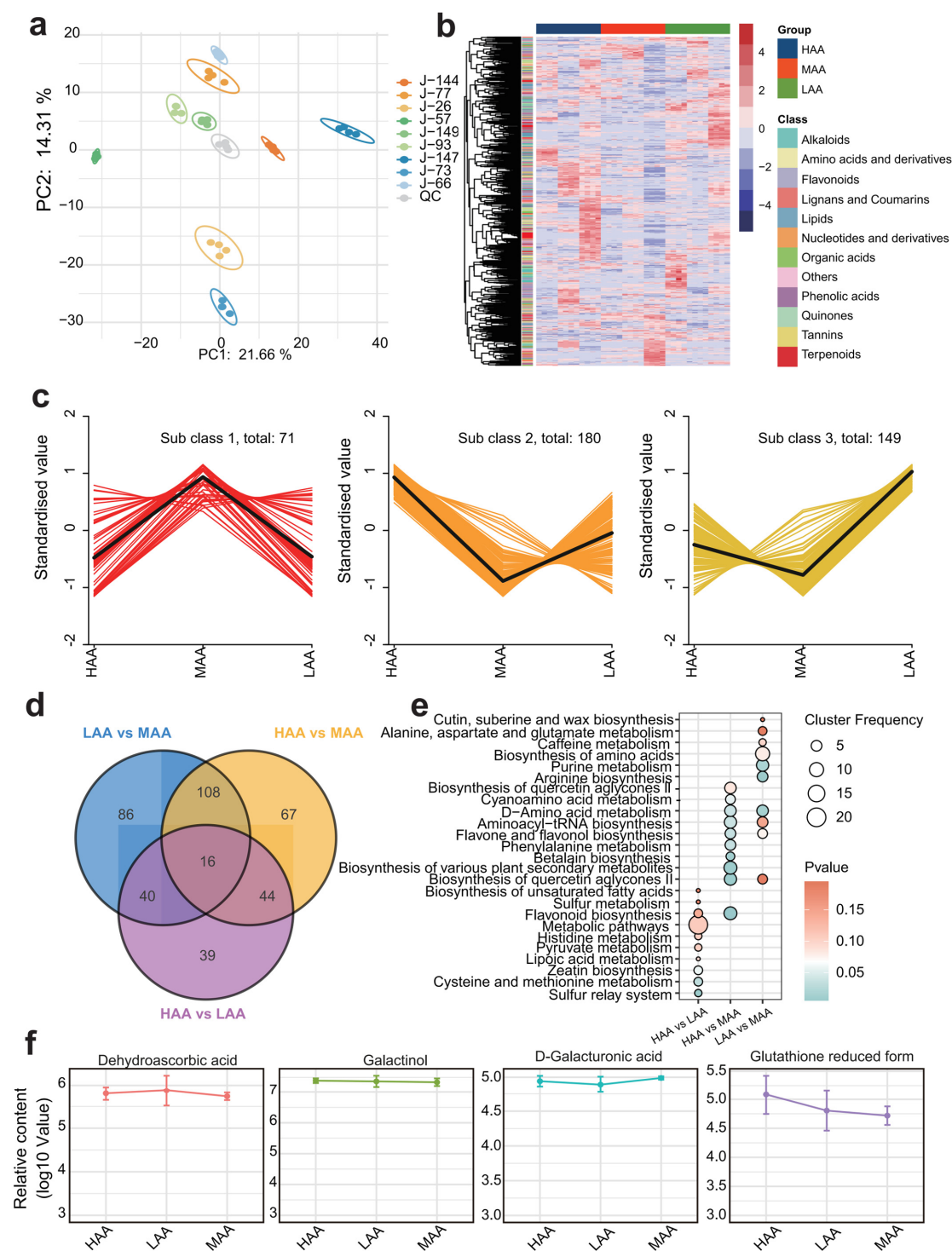


Figure 2. Metabolomic analysis of jujube fruits across three ascorbic acid content groups. (a) Principal Component Analysis (PCA) of metabolomic data. (b) Heatmap displaying metabolite profiles from the three ascorbic acid content groups. (c) k-Means clustering analysis of 550 differentially accumulated metabolites (DAMs). The x-axis represents the different groups and the Z-score of relative metabolite content. (d) Venn diagram illustrating differentially accumulated metabolites (DAMs) among jujube fruit groups. This diagram shows the overlap and specificity of DAMs identified between the LAA (low-AsA), MAA (medium-AsA), and HAA (high-AsA) groups. DAMs were defined based on $VIP \geq 1$ and $|\log_2 FC| \geq 1$. The numbers indicate group-specific and shared metabolites. (e) KEGG enrichment analysis of DAMs across different groups. (f) Dynamic changes in potential key metabolites involved in the sugar metabolism and starch degradation pathway. Error bars represent the standard deviation of replicates.

Further, orthogonal partial least-squares discriminant analysis (OPLS-DA) identified 400 differentially accumulated metabolites (DAMs) across the three groups (Supplemental Table S5), highlighting metabolic shifts that correlated with ascorbic acid levels. Clustering analysis (k-means) further categorized the metabolites into three distinct clusters (C1–C3), with the HAA group prominently featured in clusters C2, and the MAA group and LAA group were positioned in clusters C3 and C1, respectively (Figure 2c). Taking LAA as control, a total of 139 DAMs were identified in HAA group, with more down-accumulated metabolites (77) than up-accumulated metabolites (62) (Supplemental Figure S1d). When taking MAA as control, a total of 235 DAMs were identified in HAA group, with significantly more up-accumulated metabolites (180) than down-accumulated metabolites (55) (Supplemental Figure S1d). There were 250 DAMs which had different accumulated patterns between LAA and MAA groups, including 199 up-accumulated metabolites and 51 down-accumulated metabolites (Supplemental Figure S1d). A total of 16 DAMs were present in all three compare combinations (Figure 2d).

