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Integrated transcriptomic and metabolomic analyses delineate the biosynthetic pathways of functional components in maturing *Akebia trifoliata* fruit

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ABSTRACT

This study integrates transcriptomic and metabolomic analyses across five developmental stages of *Akebia trifoliata*, a climacteric fruit, to map the molecular mechanisms of fruit maturation. We identified key transcription factors, including NAC, MYB, and AP2/ERF families, and genes orchestrating a stage-specific metabolic transition. Early ripening stages were defined by flavonoid biosynthesis for protection and the initiation of starch degradation, supported by elevated expression of sucrose synthase, amylases, and alpha-amylase. Mid-stages featured enhanced phenolic synthesis, significant protein turnover, alongside differentially expressed cytochrome, E3 ubiquitin ligase, and glucan hydrolases. Final maturation was marked by a dramatic induction of invertase, leading to a surge in sucrose and fructose accumulation responsible for the fruit's characteristic sweetness. This work provides a comprehensive roadmap of ripening, revealing the coordination of flavonoid, hormonal, and carbohydrate pathways. These findings offer promising candidate genes and key metabolic markers associated with fruit quality traits. These findings provide a valuable genomic resource and lay the groundwork for future functional studies aimed at improving the nutritional value and sweetness of *A. trifoliata* through modern breeding techniques.

1. Introduction

The ripening process of fruits is a highly coordinated and complex series of biochemical, physiological, and molecular events that transforms immature fruit into a mature, palatable, and nutritious product (Barry, 2010; Martel & Giovannoni, 2007; Osorio & Fernie, 2013; Pareek, 2016; Zenoni et al., 2023). Central to this process are the hormonal regulators, particularly ethylene, which is well-known for its role in regulating ripening in climacteric fruits (Alexander & Grierson, 2002; Tipu & Sherif, 2024; Wang et al., 2022). Along with ethylene, other hormones such as auxins, abscisic acid (ABA), and gibberellins also contribute to the regulation of ripening (Gouthu & Deluc, 2015; Gupta et al., 2022; Kou et al., 2021; Park & Malka, 2022; Soto et al., 2013), though their roles may vary depending on the fruit species. The interaction between these hormones activates a cascade of gene expression changes that govern key metabolic pathways. These pathways include the breakdown of cell wall components, starch degradation, sugar accumulation, pigment biosynthesis, and the production of volatile compounds that contribute to the characteristic aroma and flavor of ripe fruits (Bhatla & Lal, 2018; Negi & Handa, 2008; Osorio et al., 2013; Pareek, 2016). At the core of these transcriptional cascades are key transcription factor (TF) families, including NAC, MYB, and AP2/ERF, which orchestrate stage-specific gene expression (Durán-Soria et al., 2020; Forlani et al., 2019). In model climacteric fruits like tomato, NAC TFs such as SlNAC1 and NOR function as master regulators that are essential for initiating the entire ripening program (Gao et al., 2018; M. Liu et al., 2024; Ma et al., 2014a). Similarly, MYB TFs are well-characterized for their role in controlling pigment accumulation (Hu et al., 2016; Xie et al., 2017), with examples like MdMYB1 in apple and VvMYBA1 in grape directly activating anthocyanin biosynthesis genes (Azuma et al., 2007; Walker et al., 2007). While these paradigms are well-established, it remains unclear whether such regulatory modules

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are universally conserved or if non-model fruits like *A. trifoliata* utilize distinct TFs or modified regulatory networks to control their unique ripening characteristics.

One of the first visible changes during ripening is the softening of fruit, which is mediated by the breakdown of cell wall components (Negi et al., 2008). Genes encoding enzymes such as polygalacturonase (PG), pectin methylesterase (PME), and cellulase are upregulated during this stage, facilitating the breakdown of pectin and cellulose, leading to the softening of the fruit's texture (Hou et al., 2019; H. Liu et al., 2018; Xue et al., 2020). At the same time, starch stored in the fruit is converted into sugars, contributing to the sweetness of ripe fruit (Cordenunsi-Lysenko et al., 2019; Gomez et al., 2002; J. Yu et al., 2022). This conversion is regulated by enzymes like ADP-glucose pyrophosphorylase (AGPase) and invertase, which break down starch and other polysaccharides into simpler sugars such as glucose, fructose, and sucrose. The increase in sugar content during ripening is essential for the development of fruit sweetness and flavor (Durán-Soria et al., 2020).

In addition to changes in texture and sweetness, fruit ripening is marked by the accumulation of pigments, which are primarily responsible for the color change in many fruits (Nagy et al., 2016; Rodrigo et al., 2013; Zhao et al., 2015). Carotenoids, such as beta-carotene and lycopene, accumulate in fruits like tomatoes and mangoes (H.-E. Khoo et al., 2011; Liang et al., 2020; Setyorini, 2021), while anthocyanins contribute to the red, purple, and blue colors in fruits such as berries and grapes (Glover & Martin, 2012; He et al., 2010; H. E. Khoo et al., 2017). The upregulation of genes such as phytoene synthase (*PSY*) for carotenoid biosynthesis and chalcone synthase (*CHS*) for anthocyanin biosynthesis is crucial for these color changes (Schijlen et al., 2007; M. Wu et al., 2020; Y. Zhang et al., 2022). The increased pigmentation not only makes the fruit visually appealing but also contributes to the fruit's antioxidant properties (Lu et al., 2021). Along with these pigments, volatile compounds that contribute to the aroma of ripe fruit are synthesized through the activity of genes involved in fatty acid, amino acid, and terpene metabolism, such as lipoxygenase (*LOX*) and alcohol dehydrogenase (*ADH*) (Defilippi et al., 2009; Osorio et al., 2010).

The integration of gene expression and metabolomic changes during ripening provides a comprehensive view of the molecular basis of fruit maturation (Guo et al., 2020; P. Li et al., 2023). As genes are expressed, they regulate metabolic processes that lead to changes in the fruit's biochemical composition, including sugars, organic acids, pigments, and volatile compounds (B. Zhang et al., 2010). For example, the decrease in organic acids, such as citric acid and malic acid, contributes to the sweet-tart balance that is characteristic of many fruits, while the increase in sugars enhances sweetness (M.-C. Wu et al., 2011). Metabolomic profiling, in combination with transcriptomic data, allows for a deeper understanding of how these changes occur in concert, offering insights into the regulatory mechanisms that control fruit ripening. This integrated approach also provides valuable information that can be used to optimize fruit quality, extend shelf-life, and improve harvest timing, ultimately benefiting both consumers and agricultural practices.

A. trifoliata is a fruit of significant ecological and nutritional value, known for its unique flavor, medicinal properties, and potential health benefits (Nazir et al., 2024; S. Zou et al., 2022). However, despite its importance, there is a lack of detailed research and information regarding its ripening process, the underlying molecular mechanisms, and the factors that influence its flavor, texture, and nutritional composition. The limited studies available focus primarily on its ecological characteristics, while its metabolic and transcriptomic profiles during ripening remain poorly understood. This gap in knowledge hinders the potential for improving fruit quality, optimizing harvest timing, and enhancing its commercial value. Metabolic and transcriptomic profiling of *Akebia* fruit is therefore crucial to fill this void, offering a deeper understanding of the fruit's biochemical processes and laying the groundwork for future studies that could unlock its full agricultural and medicinal potential.

The objective of this study is to explore the transcriptomic and metabolomic dynamics during the ripening of *A. trifoliata* fruits. By collecting samples at different ripening stages, we aim to elucidate the genetic and metabolic alterations that contribute to the transformation of the fruit from an unripe to a mature and edible state. Specifically, we focus on key markers of ripening, such as changes in sugar content, organic acids, and volatile compounds, as well as the expression of genes associated with these metabolic shifts.

2. Materials and methods

2.1. Sample collection

Pulp samples from three species of *Akebia*—*A. trifoliata* (AKT)—were collected at Lushan botanical Garden, Nanchang, China, grown under similar environmental conditions. The plants were established concurrently in 2022 and grown under identical field conditions, allowing for open pollination. Fruits were harvested at five distinct developmental stages of phase IV (Suspended expansion stage according to Yang et al., (Yang et al., 2021)) at 136,148,158,166,174 days after full bloom (DAFB). These developmental stages were designated as FA, FB, FC, FD, and FE, respectively. Ripening stage was defined physiologically as the initial appearance of natural longitudinal cracking along the ventral suture, which typically occurs upon ripening in this genus (S.-Y. Zou et al., 2023). To ensure genetic uniformity, all fruits were harvested from age-matched plants belonging to the same clonally propagated line. For each developmental stage, three biological replicates were prepared by pooling the pulp tissue from six individual fruits, with two individual fruits harvested from a single plant within this clonal population. This resulted in a total of three independent biological replicates for each stage ($n = 3$ per group) used for subsequent metabolomic analysis. Fresh samples were immediately flash-frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. UPLC-MS/MS based metabolite profiling

The UPLC-MS/MS analysis, including metabolite identification and quantification, was performed as a commercial service by Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China).

Frozen *Akebia* pulp samples (50 mg each) were lyophilized using a Scientz-100F freeze-dryer and then ground to a fine powder using a mixer mill (MM 400, Retsch) at 30 Hz for 1.5 min. The powdered sample was extracted with 1200 μL of pre-chilled (−20 °C) 70 % (*v*/v) aqueous methanol. The mixture was vortexed for 30 s every 30 min for a total of 6 times. Following extraction, the samples were centrifuged at 12,000 rpm for 3 min (Relative centrifugal force (g) 13,200) at 4 °C. The supernatant was collected and filtered through a 0.22 μm microporous membrane prior to UPLC-MS/MS analysis. Quality control (QC) samples were prepared by pooling from all extraction samples. Metabolite extraction was performed by Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China).

Metabolite profiling was performed using a UPLC-ESI-MS/MS system, consisting of an ExionLC™ AD UPLC system coupled to a QTRAP® 6500+ (SCIEX, USA). Chromatographic separation was achieved on an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The mobile phase consisted of (A) water with 0.1 % formic acid and (B) acetonitrile with 0.1 % formic acid. The gradient elution program was as follows: 0–9 min, 5 % B to 95 % B; 9–10 min, 95 % B; 10–11.1 min, 95 % B to 5 % B; 11.1–14 min, 5 % B. The flow rate was 0.35 mL/min, the column temperature was maintained at 40 °C, and the injection volume was 2 μL.

Mass spectrometry was performed in both positive and negative electrospray ionization (ESI) modes. The ESI source temperature was set to 500 °C. Ion spray voltage was +5500 V (positive mode) and − 4500 V (negative mode). Ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 50, 60, and 25 psi, respectively. Data acquisition was performed in Multiple Reaction Monitoring (MRM) mode. De-clustering potential (DP) and collision energy (CE) were optimized for each MRM

transition.

Metabolites were identified by comparing their MRM transitions (precursor/product ion pairs), retention times, and fragmentation patterns with entries in the Metware in-house database (MWDB) (Metware Biotechnology Co., Ltd., Wuhan, China (https://www.metware.cn)). Metabolite quantification was based on the peak areas of their respective MRM transitions.

Raw MS data were processed for peak detection, alignment, and area extraction. The resulting data matrix was subjected to multivariate statistical analysis. Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were performed using R software (package MetaboAnalystR; https://www.metaboanalyst.ca) to visualize group separation and identify differential metabolites. Metabolites with a Variable Importance in Projection (VIP) score > 1.0 from the OPLS-DA model, a $|\log_2(\text{Fold Change})| \geq 1$, and a p-value <0.05 (from Student's t-test or ANOVA) were considered significantly differential. Differential metabolites were mapped to KEGG pathways for functional interpretation.

2.3. Transcriptomics profiling

The complete transcriptomic analysis, from library preparation and Illumina sequencing to the bioinformatic processing of raw data, was performed as a commercial service by Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China). This included quality control, alignment to the reference genome, transcript assembly, and the initial quantification of gene expression levels. Details are listed below.

