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Transcriptomic and Metabolomic Analysis Reveals the Role of Exogenous GA₃ in Regulating Strawberry Fruit Development via Auxin Signaling

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Abstract

Gibberellins (GAs) are pivotal phytohormones regulating fruit development, yet the molecular mechanisms by which they modulate strawberry fruit size and quality remain elusive. Here, we investigated the effects of exogenous GA₃ on the fruit development of the cultivated strawberry (*Fragaria × ananassa* ‘Benihoppe’). Treatment with 20 mg/L GA₃ significantly promoted fruit enlargement, advanced ripening by 7 days, and increased fruit weight and dimensions. Integrated transcriptomic and targeted metabolomic analyses revealed that GA₃ application induced a substantial reprogramming of the endogenous hormone landscape, notably triggering a significant increase in auxin (IAA) levels. Transcriptome profiling identified numerous differentially expressed genes (DEGs), with KEGG enrichment analysis highlighting the “plant hormone signal transduction” pathway as the most significantly enriched. Further analysis pinpointed key DEGs involved in auxin signaling (*AUX/IAA*, *ARF*, *GH3*, and *SAUR*) and gibberellin perception (*GID1*), suggesting a central role for auxin-mediated processes in GA₃-induced fruit expansion. We propose that exogenous GA₃ promotes strawberry fruit development through a synergistic mechanism: (1) enhancing the biosynthesis of endogenous bioactive GAs; (2) activating auxin signal transduction pathways to drive cell expansion; and (3) attenuating the repression of DELLA proteins. Our study unveils the critical role of the GA₃-IAA signaling axis in strawberry fruit development, providing a theoretical foundation for harnessing hormone regulation to improve fruit yield and quality in strawberry production.

Keywords: auxin signaling; gibberellin; strawberry; transcriptome



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1. Introduction

Cultivated Strawberry (*Fragaria × ananassa*) is a perennial herbaceous plant belonging to the genus *Fragaria* within the family Rosaceae and is characterized by its compact growth habit, ease of cultivation, rapid propagation, prolonged fruiting period, and high economic value [1]. The edible part of the strawberry fruit primarily originates from the receptacle, whose development directly influences fruit quality and yield. The ‘Benihoppe’ strawberry, an early-maturing Japanese cultivar, is widely cultivated due to its desirable fruit flavor, high yield, extended harvest period, and broad adaptability [2].

Fruit serves as a fundamental model for studying plant growth and development, which can be categorized into five distinct stages: cell division, cell expansion, fruit development, ripening, and senescence [3]. In strawberries, fruit coloration and softening are critical hallmarks of ripening, with earlier maturation enhancing economic benefits. Strawberry ripening is intricately regulated by both endogenous and environmental cues, including phytohormones, nutrient availability, and external factors. Phytohormones such as auxins, strigolactones (SLs), and cytokinins (CKs) participate in the regulatory network of fruit ripening [4], though their interactive mechanisms remain to be fully elucidated.

Beyond gibberellins (GAs), accumulating evidence highlights the pivotal roles of other phytohormones, including abscisic acid (ABA), auxins, and brassinosteroids (BRs), in modulating fruit quality and ripening [5]. Ethylene accelerates apple ripening by stimulating chlorophyll degradation and anthocyanin accumulation [6]. It also regulates ripening via cell wall modulation; for instance, ethylene application enhances the activities of polygalacturonase (PG), xyloglucan endotransglucosylase (XET), pectin methylesterase (PME), and cellulose synthase, thereby promoting grape berry softening [7]. Similarly, ethylene increases PG, PME, and cellulase (Cx) activities to advance raspberry ripening [8]. Exogenous ABA accelerates citrus fruit color transition by altering chlorophyll degradation and anthocyanin biosynthesis, as also observed in litchi [9]. ABA further influences fruit texture, primarily determined by cell wall composition [10]. In SINCEd-RNAi transgenic tomatoes, elevated pectin content correlates with firmer fruit texture [11]. Conversely, auxin (IAA) acts as a negative regulator of strawberry ripening [12]. Exogenous auxin delays ripening by downregulating genes associated with anthocyanin synthesis, cell wall hydrolysis, and sugar metabolism [13]. Additionally, cytokinins (CKs), jasmonic acid (JA), and brassinosteroids have been implicated in strawberry ripening regulation [14–16].