While inspecting the functions of DAMs, we found that the DAMs were involved in diverse metabolism and biosynthesis pathways (Figure 2e). These analyses revealed that the metabolism pathways of histidine, pyruvate, lipoic acid, zeatin, cysteine and methionine as well as the biosynthesis of unsaturated fatty acids, flavonoid, and zeatin in the comparison between HAA and LAA groups, and biosynthesis or metabolism of phenylalanine, flavone and flavonol were identified in the comparison between HAA and MAA groups (Figure 2e, Supplemental Table S6). We also investigated the relative content of several potential key metabolites, including dehydroascorbic acid, galactinol, D-galacturonic acid, and glutathione reduced form. These metabolites have previously been reported to be significantly correlated with ascorbic acid content. However, in our study, except for glutathione reduced form, the relative content of most metabolites showed little variation among different varieties of jujube (Figure 2f). In contrast, glutathione reduced form exhibited a significant positive correlation, with the highest content in the HAA group, and the content in the MAA and LAA groups was significantly lower than in the HAA group (Figure 2f). These metabolic and biosynthesis pathways underscore the biochemical differences driving the varying ascorbic acid contents in jujube cultivars.

3.3. Transcriptomic Changes in Different Jujube Cultivars

To uncover the molecular mechanisms underlying ascorbic acid content in jujube cultivars, we performed global transcriptomic analysis on the HAA, MAA, and LAA groups. An average of 7.15 Gb raw reads were obtained, and the average Q20 and Q30 values of those samples were 97.77 and 93.56%, respectively (Supplemental Table S7). This substantial dataset provided the foundation for all downstream analyses, with over 95% of reads successfully mapping to the jujube reference genome [36] with 95.67% mapping rate (Supplemental Table S8), demonstrating the exceptional quality of our sequencing results. Pearson analysis indicated that there was high repeatability (Supplemental Figure S2a). PCA of gene expression data revealed that PC1 (18.36%), PC2 (14.29%), and PC3 (10.47%) collectively explained 43.12% of the total variance and clearly separated the nine sample groups (Figure 3a), consistent with the metabolite PCA results.