Total RNA was extracted from the *Akebia* pulp samples using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were assessed using a Qubit 4.0 Fluorometer and a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) or Qsep400 system, ensuring RNA Integrity Number (RIN) values were above a specified threshold (e.g., > 7.0).

For library construction, mRNA was enriched from total RNA using oligo(dT) magnetic beads. The enriched mRNA was then randomly fragmented using a fragmentation buffer. First-strand cDNA was synthesized using random hexamer primers and reverse transcriptase, followed by second-strand cDNA synthesis using DNA Polymerase I and RNase H. The double-stranded cDNA fragments were end-repaired, A-tailed, and ligated to sequencing adapters. Ligated fragments were size-selected and PCR-amplified to generate the final cDNA libraries.

The quality of the constructed libraries was assessed using an Agilent Bioanalyzer 2100 system. Qualified libraries were sequenced on an Illumina sequencing platform (e.g., NovaSeq 6000) generating 150 bp paired-end reads. All RNA extraction, library preparation, and sequencing services were performed by Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China).

Raw sequencing reads were processed using fastp (v0.23.2) (Chen et al., 2018) to remove adapter sequences, low-quality reads (Phred score < 20 over 50 % of bases), and reads containing more than 10 % N bases, yielding clean reads. The clean reads were then mapped to the *A. trifoliata* reference genome (Assembly ASM1797944v1, GenBank accession GCA_017979445.1) using HISAT2 (v2.2.1) (Kim et al., 2015) with default parameters. The average unique alignment rate of 82 % is considered robust for a non-model species like *A. trifoliata*, whose reference genome may contain repetitive regions or have incomplete annotations. To ensure a comprehensive analysis that includes potentially unannotated or novel transcripts, we utilized StringTie (v2.1.6) (Pertea et al., 2015) for transcript assembly following alignment. The expression levels of both reference-annotated and newly assembled transcripts were then quantified, allowing for a more complete assessment of the fruit's transcriptome during ripening.

Gene expression levels were quantified as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) using featureCounts (v2.0.3) (Liao et al., 2014) or StringTie (v2.1.6) (Pertea et al., 2015). Differential expression analysis between developmental stages was performed using DESeq2 (v1.22.1) (Love et al., 2014). Genes with a $|\log_2(\text{Fold Change})| \geq 1$ and a False Discovery Rate (FDR) < 0.05 were considered differentially expressed genes (DEGs).

Functional annotation of DEGs was performed by aligning sequences against public databases including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), NCBI non-redundant protein sequences (NR), Swiss-Prot, TrEMBL, and KOG/COG using DIAMOND (v. specified in PDF if available) (Buchfink et al., 2015). GO and KEGG pathway enrichment analyses were conducted using clusterProfiler (v4.6.0) with a significance threshold of $p < 0.05$.

2.4. Statistical analysis

For transcriptomics, FDR was used to correct p-values in differential expression analysis. For metabolomics, Student's t-test or ANOVA was used for univariate statistical analysis of metabolite levels, and multivariate analyses (PCA, OPLS-DA) were employed to assess group differences. Statistical significance was generally set at $p < 0.05$ or FDR < 0.05 as appropriate. Data visualization was performed using R packages.

3. Results

3.1. Overview of transcriptomic data

The sequencing process produced a total of 140.52 Gb of clean data across 15 samples. Each sample contributed at least 7 Gb of clean reads, with a Q30 base percentage exceeding 96 % in all samples, ensuring high-quality sequencing results. Following rigorous quality control measures, such as the removal of adapter sequences, low-quality reads, and contaminants, the data was processed to retain high-quality reads suitable for analysis.

Subsequently, the clean reads were aligned to a reference genome, achieving an average alignment rate of 86 %. Of these, 82 % of the mapped reads were uniquely aligned, providing a robust dataset for subsequent differential expression and pathway analysis. These quality control steps ensure that the transcriptomic data is accurate and reliable, offering a solid foundation for further biological insights.

To assess the overall structure of the transcriptomic data, Principal Component Analysis (PCA) was performed. The PCA plot revealed a clear developmental trajectory primarily along Principal Component 1 (PC1) accounted for 29.64 % of the total variance, while the second principal component (PC2) explained 18.3 % (Fig. 1A). While the early stages (FA and FB) showed some overlap, reflecting a gradual and continuous transcriptomic shift, a clear separation was observed between the early (FA, FB) and late (FD, FE) stages of development. This two-dimensional representation indicates that a significant portion of the variation in gene expression is captured by the first two principal components, suggesting clear differentiation among the stages in the dataset. Furthermore, Pearson's correlation coefficient was used to evaluate the biological reproducibility between replicates. The correlation plots demonstrated strong consistency across biological replicates, with correlation coefficients close to 1, confirming the reliability of the experimental design and the robustness of the gene expression profiles (Fig. S1).

The differential gene expression analysis between various developmental stages of fruit revealed a dynamic and complex regulatory landscape as the fruit progresses from earlier stages (FA) to maturity (FE). In the comparison between FA and FE, a total of 8553 genes were found to be differentially expressed, with 4389 genes upregulated and 4164 genes downregulated in the mature fruit (FE), suggesting substantial molecular changes during the transition from earlier developmental stages to full maturity (Fig. 1B and C). This indicates that a significant portion of the genome is activated or suppressed in response to developmental cues during fruit maturation.

When comparing other developmental stages to FA, similar trends

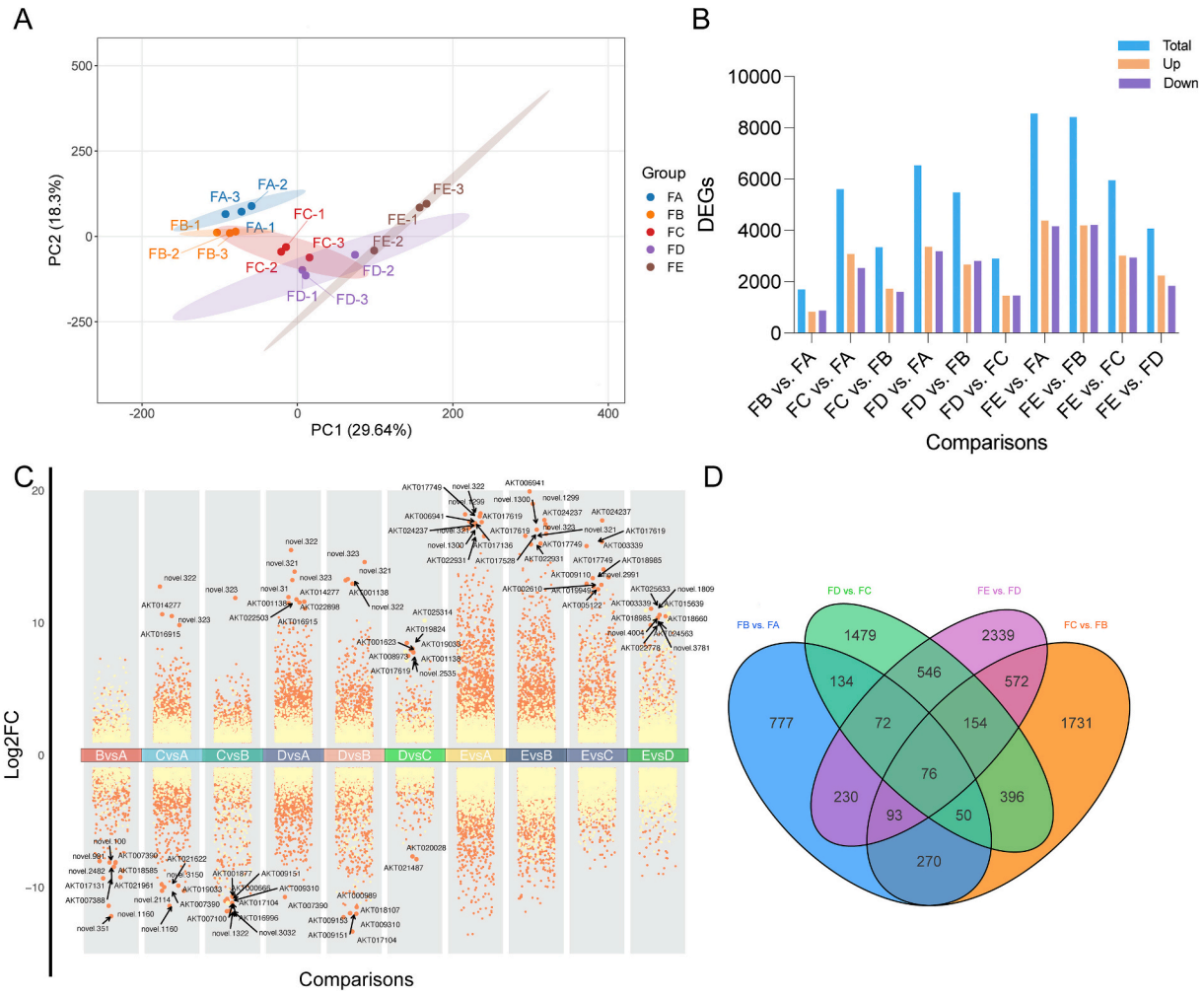

Fig. 1. Multifaceted analysis of transcriptomic changes during ripening of *Akebia trifoliata* fruit pulp. A) Principal Component Analysis (PCA) plot showing the overall variability of gene expression across different ripening stages. B) Bar plot depicting the number of differentially expressed genes (DEGs) identified in pairwise comparisons between ripening stages. C) Scatter plot of average Log_2FoldChange (Log_2FC) values of DEGs for each pairwise comparisons (FA, FB, FC, FD, and FE). DEGs are plotted based on their average Log_2FC values, with green dots representing genes with a false discovery rate (FDR) <0.0001 and orange dots representing genes with an FDR ≥ 0.0001. Key genes with significant expression changes at specific stages are labeled. D) Venn diagram illustrating the overlap of DEGs across four comparisons, including FB vs. FA, FC vs. FB, FD vs. FC, and FE vs. FD, highlighting shared and unique genes between temporal stages. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were observed, emphasizing the gene regulatory shifts as fruit development progresses. In the FC versus FA comparison, 5612 genes were differentially expressed, with 3081 upregulated and 2531 downregulated in the more developed fruit (FC), showing a similar but slightly less pronounced shift compared to FE. A comparison between FB (fruit between FA and FC) and FA revealed 1702 differentially expressed genes, with a relatively balanced split of 829 upregulated and 873 downregulated, suggesting that temporal expression patterns at the earlier stages but become more pronounced as fruit development advances.

In the comparison between FD and FA, a total of 6544 differentially expressed genes were identified, with 3361 genes upregulated and 3183 downregulated, marking a more distinct gene expression shift than between FB and FA. This further reinforces the idea that as fruit development proceeds through stages such as FC and FD, gene expression becomes increasingly complex and diverse. Interestingly, the number of differentially expressed genes decreases in the comparison of FE to other stages, particularly between FE and FD (4082 genes, with 2247 upregulated and 1835 downregulated), suggesting that many of the key changes in gene expression occur prior to full maturation.

3.2. Temporal dynamics of differential gene expression

The differential gene expression (DEG) analysis across multiple developmental stages of fruit revealed significant temporal changes in gene expression that regulate the transition from early growth to full maturation (Table S1 and Fig. S2).

A set of 76 overlapping DEGs was consistently identified as differentially expressed in four key comparisons: FB vs FA, FC vs FB, FD vs FC, and FE vs FD, highlighting their central regulatory roles in the continuous maturation process (Fig. 1D and Table S2). These comparisons represent distinct time points during fruit development, capturing the progression of molecular changes as the fruit moves from early stages to maturity. By analyzing these temporal transitions, we observe distinct expression patterns of the identified DEGs (Fig. 2A), which are key regulators in the maturation process, emphasizing their role in orchestrating the complex biological events that occur as the fruit progresses towards full maturity.