Bioactive gibberellins (GAs), diterpenoid phytohormones with complex biosynthetic pathways, orchestrate nearly all developmental processes throughout the plant life cycle. In higher plants, biologically active GAs include GA₁, GA₃, GA₄, and GA₇ [17]. GAs are critical in strawberry fruit development, particularly in cell expansion and fruit size determination [18]. Overexpression of *OsGRF* in rice (*Oryza sativa*) reduces GA levels, resulting in thicker stems, narrower leaf angles, and improved lodging resistance [19]. GA signaling is essential for Arabidopsis seed germination, as mutations in *SLY1* (*SLEEPY1*) intensify seed dormancy [20]. *MicroRNA156*, a key yield regulator, enhances seed dormancy by suppressing GA pathways via upregulation of its target *IDEAL PLANT ARCHITECTURE 1* (*IPA1*) [21]. GA-mediated flowering regulation is widely studied in horticultural crops. Exogenous GA suppresses transcription of the apple flowering-related gene *MdSPL3*, while GA treatment during citrus flowering induction downregulates *FLOWERING LOCUS T* (*FT*), *APETALA1* (*API*), and floral organ-specific genes [15,22]. Both climacteric and non-climacteric fruits exhibit high GA production during flower, young fruit, and immature fruit development. In strawberries, bioactive GA₁, GA₃, and GA₄ are detected during fruit development, with GA₄ peaking in receptacle tissues at the white stage [23]. GAs also modulate abiotic stress responses, as reduced GA levels or disrupted signaling promote plant survival under cold and salt stress [24].

Throughout their life cycle, plants must precisely coordinate their intrinsic developmental programs with fluctuating external environments. This successful adaptation largely relies on the integration of multiple hormonal signals into complex transcriptional regulatory networks. Recent research has increasingly highlighted the central hub role of specific transcription factors in this process. A temporal transcriptomic study of soybean flowering revealed that floral organ initiation and maturation are not governed by a single hormone but are orchestrated by a dynamic and sequential hormone signaling network. The study observed that distinct developmental stages from organ initiation (by active

gibberellin and cytokinin signaling), through peak flowering (by upregulation of jasmonic acid, abscisic acid, and ethylene signaling), to senescence (by enhanced abscisic acid signaling) are dominated by different hormonal combinations, forming a clear “hormonal relay” model. Key nodes coordinating this process include MADS-box transcription factors and the root-specific gene *GmNMH7*, which was unexpectedly highly expressed in floral tissues [25]. Furthermore, research in the model plant *Arabidopsis thaliana* has provided deeper molecular insights. The HD-Zip transcription factor *ATHB1* was found to directly integrate multiple stress and hormone signals. Its expression is induced by ethylene but repressed by jasmonate, and the protein itself modulates the basal expression levels of key genes in the jasmonate and salicylic acid signaling pathways (such as *JAZs*, *WRKYs*, and *NACs*). This regulation sets the baseline state of plant sensitivity to stress, thereby finely tuning the balance between growth and defense [26]. The temporal integration and dynamic reorganization of hormone signaling pathways, driven by specific transcription factors, represent a universal and core regulatory strategy enabling plants to achieve developmental transitions and stress adaptation.