Our differential gene expression analysis revealed substantial transcriptional variation among cultivars, identifying 1209, 2279 and 2382 differentially expressed genes (DEGs) with $|\log_2\text{FC}| \geq 1$ and $\text{FDR} < 0.05$ for each comparison, respectively (Supplemental Figure S2b, Supplemental Table S9). Particularly noteworthy was the comparison between HAA and MAA cultivar groups, which showed 1315 downregulated and 964 upregulated genes (Supplemental Table S9). Notably, the LAA_vs_MAA comparison exhibited the most

substantial transcriptional divergence, with 713 unique DEGs (Figure 3b), suggesting profound molecular differences between these two groups. In contrast, the HAA_vs_LAA and HAA_vs_MAA comparisons showed more moderate changes, with 360 and 636 unique DEGs (Figure 3b), respectively. Importantly, 107 DEGs were common to all three comparisons, potentially representing core regulatory elements involved in fundamental biological processes across conditions (Figure 3b).

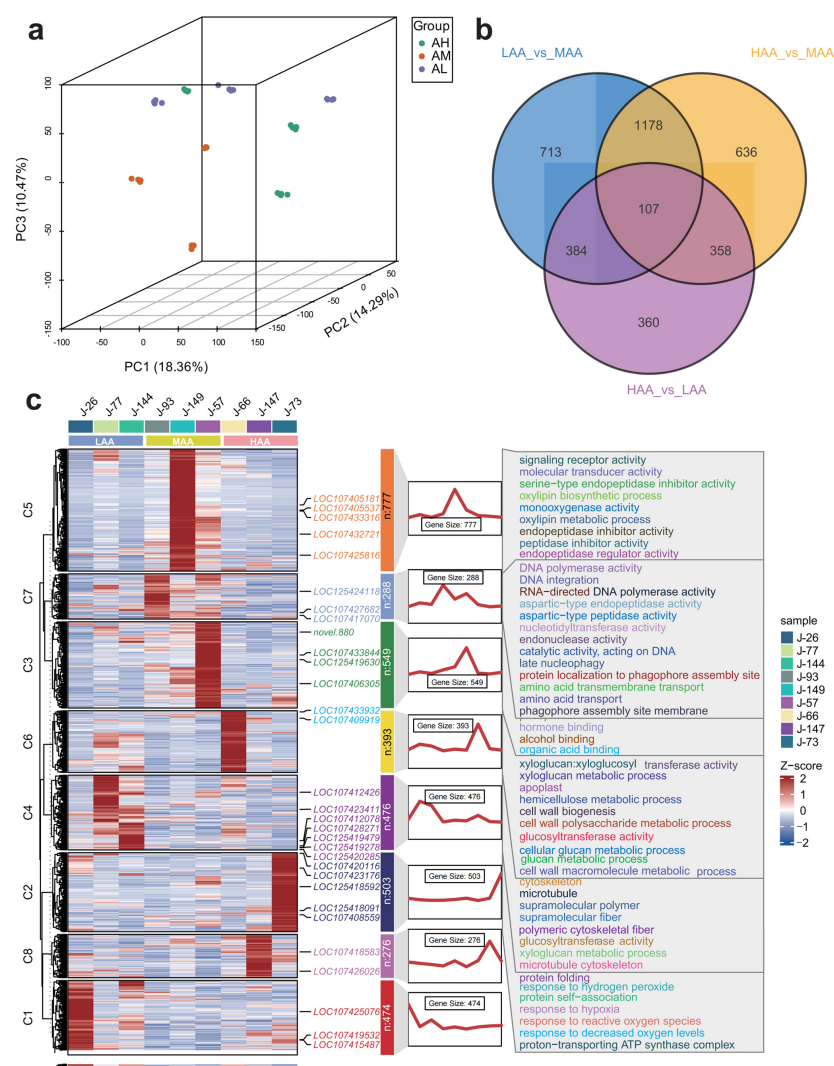


Figure 3. Global expression profile of three ascorbic acid content groups. **(a)** Principal Component Analysis (PCA) of the transcriptome data. **(b)** Venn diagram displaying differentially expressed genes (DEGs) among jujube fruit groups. This diagram visualizes the overlap and specificity of DEGs identified between the LAA (low-AsA), MAA (medium-AsA), and HAA (high-AsA) groups. DEGs were defined using a threshold of $|\log_2 \text{fold change}| \geq 1$ and a false discovery rate (FDR) < 0.05 . Numbers indicate the count of unique and shared DEGs across the comparisons. **(c)** ClusterGVis analysis of differentially expressed genes (DEGs) in jujube fruit. A heatmap shows the expression profiles of DEGs, which are grouped into distinct clusters based on their temporal or group-specific expression patterns. Each row corresponds to a gene, each column to a sample, and colors represent relative expression levels (normalized by Z-score). Line plots depict the average expression trends of genes within each cluster across the different jujube groups (LAA, MAA, HAA), with shaded areas representing standard deviations. Additionally, Gene Ontology (GO) enrichment analysis is performed for each expression cluster, highlighting the top significantly enriched biological processes. Clusters with distinct expression dynamics are linked to specific functional categories, thereby offering insights into the regulatory mechanisms that underlie ascorbic acid accumulation.