For instance, *AKT000026*, involved in lysine degradation, demonstrated continuous upregulation across all comparisons, suggesting its pivotal role in metabolic processes essential for fruit ripening. Its sustained expression throughout the stages indicates that it is involved in

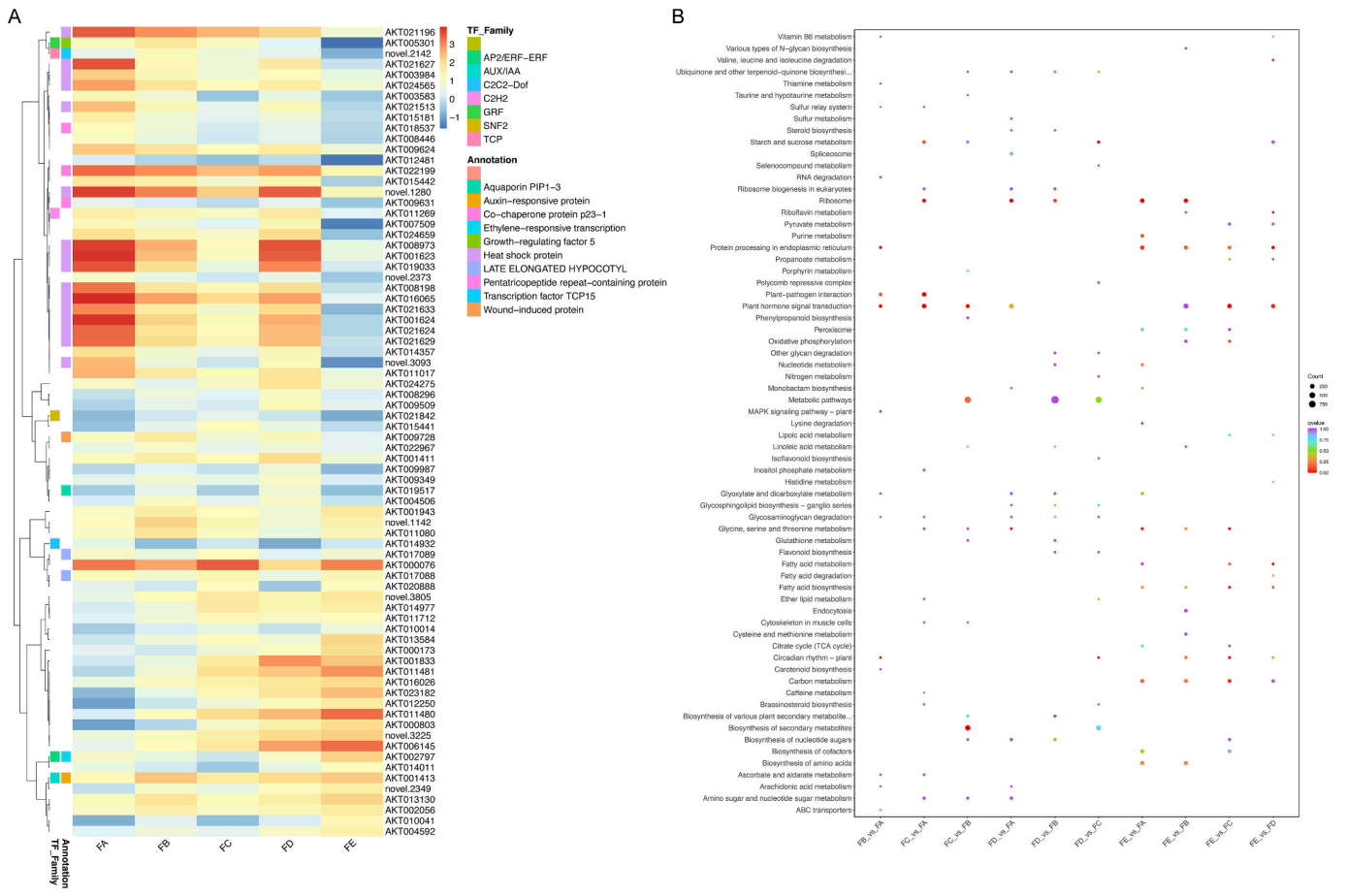

Fig. 2. Expression dynamics of DAMs in *Akebia* fruit development A) Expression profile of 76 overlapped DEGs identified in temporal transition of akebia fruit ripening. A complete list of these 76 genes, including their functional annotations, can be found in Supplementary Table S2, B) KEGG pathway enrichment analysis for all the DAMs in each pairwise comparison.

metabolic shifts that support biochemical changes during maturation. Similarly, *AKT000008*, associated with RNA polymerase, displayed a particularly strong increase in expression in the later stages of development, especially from FD to FE, indicating its role in transcriptional regulation and the activation of ripening-related genes during fruit maturation. The upregulation of *AKT000025*, a RING-type E3 ubiquitin ligase, was observed primarily during the transitions from FC to FD and FD to FE, suggesting its role in protein degradation and post-translational modifications that are critical for the final stages of fruit ripening, including the remodeling of cellular structures.

In addition to these core genes, *AKT000011*, a predicted lipase, and *AKT000012*, a hypothetical protein, exhibited differential expression in early developmental stages (FB vs FA) and mid-to-late stages (FD vs FC), respectively. The upregulation of *AKT000011* in early stages is indicative of the initiation of lipid metabolism, which is essential for membrane formation as the fruit begins to develop. *AKT000012*, although uncharacterized, exhibited significant expression in the transition from FC to FD, suggesting it may play a role in processes activated as the fruit matures.

Further exploitation of 76 overlapped DEGs highlighted genes are involved in critical processes such as hormonal regulation, cell wall modification, and stress responses (Fig. 2C). Notably, genes like Auxin-responsive protein IAA17 (*AKT001413*) and Ethylene-responsive transcription factor ERF110 (*AKT002797*) are implicated in auxin and ethylene signaling pathways, both of which play vital roles in initiating and regulating fruit ripening. Additionally, Transcription factor TCP15 (*novel.2142*) is involved in cell wall modification, contributing to fruit softening, a key characteristic of ripening. Genes related to stress response, such as 17.3 kDa class II heat shock protein (*AKT001623*) and 22.7 kDa class IV heat shock protein (*AKT008973*), help maintain protein stability and cellular integrity during the ripening process. Furthermore, genes like Phospholipid:diacylglycerol acyltransferase 1 (*AKT000173*) highlights the metabolic shifts, particularly in sugar and lipid metabolism, which contribute to the accumulation of sugars and flavor development in ripe fruit.

The KEGG pathway analysis of the 76 overlapped DEGs further revealed significant enrichment in several pathways critical to fruit maturation. Genes such as *AKT000008* were enriched in the RNA polymerase pathway (ko03020), emphasizing their role in transcriptional regulation during ripening. *AKT000173* and other genes linked to lipid metabolism (ko00561) were identified, reflecting their essential roles in membrane formation and the accumulation of oils during the maturation process. Additionally, *AKT014011* was associated with amino acid metabolism (K15015), underscoring the importance of amino acid degradation in the biochemical changes during fruit ripening. Furthermore, genes such as *AKT003984* and *AKT021196*, involved in protein folding and post-translational modification, indicate that protein remodeling is essential during the final stages of fruit ripening, contributing to texture and structure.

Temporal changes in gene expression were also observed across the stages of fruit development, with specific genes upregulated at different transition points. Between FA and FB, *AKT000011*, a predicted lipase, was upregulated, marking the initiation of lipid metabolism crucial for membrane formation. As the fruit progressed from FB to FC, *AKT000025* was upregulated, signaling the need for increased protein degradation and remodeling as the fruit matured. The transition from FC to FD

showed further upregulation of *AKT000008*, indicating enhanced transcriptional regulation as the fruit neared its final stages. Finally, the transition from FD to FE revealed a continued increase in the expression of *AKT000026* and *AKT000008*, which underscores their sustained roles in amino acid metabolism and transcriptional control during the final ripening phase.

The analysis further revealed a group of genes with high expression at the early stages of fruit development (FA), which gradually decrease as the fruit matures (FE). Notably, many of these genes are associated with heat shock proteins (HSPs), including HSP70 family members and small heat shock proteins. Genes such as *AKT001623*, *AKT001624*, *AKT016065*, *AKT021196*, and *AKT021513* belong to these families, and they exhibit a significant decline in expression from early developmental stages to full maturity. Heat shock proteins are essential for protecting cells under stress conditions by acting as molecular chaperones, facilitating protein folding, and preventing protein aggregation. Their elevated expression during early fruit development suggests their role in managing the stresses associated with rapid cell division, growth, and metabolic activity in the early stages. As the fruit matures and the stress levels stabilize, the expression of these heat shock proteins decreases, reflecting a shift in cellular processes as the fruit transitions into its final ripening phase. This downregulation highlights the transition from stress response mechanisms to those regulating ripening and maturation, underscoring the critical role of these proteins in early fruit development and their eventual reduction as the fruit reaches full maturity.

The enriched pathways involved in RNA polymerase (ko03020), lipid metabolism (ko00564), amino acid metabolism (ko00260), and protein folding and post-translational modifications (ko04141) underscore the importance of transcription regulation, lipid accumulation, and protein remodeling in the fruit ripening process. These findings provide critical insights into the molecular mechanisms driving fruit maturation, offering potential targets for further research aimed at improving fruit quality and controlling the ripening process.

3.3. Regulation of starch and sucrose metabolism during Akebia fruit ripening

Starch and sucrose metabolism genes displayed stage-specific expression patterns closely aligned with the physiological progression of *Akebia* fruit ripening (Table S1). Sucrose synthase (*AKT000057*) was strongly upregulated at FB (15.12 FPKM) compared to FA (1.22 FPKM), facilitating early sucrose cleavage and contributing to sugar availability for cellular expansion. Its sharp decline by FE (0.07 FPKM) suggests a shift away from sucrose catabolism at full maturity. Similarly, beta-amylase (*AKT000959*) peaked early (FB: 22.24 FPKM) and decreased steadily through ripening, supporting early starch breakdown into maltose.

Mid- to late-ripening stages showed enhanced expression of glucan endo-1,3-beta-glucosidase (*AKT001060*), peaking at FD (4.01 FPKM), and endoglucanase (*AKT000133*), with highest levels at FE (1.30 FPKM), indicating roles in carbohydrate remodeling and sugar release. PDCB5 (AKT000733), a plasmodesmata callose-binding protein, showed stable expression until FD, then dropped at FE (0.27 FPKM), potentially reflecting reduced symplastic transport as ripening concludes.

Among the most dramatically induced genes, invertase (*AKT019260*) increased from FA (22.70 FPKM) and FB (29.96 FPKM) to extreme levels at FD (2740.87 FPKM) and FE (4154.26 FPKM), driving sucrose hydrolysis and hexose accumulation that enhances sweetness. Alpha-amylase 3 (*AKT011802*) remained highly expressed throughout, peaking at FC (1118.54 FPKM) and maintaining elevated expression at FE (1662.14 FPKM), indicating sustained starch degradation. In contrast, beta-amylase 1 (*AKT006405*) was highest at FA (1424.10 FPKM) and declined progressively (FE: 566.81 FPKM), marking its action in early starch mobilization.

Late-stage-specific increases were observed in beta-amylase 3 (AKT017619) and inactive beta-amylase 9 (*AKT012726*) at FE (828.47 and 1599.16 FPKM), suggesting roles in terminal carbohydrate conversion or regulation. Supporting this, alpha-glucan phosphorylase (*AKT009883*) and glucan-branching enzyme (*AKT005156*) peaked between FC and FE, coordinating starch remodeling and energy release. Additionally, glucan endo-1,3-beta-glucosidase (*AKT002120*) remained elevated through FE (445.82 FPKM), possibly linking sugar turnover to stress adaptation.