Phytohormone applications are widely employed to enhance crop yield and quality. Recent studies have focused on exogenous GA₃ in regulating plant growth and development. Strawberries are non-climacteric fruits with heightened sensitivity to exogenous hormones, and the enlargement of their receptacle is a pivotal determinant of yield and quality. This study therefore investigated the molecular mechanisms underlying GA₃-mediated strawberry fruit development using ‘Benihoppe’ strawberries. By applying exogenous GA₃ and utilizing integrated approaches including comparative transcriptomics and HPLC-based phytohormone quantification, we aimed to: determine the physiological impacts of GA₃ on fruit size, weight and ripening process; uncover dynamic changes in endogenous hormone homeostasis and genome-wide gene expression patterns during fruit development through combined transcriptome and targeted hormone metabolome analysis; and decipher the key molecular mechanisms, particularly the auxin signaling-mediated cell expansion pathway, through which GA₃ regulates fruit development. These findings elucidate the cooperative GA₃-auxin signaling network and provide new theoretical perspectives for molecular quality regulation in strawberry.

2. Materials and Methods

2.1. Plant Materials and Exogenous GA₃ Spraying Treatment

The experiment was conducted on the octoploid cultivated strawberry (*Fragaria × ananassa*) ‘Benihoppe’. The experimental plants were cultivated in a greenhouse at Xijiang Ecological Park (119.49° N, 32.28° E) under controlled environmental conditions: temperature maintained at 15–25 °C, relative humidity at 40%, and a daily photoperiod of 13 h. The growth substrate consisted of peat, vermiculite, and perlite mixed in a 3:1:1 ratio, providing optimal aeration, uniform texture, and adequate buffering capacity. The substrate pH was maintained between 5.5 and 6.5, with an EC value below 0.5 mS/cm. Plants were irrigated with a modified Yamazaki nutrient solution with a total nitrogen concentration of 6 mol/L, applied twice daily. The EC of the nutrient solution was maintained at approximately 0.7 during the seedling stage, increased to 1.0 after active plant growth was established, and gradually raised from 1.0 to 1.2 during fruit expansion through maturity (Supplemental Figure S1).

Gibberellin was dissolved in 10 mL of 95% ethanol (China National Medicines Co., Ltd., Beijing, China) as a stock solution, which was subsequently diluted to generate gradient concentrations of 10, 20, and 30 mg/L. To each solution, 3 mL/L of Tween-20 was added as a surfactant. For each concentration, 50 well-developed, medium-sized fruits with uniform green coloration were selected. The first spray application was administered at 7 days after full bloom (DAF), followed by a second spray at 14 DAF at the same concentration. Each

fruit was individually labeled. A hand-held sprayer was used to apply the solution as a fine mist over the entire fruit surface until runoff. Fruit sampling was conducted at 14, 21, 28, 35, and 42 DAF, with 10 fruits collected at each time point until full maturity. The transverse diameter, longitudinal diameter, and individual fruit weight were measured and recorded. The fruits were then cut into pieces, immediately frozen in liquid nitrogen, and stored at -80°C for subsequent transcriptome sequencing and hormone content analysis.

2.2. RNA Extraction and Transcriptome Sequencing

Total RNA was isolated with the Plant RNAprep Pure Micro Kit from TIANGEN (Beijing, China). RNA quality control, including concentration and integrity assessment, was performed on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A total of 3 μg of qualified RNA served as the starting material for library construction, which was carried out following the instructions of the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). To obtain cDNA fragments of approximately 200 bp, the library was purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA) [27]. Adapter-ligated DNA fragments were then enriched through a 15-cycle PCR amplification with Illumina-specific primer cocktails. The resulting PCR products were subsequently purified (AMPure XP system) and quantitatively assessed with an Agilent high-sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent, Suzhou, China). Finally, the prepared libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA).

2.3. Screen for Differentially Expressed Genes

The clean reads were aligned to the strawberry reference genome (https://www.rosaceae.org/species/fragaria_vesca/genome_v4.0.a2, accessed on 21 January 2025) using HISAT2 software (version: 2.0.1-beta). After obtaining uniquely mapped reads, we used the featureCounts software (version: v1.6.0) to count the number of reads mapped to each gene based on the genome annotation file. The normalization method employed was FPKM (Fragments Per Kilobase of exon per Million mapped fragments). To compare gene expression differences between different samples, differential expression analysis was performed using the R package edgeR (version 3.8.1). Genes with an FDR value less than 0.05 and an absolute $\log_2\text{FC}$ greater than 1 were considered significantly differentially expressed genes (DEGs).