The KEGG enrichment analysis reveals potential key pathway responses across experimental comparisons. Phenylpropanoid biosynthesis emerges as the most significantly enriched pathway, containing the highest number of differentially expressed genes, indicating its central role in the observed transcriptional changes (Supplemental Figure S2c, Supplemental Table S10). Immune-related pathways, particularly plant-pathogen interaction, show strong but condition-specific activation patterns (Supplemental Figure S2c). Metabolic pathways like α -linolenic acid and starch/sucrose metabolism demonstrate consistent involvement across multiple comparisons, while hormone signaling pathways exhibit more variable responses (Supplemental Figure S2c). This pattern reflects cultivar-specific differences in secondary metabolism and stress responses.

Subsequently, we performed K-means clustering analysis on the standardized expression data to group genes with similar expression profiles. The genes in clusters C1 and C4, primarily associated with the HAA group, were significantly enriched in pathways involved in systemic acquired resistance, glucosyltransferase activity, and xyloglucan metabolic process (Figure 3c, Supplemental Table S11). Conversely, the HAA group predominantly clustered in C2, C6 and C8, where genes related to response to heat, response to oxygen levels, fruit ripening, and cellulose synthase activity, were more highly expressed (Figure 3c, Supplemental Table S11). Among these, *LOC107418583*, exhibiting high expression levels in the HAA group (Figure 3c), emerged as an O-glucosyltransferase rumi-like protein, which regulates O-glucosylation of Notch receptors, a critical post-translational modification in cell signaling. Another significant DEG, *LOC125418592* (FPKM 31.03-37.30 in HAA group), includes various enzymes, such as senescence marker protein 30 (SMP-30), gluconolactonase and luciferin-regenerating enzyme (LRE), potentially contributing to growth regulation and developmental differences among cultivars (Figure 3c).