Together, these expression profiles outline a biphasic regulatory pattern: early-to-mid stages prioritize starch degradation through amylases and glucanases, while late stages emphasize invertase-mediated sugar release. These changes align with metabolomic data showing increased sucrose and fructose at FE, underscoring the molecular basis for sweetness development and the coordinated role of carbohydrate metabolism in *Akebia* fruit maturation.

3.4. Transcription factor dynamics in Akebia ripening

To further extend the analysis of differentially expressed genes (DEGs), we characterized the expression of several key transcription factors (TFs) involved in regulating fruit ripening. A total of 525 TFs (Fig. 3A) were identified among the DEGs, and 165 (Fig. 3B) of these were further associated with fruit maturity, including TF families such as NAC, AP2/ERF, WRKY, MYB, and others. These TFs were analyzed across five stages of fruit maturity in *Akebia* (Fig. 3 and Table S3). The expression profiles of these TFs highlight their distinct regulatory roles during fruit development, from early maturation to full ripening, while emphasizing their involvement in processes such as stress response, cell wall modification, and pigmentation.

The expression of NAC TFs, specifically *AKT000864*, *AKT009146*, and *novel.1151*, showed distinct patterns across the stages of ripening. *AKT000864*, which exhibited moderate expression at early stages (FA: 172.61), increased significantly at later stages, peaking in the final ripening stage (FE: 220.33). This indicates its pivotal role in the late stages of ripening, possibly regulating fruit softening and stress responses. *AKT009146*, another NAC gene, showed minimal expression at all stages (FB: 7.50), with decreased expression in the final stages (FE: 2.07), suggesting a minor role in ripening. In contrast, *novel.1151*, a novel NAC gene, displayed robust expression throughout ripening, particularly peaking at FE (FE: 625.85), suggesting its significant involvement in late-stage processes like stress tolerance and fruit maturation.

AKT003828, a member of the MYB family, exhibited high expression at early stages (FA: 340.88), followed by a gradual decrease through the middle stages (FB: 387.09) and a significant drop towards the final stages (FE: 47.50). This dynamic suggests that *AKT003828* is likely involved in early ripening processes, such as pigment accumulation and secondary metabolite synthesis, which are crucial for color development and flavor formation. The decrease in expression during the final ripening stages likely reflects the cessation of these activities as the fruit matures.

The expression patterns of AP2/ERF TFs, including *AKT007306*, *AKT009029*, and *AKT023211*, varied significantly across the ripening stages. *AKT007306*, which belongs to the AP2/ERF-AP2 subfamily, exhibited a slight increase from FA to FB (FB: 25.31) but declined sharply in later stages, with no detectable expression at FE (FE: 0.00). This suggests that *AKT007306* is involved in the initiation of ripening, particularly in ethylene signaling pathways, but is not critical in the later ripening stages. Similarly, *AKT009029*, an AP2/ERF-ERF gene, maintained high expression from FA to FB (FB: 252.43) but decreased significantly during the final ripening stages (FE: 4.25), indicating its involvement in regulating ethylene production and response in the early to mid-stages of ripening. *AKT023211*, another AP2/ERF-ERF gene, followed a similar pattern, with peak expression during the middle stages (FB: 365.23) and a decline towards the final stages (FE: 0.71), suggesting a role in mid-ripening processes such as cell wall

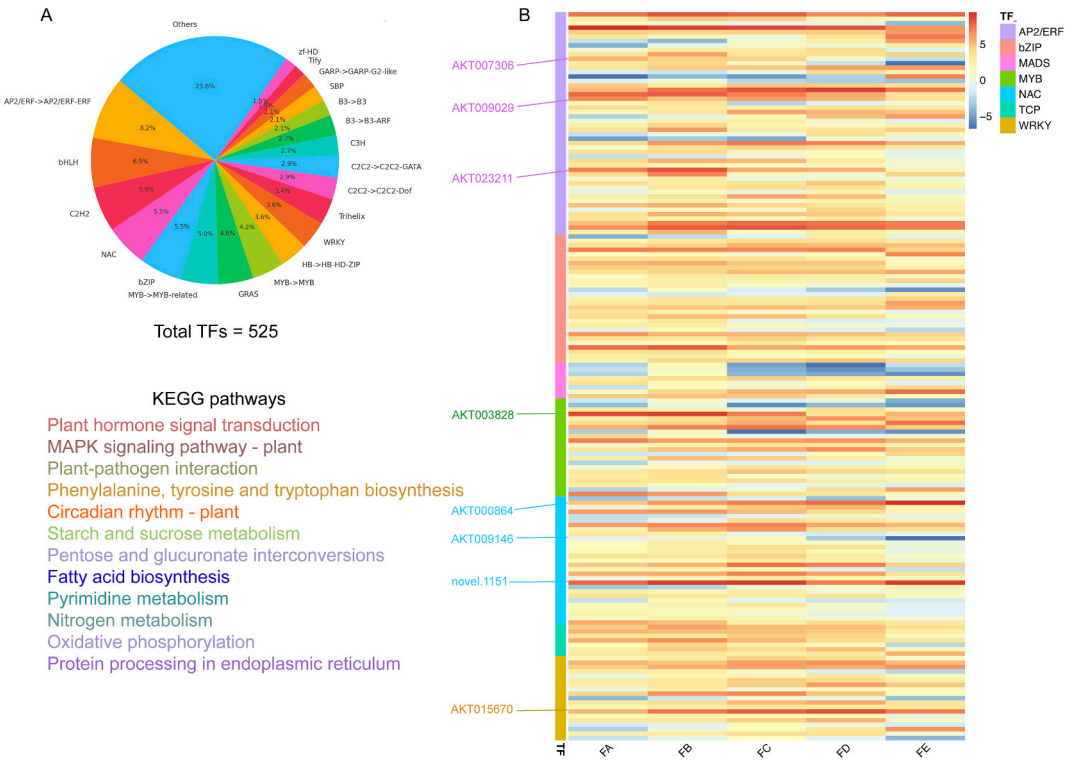

Fig. 3. Transcription factors (TFs) identified as DEGs, mediated regulation of fruit maturity in *Akebia* fruit. A) Distribution pattern of 525 TFs identified as DEGs and associated significant KEGG pathways B) Temporal expression pattern of TFs.

modification and sugar accumulation.

The expression of *AKT015670*, a WRKY TF, showed an increasing trend from early stages (FA: 25.03) to middle stages (FB: 286.72), peaking in FC (FC: 268.98), before slightly decreasing in the final stages (FE: 143.14). This suggests that *AKT015670* plays a significant role in the regulation of defense responses and other ripening-related physiological changes during fruit maturation. Its relatively high expression in the middle stages could be indicative of its role in modulating stress responses and other signaling pathways that facilitate fruit ripening.

The expression dynamics of these key TFs across different stages of fruit maturity in *Akebia* highlight their specific roles in regulating various ripening processes. NAC, MYB, AP2/ERF, and WRKY TFs contribute to different aspects of ripening, including pigment synthesis, ethylene signaling, stress response, and fruit softening. Understanding these regulatory networks provides valuable insights into the molecular mechanisms controlling fruit ripening and can inform breeding strategies aimed at improving fruit quality and ripening control in *Akebia* and other fruit crops.

3.5. Metabolic characterization governing fruit ripening

The comprehensive analysis of metabolomic data across *Akebia* fruit development stages reveals significant shifts and distinctions in metabolite composition and concentration. The metabolite class distribution (Fig. 4A) indicates that terpenoids (19.73 %) and flavonoids (14.89 %) dominate the profile, highlighting their essential roles in the fruit's physiological processes and defense mechanisms. Other significant classes include phenolic acids and alkaloids, which are crucial for the plant's stress response and pigment synthesis. The principal component analysis (Fig. 4B) successfully differentiates the developmental stages based on their metabolomic signatures, capturing 32.59 % of the variance in the first component and 13.08 % in the second. This clear segregation underscores the distinct biochemical states of the fruit at each developmental phase.

In terms of differential metabolite changes, the analysis between early and late developmental stages (Fig. 4C and D) shows significant changes in differential accumulation patterns of metabolites in different comparisons. This substantial alteration in metabolite levels suggests a vigorous reconfiguration of metabolic pathways as the fruit approaches full ripeness. The comparative Venn diagram (Fig. 4E) further illustrates the metabolic overlaps and unique features among the stages, identifying shared and distinct pathways that are active at different times of fruit development. This diagram quantifies the shared metabolites across stages, with notable overlaps indicating continuous or recurring metabolic activities necessary for fruit development and maturation.

3.6. Temporal metabolic dynamics

In the comprehensive metabolomic profiling of *Akebia* fruit, 50 differentially accumulated metabolites (DAMs) were identified, spanning critical stages of fruit development from FB vs. FA, FC vs. FB, FD vs. FC, to FE vs. FD (Fig. 5B and Table S4). Among these, key metabolites such as the alkaloid L-Pipecolate, the amino acid L-Threonine, and the flavonoid (−)-Epicatechin were highlighted for their distinct roles in the ripening process. L-Pipecolate, for instance, shows a significant increase in accumulation towards the final ripening stage, FE, indicating its potential involvement in defense mechanisms and stress responses during late-stage fruit development. L-Threonine is observed to accumulate predominantly in the earlier stages, FA and FC, underscoring its importance in protein synthesis and primary metabolic processes essential during the initial development phases. Meanwhile, (−)-Epicatechin, a flavanol, peaks during the early ripening stage, FB, contributing to the antioxidant capacity and aiding in the color development of the fruit, which is crucial for its protective functions and market appeal. These findings demonstrate the dynamic regulation and significant roles these metabolites play across different stages, providing insights into the metabolic adjustments that occur during *Akebia* fruit ripening. This rich dataset forms a basis for further investigations into improving fruit quality traits and understanding the biochemical pathways integral to fruit maturation.