3.4. Identification of Potential Genes Related to Ascorbic Acid Contents

Our comprehensive analysis of vitamin C metabolism in jujube cultivars revealed distinct patterns of gene expression and metabolic regulation underlying differential ascorbic acid (AsA) accumulation. We identified the candidate genes involved in the ascorbic acid (AsA) synthesis and recycling pathways, referencing previous reports on jujube ‘Dongzao’ [32]. Subsequently, using transcriptome data, we generated a heatmap to visualize the differentially expressed genes (DEGs) related to AsA metabolism in different jujube groups (Figure 4a). In this study, we identified 16 candidate genes associated with AsA synthesis and recycling, including hexokinase (*HXK*), D-glucose-6-phosphate isomerase (*PGI*), phosphomannose isomerase (*PMI*), phosphomannomutase (*PMM*), GDP-mannose pyrophosphorylase (*GMP*), GDP-mannose-3',5'-epimerase (*GME*), GDP-L-galactose (*GGP*), L-galactose-1-phosphate phosphatase (*GPP*), L-galactose dehydrogenase (*GalDH*), L-galactono-1,4-lactone dehydrogenase (*GalLDH*), L-gulonono-1,4-lactone oxidase (*GulLO*), and myo-inositol oxygenase (*MIOX*) for AsA synthesis, as well as ascorbate peroxidase (*APX*), ascorbate oxidase (*AO*), dehydroascorbate reductase (*DHAR*), and monodehydroascorbate reductase (*MDHAR*) for AsA recycling (Figure 4a). The transcriptomic data showed coordinated upregulation of the L-galactose pathway in HAA cultivars, with particularly strong induction of GDP-mannose-3',5'-epimerase (*GME*) and L-galactose-1-phosphate phosphatase (*GPP*). In contrast, alternative biosynthetic pathways showed either stable or reduced expression, confirming the L-galactose pathway as the dominant route for AsA biosynthesis in jujube. Correlation analysis revealed that the expression levels of *PMI2*, *PMM*, and *GMP1* were strongly positively correlated with the content of D-galacturonic acid, while *GMP1* expression also showed a high correlation with dehydroascorbic acid (DHA) levels, suggesting that *GMP1* may play a crucial role in AsA biosynthesis (Figure 4b).

a

D-Glucose $\xrightarrow{HXK}$ D-Glucose-6-P $\xrightarrow{PGI}$ D-Fructose-6-P $\xrightarrow{PMI}$ D-Mannose-6-P $\xrightarrow{PMM}$ D-Mannose-1-P $\xrightarrow{GMP}$ GDP-D-Mannose $\xrightarrow{GME}$ GDP-L-Galactose $\xrightarrow{GGP}$ L-galactose-1-P $\xrightarrow{GPP}$ L-Galactose $\xrightarrow{GalDH}$ L-Galactose-1,4-lactone $\xrightarrow{GalDH}$ Dehydroascorbate $\xrightarrow{DHAR}$ Ascorbic acid $\xrightarrow{MDHAR}$ L-Gulonono-1,4-lactone $\xrightarrow{GuILO}$ D-Glucuronic acid $\xrightarrow{MIOX}$ 1-D-myo-inositol $\xrightarrow{MIOX}$ D-Glucuronic acid

L-galactose pathway

Ascorbic acid pathway

Myo-inositol pathway

b

Cluster Dendrogram

Correlation matrix (Mantel's p, Mantel's r, Spearman's r)

c

Heatmap of gene expression across modules

d

Heatmap of metabolite levels across modules

e

Module pink

Network diagram showing interactions between genes and TFs

To further investigate the molecular mechanisms underlying ascorbic acid (AsA) accumulation in jujube, we conducted an integrated analysis of metabolomic and transcriptomic data. This comprehensive approach revealed intricate relationships between metabolic

pathways and gene expression patterns that contribute to the differential AsA content observed among HAA, MAA, and LAA cultivars. The metabolite-gene correlation network was constructed using stringent criteria ($|r| > 0.8$, $p < 0.01$), which identified three major functional clusters central to AsA metabolism. A total of 13,452 DEGs were used to conduct WGCNA (Figure 4c). After merging similar modules, 25 modules were generated, each of which comprised 114 to 2609 genes. We found that three modules were significantly correlated ($r \geq 0.7$, p -value < 0.05) with ascorbic acid and its intermediates (Figure 4d). Among these genes, the orangered 4 module was significantly correlated with galacturonic acid. The pink module was significantly correlated with dehydroascorbic acid, and the blue module was significantly correlated with reduced glutathione, respectively (Figure 4d).

Possible central genes within a module are typically regarded as representative of the module's biological function. We constructed a co-expression network to visualize these relationships (Figure 4e). Using eigengene connectivity (KME) values, the top 25 most highly connected genes in the pink module were selected to build the network, with transcription factors (TFs) identified as potential hub genes. Among these, 11 TFs were identified—including members of the ERF, WRKY, MYB, and MADS-box families—suggesting their potential roles in AsA biosynthesis (Figure 4e). These regulatory components and their interactions serve as valuable targets for molecular breeding strategies aimed at enhancing AsA content and improving the nutritional quality of jujube. The promising candidate genes and TFs identified here represent promising candidates for future functional studies and genetic improvement programs.