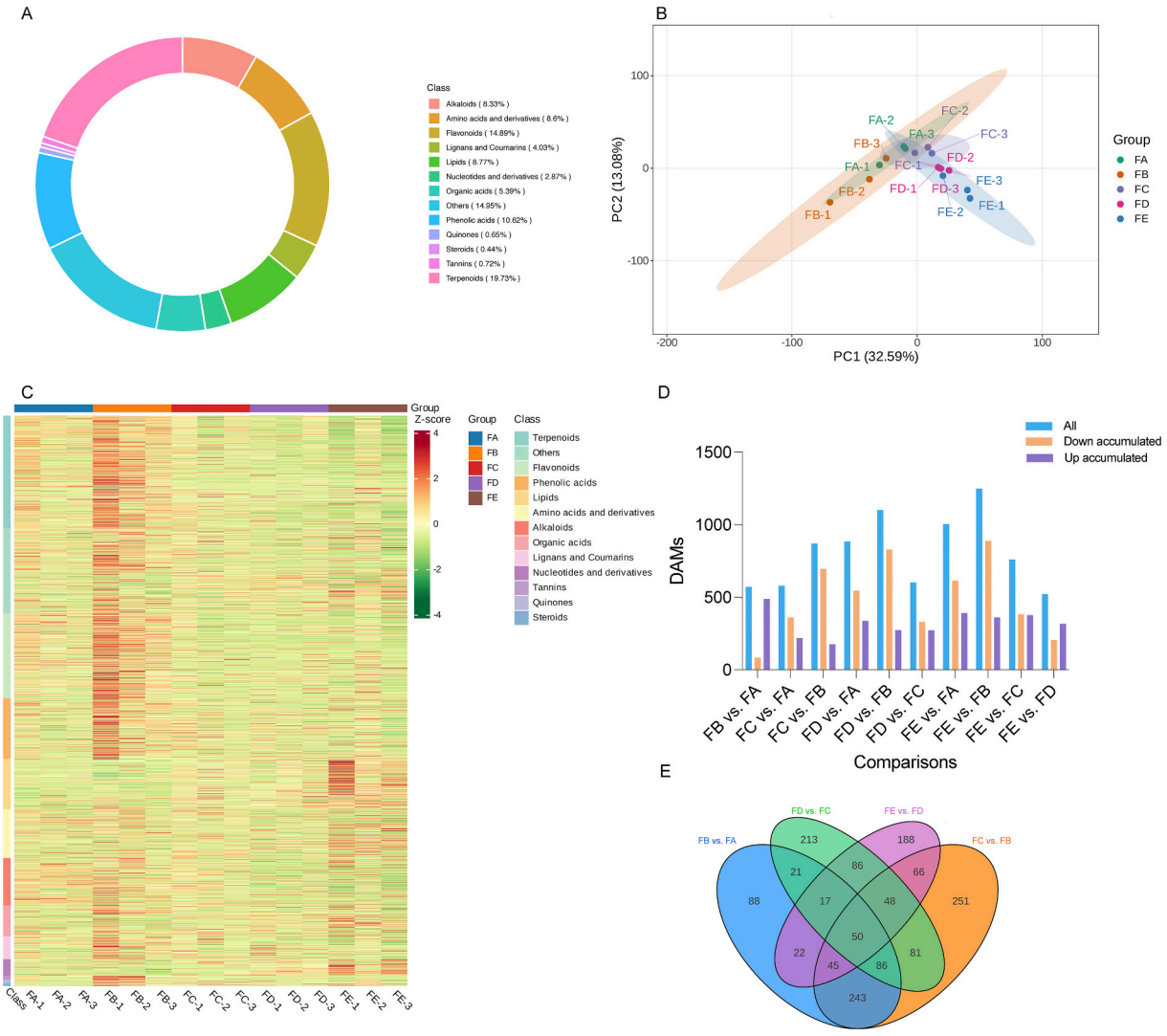

Fig. 4. Comprehensive Metabolomic Analysis of *Akebia* Fruit Development. A) Distribution of metabolite classes emphasizing the prevalence of terpenoids and flavonoids essential for physiological and defensive functions. B) PCA plot delineating the distinct metabolomic profiles of developmental stages, highlighting the substantial biochemical variance captured by the principal components. C) Heatmap illustrating overall temporal accumulation pattern of metabolites at different developmental stages D) Detailed differential metabolite analysis revealing the extensive metabolic changes as the fruit transitions from initial growth to full maturation. E) Venn diagram showing overlaps and unique metabolite distributions among stages, providing insights into the dynamic metabolic adjustments throughout *Akebia* fruit development.

The extended dataset beyond the 50 overlapped differentially accumulated metabolites (DAMs) reveals intricate stage-specific dynamics in the metabolite regulation that underpin the ripening process of *Akebia* fruit (Fig. 5A and Table S5). This comprehensive analysis offers insights into the shifts within specific classes of metabolites, providing clues on how these changes support different developmental transitions. In the early-stage comparison (FB vs FA), significant upregulation is observed in specific classes such as flavonoids and phenolic acids. For instance, Neochlorogenic acid and Cryptochlorogenic acid, both phenolic acids known for their antioxidative properties, show substantial increases, suggesting a role in protecting the young fruit from oxidative stress and aiding in early developmental processes.

As the fruit transitions from FB to FC, there is a noticeable shift in the accumulation patterns of amino acids and related compounds, which are crucial for protein synthesis and growth. Tyrosine and phenylalanine, which are precursors to numerous secondary metabolites including flavonoids, are differentially accumulated, indicating a redirection of metabolic pathways towards supporting rapid growth phases. During the FD vs FC stage, there is a marked decrease in alkaloids such as Spermidine, a polyamine involved in cell growth and differentiation, which declines significantly as the fruit begins to prepare for ripening. This suggests a reduction in cell division activities and an increase in processes that promote maturation and senescence.

In the final maturation phase (FE vs FD), sugar metabolites such as Sucrose and Fructose are highly accumulated, highlighting their critical role in sweetening the fruit, which is key to attracting seed dispersers and enhancing fruit palatability for marketability. The increase in sugars also indicates a shift towards energy storage in the form of easily metabolizable carbohydrates, essential for the ripening process. These stage-specific regulatory patterns of different classes of metabolites not only illustrate the metabolic adjustments necessary at each stage but also reflect the fruit's physiological responses to developmental cues and environmental factors. Such dynamic changes in metabolite profiles are crucial for optimizing fruit development, ensuring successful ripening, and ultimately affecting the quality and yield of the harvested fruit. The data thus provide valuable markers for breeding and cultivation strategies aimed at enhancing fruit quality traits in *Akebia*.

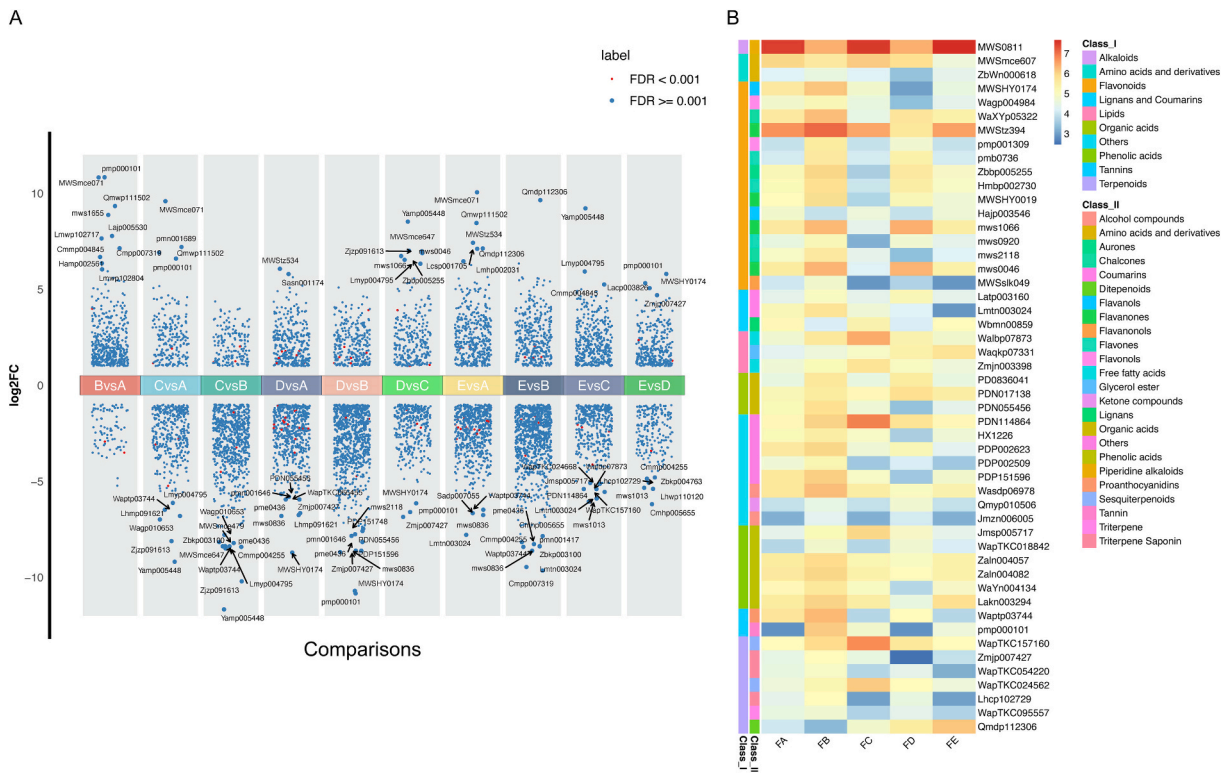

Fig. 5. Temporal Metabolic Dynamics in *Akebia* Fruit Ripening. A) Differential accumulation pattern of metabolites across all pairwise comparisons, highlighting topFC metabolites in each comparison. Metabolites are labeled with unique ID codes for clarity; full chemical names corresponding to each ID can be found in Supplementary Table S4. B) Heatmap illustrating the concentration changes of 50 differentially accumulated metabolites across developmental stages, with a focus on key metabolites that play crucial roles in developmental processes, stress responses, and ripening. A complete list of these 50 metabolites, including their ID codes, full names, and statistical details, is provided in the dedicated Supplementary Table S5.

3.7. Conjoint analysis of metabolome and transcriptome reveals key pathways in fruit ripening

The conjoint analysis integrating metabolomic and transcriptomic data across four critical stages of *Akebia trifoliata* fruit development provides an integrative view of the stage-specific biochemical and genetic shifts governing ripening (Fig. 6). This approach elucidates the complex interplay between gene expression and metabolic output as the fruit progresses towards full maturity. Among the significantly enriched pathways was 'taurine and hypotaurine metabolism. It is important to note that while this pathway was identified by the automated KEGG mapping software due to the presence of enzymes with broad substrate specificities (such as cysteine dioxygenase), the biological significance of this specific pathway in higher plants like *A. trifoliata* is likely minimal. Therefore, our subsequent analysis focuses on pathways with well-established roles in fruit development.

3.7.1. Initial stages (FA to FB): Activation of protective and precursor pathways

The initial stages of ripening are characterized by significant metabolic adjustments necessary for early development, focusing on protection and precursor synthesis (Fig. 6A). Key activated pathways include the biosynthesis of flavonoids (Kaempferol Aglycones II, Flavanols I, Quercetin Aglycones II) and other phenolic compounds (Caffeic Acid Derivatives, Phenylacetic Acid Derivatives), providing antioxidant capacity, UV protection, and potentially contributing to structural integrity and early aroma development. Our conjoint analysis pinpoints specific regulatory events underpinning these changes. Notably, the downregulation of *AKT000925* (MYB transcription factor) strongly correlated with increased levels of specific kaempferol derivatives, including Kaempferol-7-O-rhamnoside, Afzelin, 6-Hydroxykaempferol-7-O-glucoside, and Dihydrokaempferide (Fig. S6A and S3), suggesting this MYB factor may act as a repressor of kaempferol biosynthesis. Concurrently, within the broader phenylpropanoid pathway, specific gene activities were linked to precursor accumulation (Figure 6SB and S4): *AKT022999* and novel.762 (both cytochrome P450s) correlated with Dicaffeoylshikimic acid levels, while novel.2163 (NAC83 TF) was linked to 3-O-caffeoylshikimic acid and 4-Coumarate. Furthermore, novel.2163, *AKT022999*, and another cytochrome P450 correlated with 1-O-p-Coumaroylquinic acid accumulation. These specific gene-metabolite associations highlight the coordinated regulation involving MYB and NAC transcription factors alongside cytochrome P450 enzymes in directing metabolic flux towards protective flavonoids and key phenolic precursors during early fruit development.

3.7.2. Mid-stage transition (FB to FC): Enhancement of defense and ongoing phenolic synthesis

As the fruit transitions into mid-stage development (Fig. 6B), there is a continued emphasis on protection and the development of flavor/aroma profiles. Pathways such as Kaempferol Aglycones II and Phenylacetic Acid Derivatives remain active, alongside the strengthening of defense strategies indicated by the regulation of Biosynthesis of Coumarins III. Pigmentation and antioxidant pathways involving Quercetin Aglycones I and Kaempferol Aglycones I are also actively managed. Specific conjoint analysis revealed further metabolic links during this phase (Figure 6SC). For instance, *AKT004470* (Mannitol Dehydrogenase), primarily associated with mannitol metabolism, showed a significant correlation with both Dicaffeoylshikimic acid and 4-Coumarate. This suggests a potential secondary role for this enzyme in managing oxidative stress or influencing metabolic flux towards phenolic biosynthesis during this transition.

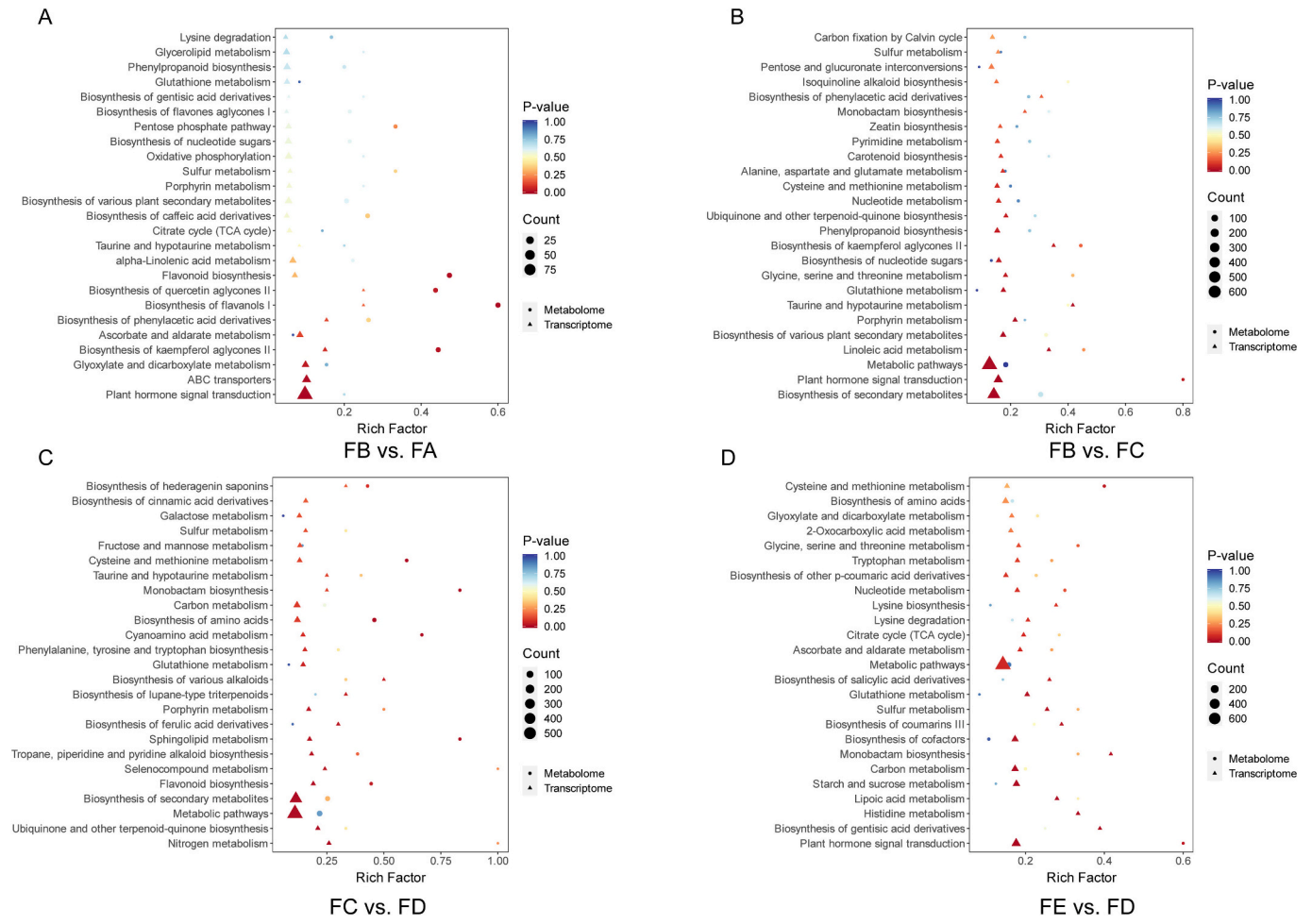

Fig. 6. KEGG pathway enrichment identified in conjoint analysis of metabolome and transcriptome under multiple comparisons A) FB vs. FA, B) FC vs. FB, C) FD vs. FC, and D) FE vs. FD.

3.7.3. Mid-to-late transition (FD vs FC): Focus on defense signaling and structural components

The transition towards late development marks a pivotal phase preparing for final maturation (Fig. 6C). General pathway analysis indicates upregulation related to cell wall strengthening (Biosynthesis of Ferulic Acid Derivatives), natural defenses (Biosynthesis of Lupane-type Triterpenoids, Hederagenin Saponins), and flavor/aroma (Cinnamic Acid Derivatives, Other p-Coumaric Acid Derivatives). Conjoint analysis during this transition highlighted significant gene-metabolite interactions within the jasmonic acid biosynthesis pathway (Fig. S6D). Strong correlations were observed between (−)-Jasmonic acid levels and the expression of diverse regulatory genes, including the transcription factor BH093 (*AKT006948*), receptor kinases (LRK10/AKT014718, novel.2920 G-type lectin), a protein-tyrosine kinase (CEPR2/*AKT008901*), calcium signaling components (CML45/*AKT008513*, BH081/*AKT006086*, CML46/*AKT011875*), an auxin transporter (LAX2/*AKT009861*), and a gene involved in germination regulation (DOG1-like/*AKT018325*). This intricate network underscores the importance of jasmonic acid signaling, coordinated by various regulatory inputs, in bolstering the fruit's defense mechanisms and potentially influencing structural integrity as it approaches the final ripening stages.

3.7.4. Concluding stages (FE vs FD): Final maturation and metabolic support

In the concluding stages, the focus shifts to finalizing ripening processes, involving adjustments in stress mitigation, defense, and structural/aromatic properties (Fig. 6D), evidenced by the regulation of pathways producing Gentisic Acid, Coumarins III, Salicylic Acid, and other phenolic derivatives. The conjoint analysis revealed critical gene-metabolite relationships supporting these final biochemical adjustments, particularly in cofactor biosynthesis (Fig. S5). Genes encoding Bifunctional riboflavin kinase/FMN adenylyltransferases (*AKT002592*, *AKT007004*) and 3-oxoacyl-[acyl-carrier-protein] synthase II (*AKT017215*, *AKT018497*), along with the uncharacterized gene *AKT001654*, all showed strong correlations with Aminoimidazole ribotide levels. These associations highlight the essential role of regulating cofactor pools (via riboflavin metabolism) and fatty acid biosynthesis to support the high energy demands and cellular activities characteristic of the final maturation phase.

3.8. Delineation of key metabolic and regulatory pathways

3.8.1. Dynamic regulation of the flavonoid biosynthesis pathway

To gain a mechanistic understanding of flavonoid accumulation, we integrated our transcriptomic and metabolomic data onto the flavonoid biosynthesis pathway (Fig. 7). This revealed a highly dynamic and stage-specific regulatory program, with a clear shift from the synthesis of precursor flavonoids in early development to their modification in later stages.

The initial steps of the pathway were strongly activated during the transition from young (FA) to green mature (FB) fruit. Key entry point genes, including 4-coumarate-CoA ligase (*4CL2*) and chalcone synthase (*CHS*), were significantly upregulated. This transcriptional induction

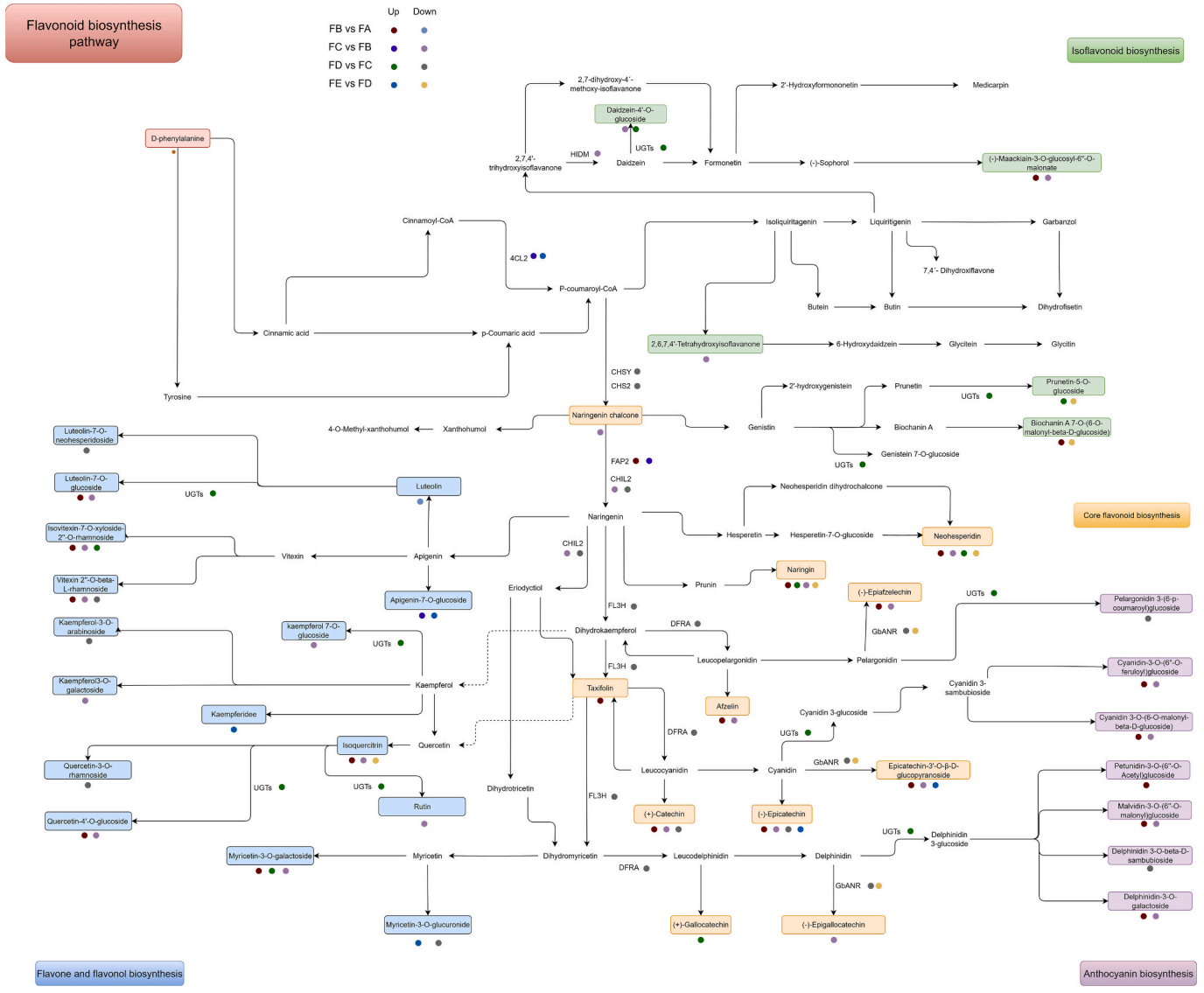

Fig. 7. Integrated view of the flavonoid biosynthesis pathway in maturing *A. trifoliata* fruit: The diagram illustrates the major branches of flavonoid biosynthesis, including core flavonoid, flavone/flavonol, isoflavonoid, and anthocyanin pathways. Metabolites that were detected in our study are shown in colored boxes, while genes are represented by their official symbols. The colored dots next to each gene or metabolite indicate its differential expression or accumulation in a specific stage comparison: Red (up) / Light Blue (down) for FB vs FA; Dark Blue (up) / Purple (down) for FC vs FB; Green (up) / Gray (down) for FD vs FC; and Teal (up) / Gold (down) for FE vs FD. This integrated map highlights the early activation of core flavonoid synthesis and the late-stage shift towards anthocyanin production. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was directly correlated with a marked increase in the accumulation of central intermediates. For instance, naringenin chalcone and its cyclized product naringenin, a critical branchpoint metabolite, showed significant accumulation in the FB vs. FA comparison. Downstream, this flux led to a surge in flavan-3-ols, with (−)-epicatechin and (+)-catechin showing strong upregulation and peaking in the mid-ripening stages (FB and FC). Similarly, several flavonols and their glycosides, such as isoquercitrin and quercetin-4'-O-glucoside, were also most abundant during these early-to-mid stages.