The genes used for qRT-PCR validation were determined by searching relevant literature for genes related to the ASA signaling pathway and other differentially expressed genes (DEGs). ERF098-like (LOC107405537), heat stress transcription factor A-5 (LOC107407375), ethylene-response factor C3 (LOC107411160), pathogenesis-related genes transcriptional activator PTI5-like (LOC107411165), cell division cycle 5-like protein (LOC107412324), scarecrow-like protein 14 (LOC107413462), myb-related protein 306 (LOC107415546), BTB/POZ and TAZ domain-containing protein 1 (LOC107418254), zinc finger CCCH domain-containing protein 64 (LOC107424000), transcription factor MYB13 (LOC107425754), trihelix transcription factor GT-1 (LOC107428405), retinoblastoma-related protein (LOC107428703), single-stranded DNA-binding protein WHY1, chloroplastic (LOC107428845), protein TIFY 6B (LOC107429275), heat stress transcription factor A-3 (LOC107432152), transcription termination factor MTERF9, chloroplastic (LOC107435498), the results showed that most genes had relatively stable expression levels among different varieties, except for a few genes (Figure 5).

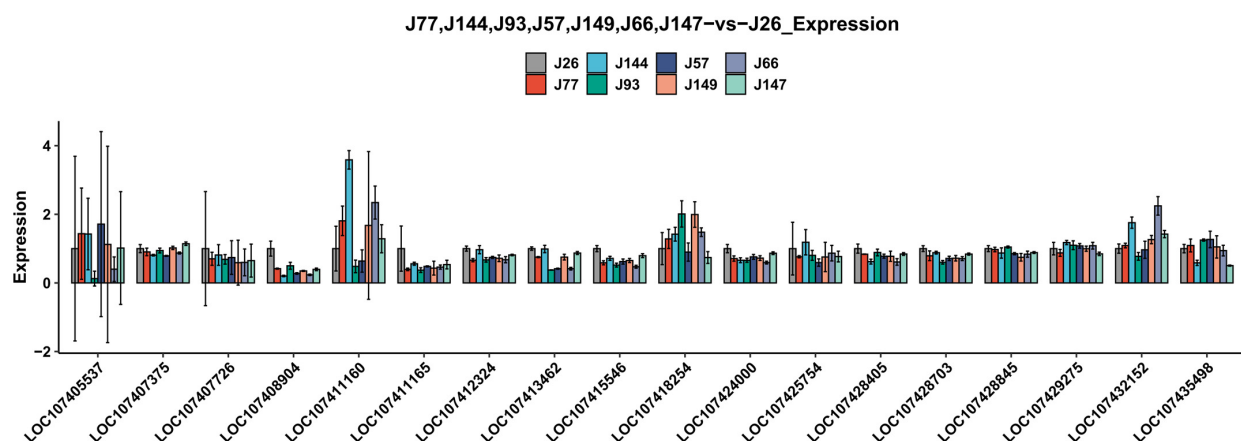


Figure 5. Quantitative real-time polymerase chain reaction (qRT-PCR) results. Indicating the relative expression level of 18 DEGs in the J26, J144, J147, J149, J57, J66, J77, and J93 cultivars.

4. Discussion

The vitamin C (VC) content of the selected jujube varieties was determined, and the nine varieties from the same environment were classified into three categories based on their VC content: high ascorbic acid (HAA), medium ascorbic acid (MAA), and low ascorbic acid (LAA). From the morphological perspective of these nine jujube varieties, jujubes from different categories also presented certain differences in acid and sugar content, while varieties within the same category were relatively similar. To further explore these differences, transcriptome and metabolome sequencing were carried out.