As the fruit transitioned towards full ripeness, the expression of many core structural genes was attenuated. For instance, chalcone isomerase (*CHIL2*), flavanone 3-hydroxylase (FL3H), and dihydroflavonol 4-reductase (*DFRA*) were downregulated in the FD vs. FC and FE vs. FD comparisons. This transcriptional downregulation corresponded with stabilizing or decreasing levels of their respective substrates and immediate products.

While core synthesis slowed, the late stages were characterized by extensive modification of existing flavonoids, primarily through glycosylation. The expression of several UDP-glycosyltransferases (*UGTs*) remained high or was specifically induced during the final ripening steps. This activity likely drives the formation of the diverse array of anthocyanins responsible for the final purple color of the mature fruit. Our data show a significant accumulation of various anthocyanin glycosides, including cyanidin-3-O-(6-O-malonyl-beta-D-glucoside), delphinidin-3-O-galactoside, and petunidin-3-O-(6''-*O*-acetyl)glucoside, specifically during the FE stage.

3.8.2. *Dynamic regulation of the hormone biosynthesis and signaling pathway*

To investigate the hormonal regulation underpinning fruit ripening, we examined the expression of key genes involved in the biosynthesis and signaling of major phytohormones (Fig. 8). The results point to a complex interplay between different hormones, with jasmonic acid, auxin, and abscisic acid signaling appearing particularly active.

The jasmonic acid (JA) pathway showed dynamic regulation throughout ripening. Key biosynthesis genes such as allene oxide

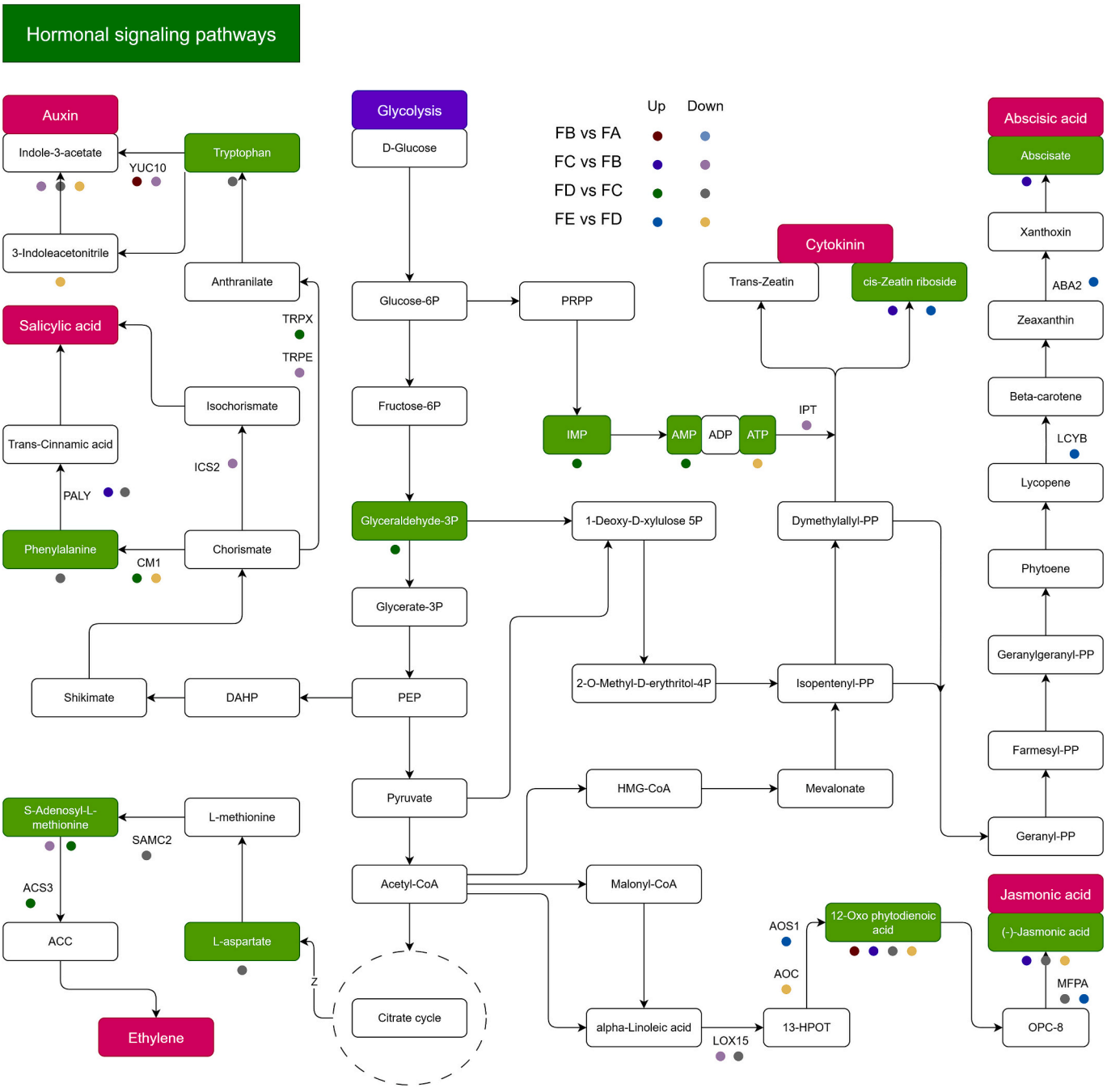

Fig. 8. Transcriptional regulation of key phytohormone biosynthesis pathways during *A. trifoliata* ripening. This schematic displays the interconnected biosynthesis pathways for major plant hormones, including auxin, abscisic acid (ABA), jasmonic acid (JA), ethylene, cytokinin, and salicylic acid. Genes with statistically significant differential expression are shown with colored dots indicating their regulation pattern in each consecutive stage comparison: Red (up) / Light Blue (down) for FB vs FA; Dark Blue (up) / Purple (down) for FC vs FB; Green (up)/Gray (down) for FD vs FC; and Teal (up) / Gold (down) for FE vs FD. The dynamic expression of genes in the JA, auxin, and ABA pathways suggests their central roles in coordinating the developmental transitions from fruit growth to maturation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

synthase (*AOS1*) and allene oxide cyclase (*AOC*) were differentially expressed, with particularly strong upregulation of several genes during the early (FB vs. FA) and late (FE vs. FD) transitions. This was correlated with a significant accumulation of the JA precursor 12-oxo-phytodienoic acid and of jasmonic acid itself, suggesting that JA signaling plays a biphasic role in both the initiation and final stages of maturation.

In the auxin biosynthesis pathway, the gene *YUCCA10* (*YUC10*), which catalyzes a rate-limiting step in tryptophan-dependent auxin synthesis, was strongly upregulated early (FB vs. FA) but downregulated in later stages. This is consistent with the known role of auxin in early fruit development and its subsequent decline during ripening.

Genes related to abscisic acid (ABA) and ethylene biosynthesis also showed stage-specific expression. The ABA biosynthesis gene *ABA2* was induced in the mid-to-late stages, while ethylene pathway genes like ACC synthase (*ACS3*) were active in the FD vs. FC transition, aligning with their established roles in promoting ripening in climacteric fruits. In contrast, genes in the salicylic acid pathway, such as *PALY* and *ICS2*, were generally downregulated as ripening progressed, suggesting a potential trade-off between growth/ripening and defense signaling.

3.8.3. A metabolic shift from starch degradation to soluble sugar accumulation

The development of sweetness is a key indicator of fruit ripeness. To elucidate the underlying biochemical changes, we mapped the expression of relevant genes onto the starch and sucrose metabolism pathway (Fig. 9). Our analysis revealed a major metabolic reprogramming event, shifting from starch degradation in early stages to a dramatic accumulation of soluble sugars, particularly fructose and glucose, at the final stage of ripening.

During early fruit development (FA to FC), genes associated with starch degradation were actively expressed. We observed upregulation of genes encoding enzymes like alpha-amylase and beta-amylase, which break down starch into malto-oligosaccharides (MOS) and maltose. Concurrently, genes for converting these products into glucose-1P and glucose-6P were also active, indicating that stored starch was being mobilized to provide energy and carbon skeletons for the growing fruit.

Sucrose metabolism appeared to be dynamic throughout ripening. Genes for sucrose synthesis, such as those encoding sucrose phosphate synthase (SPS), showed moderate expression, suggesting that sucrose was actively being produced and likely served as a transport sugar. However, the most striking transcriptional event occurred during the final ripening transition (FE vs. FD). We observed a dramatic and significant upregulation of genes encoding invertases, the enzymes responsible for the irreversible hydrolysis of sucrose into glucose and fructose.

This late-stage induction of invertase activity directly correlated with the significant accumulation of fructose and glucose, which are the primary contributors to the sweet taste of the mature fruit pulp. Additionally, genes involved in the raffinose family oligosaccharide (RFO) pathway, leading to the synthesis of raffinose and galactinol, were also differentially expressed, suggesting a role for these sugars, possibly in stress tolerance or signaling.

The ripening of Akebia fruit is governed by a temporally coordinated cascade of gene expression and metabolic pathway shifts, as revealed by integrated transcriptomic and metabolomic analysis (Fig. 8). Early stages (FA–FB) are marked by activation of flavonoid and phenylpropanoid biosynthesis for protection, alongside the initiation of starch degradation through elevated expression of sucrose synthase and amylases, supplying energy and sugar precursors. Mid-stages (FB–FD) emphasize defense signaling and phenolic compound production, with continued carbohydrate mobilization supported by glucanases and alpha-amylase activity. In the final stage (FE), a pronounced upregulation of invertase and other sugar-converting enzymes coincides with the peak accumulation of sucrose, glucose, and fructose, enhancing

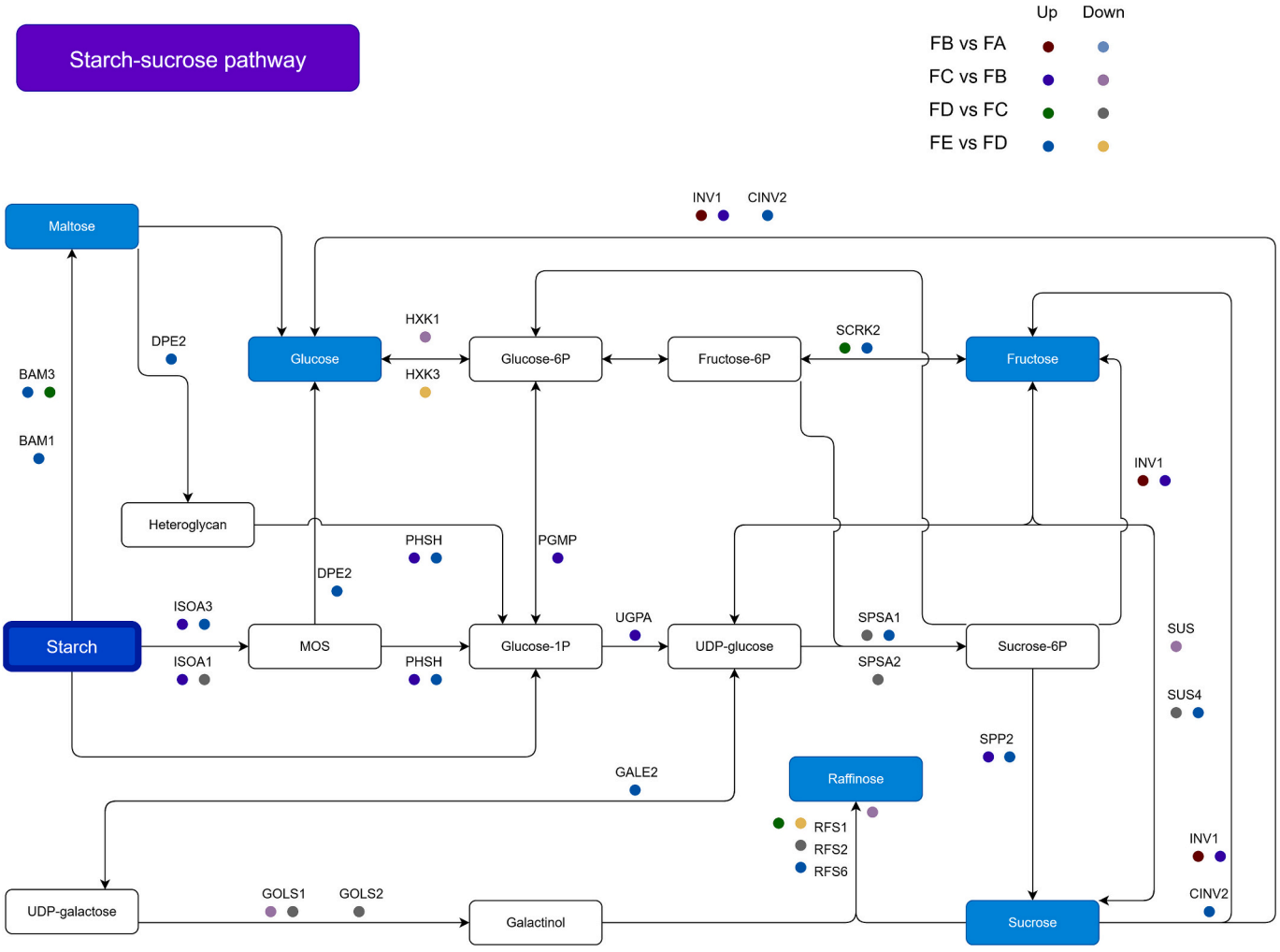

Fig. 9. Coordinated Regulation of the Starch and Sucrose Metabolism Pathway During Fruit Ripening. The schematic shows the central pathways of carbohydrate metabolism, including starch degradation, sucrose synthesis and hydrolysis, and the raffinose family pathway. Metabolites detected in this study are shown in boxes. The colored dots next to genes (on arrows) or metabolites (in boxes) indicate their differential expression or accumulation in each consecutive stage comparison: Red (up) / Light Blue (down) for FB vs FA; Dark Blue (up)/Purple (down) for FC vs FB; Green (up) / Gray (down) for FD vs FC; and Teal (up) / Gold (down) for FE vs FD. The pathway highlights the critical, late-stage upregulation of invertase, which drives the conversion of sucrose to fructose and glucose, the primary determinant of sweetness in the mature fruit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sweetness and signaling full ripening. These stage-specific regulatory events, integrating sugar metabolism with structural and defense-related changes, underpin the biochemical transitions that define *A. trifoliata* fruit quality. The findings provide a valuable foundation for functional research and targeted breeding strategies.

4. Discussion

This study provides a comprehensive temporal analysis of the transcriptomic and metabolomic changes occurring during *Akebia trifoliata* fruit ripening, offering novel insights into the molecular mechanisms underlying this developmental process. Our results reveal a highly coordinated series of events across four distinct ripening stages (FA, FB, FC, FE), highlighting the dynamic interplay between gene expression and metabolite accumulation.

The early stages of ripening (FA) were characterized by elevated expression of genes associated with stress responses, potentially reflecting the rapid growth and developmental changes occurring at this time (Kou et al., 2021). The observed high levels of L-threonine during this phase further support active primary metabolism and protein synthesis necessary for fruit development. As the fruit transitioned into the early ripening stage (FB), we noted the upregulation of specific transcription factors and the initiation of secondary metabolic pathways, as evidenced by the increased accumulation of flavonoids like (−)-epicatechin and phenolic acids. This aligns with the established roles of these compounds in providing antioxidant protection and contributing to early color development (Jia et al., 2024; J. Liu et al., 2023; Muhammad et al., 2024).

The middle ripening stages (FC and FD) witnessed significant transcriptional reprogramming, marked by the differential expression of 76 core genes across all transitions and the dynamic activity of key TF families, including NAC, MYB, AP2/ERF, and WRKY. The upregulation of *AKT000011* (lipase) in early transitions suggests the initiation of lipid metabolism, potentially crucial for membrane formation (Jin et al., 2024). The subsequent increase in *AKT000025* (E3 ubiquitin ligase) points towards the importance of protein turnover in preparing for later ripening events (Y. Wang et al., 2023; Y. Yu et al., 2020). The significant late-stage induction of the NAC TF *AKT000864*, peaking at the fully mature (FE) stage, mirrors the expression of canonical ripening master regulators like tomato's SlNAC1 and NOR (No-ripening). This conserved pattern strongly suggests that *AKT000864* plays a pivotal, conserved role in promoting the final stages of climacteric ripening, likely by activating downstream genes related to softening, aroma, and sugar accumulation. In contrast, the expression profile of the MYB TF *AKT003828* presents a more novel pattern. Unlike anthocyanin-promoting MYBs in apple (MdMYB1) and grape (VvMYBA1), which are upregulated during color development in mid-to-late ripening, *AKT003828* was highly expressed in the earliest stage (FA) and its expression steadily declined as the fruit matured. This inverse pattern suggests a distinct function. While our data cannot confirm a direct regulatory link—which would require experimental validation through methods such as ChIP-seq or Y1H assays—this inverse expression pattern makes *AKT003828* a compelling candidate for a transcriptional repressor of the flavonoid pathway during the later stages of ripening. Notably, the peak expression of certain AP2/ERF TFs supports their known involvement in ethylene signaling, a cornerstone of climacteric ripening (Thilakarathne et al., 2025), although the specific role of ethylene in *A. trifoliata* warrants further investigation (Thirugnanasambantham et al., 2015). The observed changes in amino acid metabolism during these stages likely provide precursors for various secondary metabolites (Martínez-Rivas & Fernie, 2024).

The final ripening stage (FE) was characterized by a surge in sugar metabolites (sucrose and fructose), consistent with the development of fruit sweetness that is critical for palatability and seed dispersal (Cordenunsi-Lysenko et al., 2019; Durán-Soria et al., 2020). The continuous upregulation of *AKT000026* (lysine degradation) and the strong induction of *AKT000008* (RNA polymerase) in later stages suggest ongoing metabolic adjustments and transcriptional regulation until full maturity (Gambhir et al., 2024; X. Li et al., 2022; W. Wang et al., 2023). The late activation of specific NAC TFs, such as *AKT000864* and novel.1151, may contribute to the final stages of fruit softening and stress tolerance (Ma et al., 2014b).

Conjoint analysis of our transcriptomic and metabolomic data revealed a temporally coordinated activation of key metabolic pathways. Early ripening stages showed enrichment in flavonoid and phenylpropanoid biosynthesis, likely contributing to antioxidant capacity and pigment development (Savoi et al., 2016; Singh et al., 2010; Zhong et al., 2022). The modulation of defense-related pathways involving coumarins suggests an active response to environmental challenges throughout ripening (Al-Khayri et al., 2023; Nath et al., 2023). In the later stages, pathways associated with cell wall modification and the biosynthesis of aroma compounds become prominent, contributing to the characteristic texture and flavor of ripe *A. trifoliata* fruit (Goulao & Oliveira, 2008; Paniagua et al., 2017).

Interestingly, the expression patterns of HSP-related genes, highly expressed in early development and downregulated towards maturity, suggest a critical role in managing initial growth stresses, followed by a shift towards ripening-specific processes (Ji et al., 2019; C. Zhang et al., 2023). This trend suggests a crucial role for HSPs in managing cellular stress associated with rapid cell division and expansion in young fruit (Aghdam et al., 2013; Fanwoua et al., 2012; Y.-H. Liu et al., 2016). This finding presents an intriguing contrast to the roles of HSPs reported in some other climacteric fruits (Ré et al., 2017). For instance, in tomato, specific small HSPs have been shown to accumulate during ripening, where they are thought to function as molecular chaperones that protect and stabilize ripening-related enzymes, such as phytoene synthase, which is critical for carotenoid biosynthesis (Neta-Sharir et al., 2005; Upadhyay et al., 2020). Similarly, in ripening bananas, HSP expression is often induced in response to ethylene and is associated with maintaining cellular integrity during the dramatic metabolic changes of maturation (T. Li et al., 2015). The downregulation of HSPs in maturing *A. trifoliata*, aligning with previous reports (Niu et al., 2021), may therefore signify a key developmental transition, marking a shift away from a generalized stress-response program towards the execution of highly specific, genetically programmed ripening pathways. Moreover, downregulation coincides with the upregulation of other stress-related pathways in later stages, indicating a dynamic adaptation to the changing physiological demands of the developing fruit (Chen et al., 2024; Tipu et al., 2024).

Our integrated analysis of the ripening pulp provides a complementary perspective to recent studies that have investigated the molecular basis of fruit cracking in *A. trifoliata* peel. Our findings align well with those of Jiang et al. (Jiang et al., 2023), who also used a multi-omics approach on the peel and identified cell wall degradation and phenylpropanoid metabolism as central processes. They similarly noted the upregulation of polygalacturonase and pectate lyase as key events leading to cracking. Further reinforcing this, the work by Nazir et al. (Nazir et al., 2024) provided a spatial dimension by demonstrating that these enzymatic activities, particularly polygalacturonase and β-galactosidase, are significantly more elevated in the cracking-prone ventral suture compared to the more resilient dorsoventral tissue.

While this study provides a comprehensive overview of the transcriptomic and metabolomic landscape of maturing *A. trifoliata* fruit, it is important to acknowledge its limitations. Our findings are primarily based on correlational evidence derived from expression and metabolite profiling. Although our integrated analysis allows for the generation of strong, data-driven hypotheses about gene function and regulatory networks, direct experimental proof of these proposed relationships is still required. For example, the precise regulatory links between the identified transcription factors (e.g., *AKT000864* NAC and *AKT003828* MYB) and their putative downstream targets in the sugar and flavonoid pathways need to be confirmed. Future research should therefore focus

on functional genomics approaches to validate our key candidate genes. Techniques such as Virus-Induced Gene Silencing (VIGS) could be employed to assess the impact of knocking down specific TFs on fruit phenotype, while Yeast-One-Hybrid (Y1H) or dual-luciferase reporter assays could be used to confirm direct TF-promoter interactions. Such studies will be crucial for translating the candidate genes identified here into bona fide targets for the genetic improvement of *A. trifoliata*.

In conclusion, this study provides a comprehensive molecular framework for understanding the ripening process in *A. trifoliata*. The identified key genes, transcription factors, and metabolic pathways offer valuable targets for future research aimed at manipulating fruit quality traits and extending shelf life in this nutritionally and ecologically important fruit. Further studies focusing on the hormonal regulation, particularly the role of ethylene and other plant hormones, and the functional characterization of the identified DEGs and DAMs will further enhance our understanding of the intricate mechanisms governing *A. trifoliata* fruit maturation.

CRediT authorship contribution statement

Mian Faisal Nazir: Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Tianjiao Jia:** Software, Methodology, Investigation. **Chen Feng:** Validation, Supervision, Investigation. **Edgar Manuel Bovio-Zenteno:** Validation, Methodology, Investigation. **Yi Zhang:** Methodology, Investigation, Data curation. **Longyu Dai:** Software, Resources, Methodology, Investigation. **Jie Xu:** Visualization, Validation, Methodology, Investigation. **Yafang Zhao:** Visualization, Validation, Resources, Methodology, Investigation. **Shuaiyu Zou:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2025.100312.

Data availability

Data will be made available on request.

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