Ascorbic acid (AsA) can promote the regeneration of reduced phenolics, indicating that these antioxidant systems may function synergistically rather than independently. The authors reported that the HAA group had higher abundances of metabolites associated with antioxidant defense, such as phenolic acids, terpenoids, and flavonoids (Figure 2b). These secondary metabolites can either facilitate AsA metabolism or interact with AsA. Considering the functional interrelationship between AsA and phenolic compounds in redox homeostasis is of great significance. For example, AsA promotes the regeneration of reduced phenolics, suggesting that these antioxidant systems may act in synergy rather than operate independently. Moreover, AsA is involved in the AsA–glutathione (GSH) cycle, further highlighting its central role in the antioxidant network. AsA mitigates oxidative stress in seeds during natural dehydration and germination by scavenging ROS [37]. Transcriptomic analysis identified candidate differentially expressed genes (DEGs) and regulatory pathways associated with ascorbic acid (AsA) biosynthesis. The significant up-regulation of GDP-L-galactose phosphorylase (GPP) and GDP-D-mannose 3',5'-epimerase (GME) in HAA cultivars emphasizes their central role in determining AsA content. DEGs such as MYB transcription factors (MYB TFs), heat shock transcription factors (HSF TFs), and WRKY, which are also associated with resistance to high temperature, low temperature, salt stress, and involvement in plant biosynthesis, were significantly enriched with differential expression levels. Additionally, AsA regulates rice seed germination by influencing gibberellin biosynthesis and amylase gene expression, thereby affecting gibberellin (GA) content and amylase activity.

Figures 3c and 5 showed expression changes in genes related to the AsA synthesis pathway and differentially expressed disease-resistance-related genes (such as PTI5-like, a pathogenesis-related gene transcriptional activator of the AP2/ERF superfamily). The AP2/ERF superfamily is a plant-specific transcription factor family with an AP2/ERF DNA-binding domain of approximately 60–70 amino acids. PTI5-like proteins contain conserved motifs such as YRG and RAYD, which are crucial for DNA binding and pathogenesis-related (PR) gene transcriptional activation [38,39]. Moreover, this type of transcription factor can specifically recognize the GCC-box cis-element (TAAGAGCCGCC) in the promoter of ethylene-induced PR genes, thereby precisely regulating the expression of defense-related genes. In addition, multiple ethylene-response factor (ERF) family genes are differentially expressed in the transcriptome, suggesting that AsA biosynthesis may be regulated by hormones such as ethylene. Interestingly, the maturation periods of the nine varieties are not completely consistent, which may be related to the fact that during the stress-resistance process, some ERF genes inhibit ethylene synthesis [40], delay fruit ripening, and increase VC content. In this study, ERFs as differentially expressed genes were enriched, and the disease resistance pathway was discovered in HAA groups. There are few studies on ERF regulating the AsA synthesis pathway to change the disease resistance of jujube, which provides a novel and interesting direction for subsequent research. The HAA genome may participate in the ASA-GSH regulatory cycle by inducing the expression of multiple resistance genes, thereby enhancing the disease resistance of Chinese jujube, which may also be the innovation of the article.

In addition to ASA, other antioxidant systems, particularly flavonoids and anthocyanins, also make significant contributions to cellular redox balance during fruit development and ripening. In this study, HAA groups have higher terpenoids and flavonoids compared with other groups. These compounds can partially compensate for antioxidant capacity, and a negative correlation between ascorbic acid (AsA) content and pigmentation has been reported in some fruit systems. This functional complementarity should be considered when explaining differences in ascorbic acid levels among different varieties.

The identification of gene co-expression modules and promising hub genes offers valuable insights into the genetic regulation of ascorbic acid metabolism. These results are consistent with findings in other fruit crops such as tomato and kiwifruit, supporting the evolutionary conservation of AsA biosynthetic pathways. Future research should aim to functionally validate these candidate genes using genome-editing approaches like CRISPR-Cas9, paving the way for the development of nutritionally enhanced jujube varieties.

5. Conclusions

Our integrated multi-omics approach has elucidated the potential key genetic and metabolic mechanisms underlying ascorbic acid accumulation in jujube fruits. By identifying candidate regulatory genes like GPP, ERF and PTI5, this study offers development prospects and targets for molecular breeding aimed at improving jujube quality and antidisease properties.

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