2.4. RNA-Seq Data Analysis and Annotation

Enrichment analysis was performed on genes corresponding to GO annotations, with significant enrichment results filtered using a p -value < 0.05 . The clusterProfiler R package (version 3.8.1) was used to analyze the differentially expressed genes for Gene Ontology (GO) enrichment. KEGG is an important database resource for in-depth understanding of the high-level functions and utilities of biological systems. It specifically focuses on cells, organisms, and ecosystems, relying on molecular-level information. This includes large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>, accessed on 21 January 2025). We used the clusterProfiler R package to evaluate the statistical enrichment of differentially expressed genes in KEGG pathways.

2.5. Metabolite Profiling of Hormone Content Using UPLC-MS/MS

The central tissue of strawberry fruits was sampled with three biological replicates per experimental group. Fruit samples were pulverized in liquid nitrogen, and 200 ± 1 mg of the resulting powder was homogenized in 1.0 mL of extraction solvent (methanol/water/formic acid, 15:4:1). The mixture was subjected to low-temperature ultrasonic extraction for 10 min,

followed by overnight extraction at 4 °C. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected. The residue was re-extracted with 500 µL of extraction solvent under the same ultrasonic conditions, and the combined supernatants were concentrated at 4 °C and 300 rpm. The dried extract was reconstituted in 200 µL of 80% methanol, vortexed for 5 min, and centrifuged at 4 °C and 12,000 rpm for 5 min. The supernatant was filtered through a 0.22 µm microporous membrane and transferred to vials for UPLC-MS/MS analysis. The analytical platform consisted of an ultra-performance liquid chromatography system Shim-pack UFLC SHIMADZU CBM30A (Shimadzu Corporation, Kyoto, Japan) coupled with a QTRAP® 4500+ tandem mass spectrometer (AB Sciex, Framingham, MA, USA) [28]. For MRM detection, ion transitions were monitored using optimized declustering potential (DP) and collision energy (CE) parameters.

2.6. Statistical Analysis

The $2^{-\Delta\Delta CT}$ method was used for data analysis, and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used for data visualization. To analyze significant differences, IBM SPSS v30 (IBM SPSS Software Inc., San Diego, CA, USA) was used. Principal Component Analysis (PCA) of gene expression profiles from different samples was performed using the `prcomp` function in R v4.1.2, with Z-score-scaled FPKM values as input. The PCA score plot of gene expression profiles, box plots of gene expression levels and metabolite contents, the volcano plot of DEGs, and bar plots for GO enrichment and KEGG pathway enrichment of DEGs were all generated using the R package `ggplot2` v3.3.4. Pearson correlations between the metabolomic profiles of all samples were visualized as a heatmap using the R package `corrplot` v0.92. The heatmap displaying the metabolomic profiles was generated using the R package `ComplexHeatmap` v2.9.4. Differences in auxin metabolite content in strawberry fruits were determined using the Least Significant Difference (LSD) test, with a p value < 0.05 considered statistically significant.

3. Results

3.1. The Role of GA3 in Berry Growth During Fruit Development

To assess the impact of GA3 on early fruit development, the transverse and longitudinal diameters of ‘Benihoppe’ strawberry fruits were measured at 14, 21, 28, 35, and 42 days after flowering (DAF). GA3 application significantly promoted fruit enlargement, with treated fruits exhibiting a greater transverse diameter than the controls throughout the experimental period (Figure 1A). From 28 to 42 DAF, both diameters were significantly increased by GA3 treatment (Figure 1B,C). Notably, although the transverse diameter of GA3-treated fruit remained larger at 28 DAF, the magnitude of difference from the control began to narrow (Figure 1B). Figure 1D revealed that fruit weight exhibited highly significant differences ($p < 0.01$) compared to the control group from 21 to 42 days after treatment, indicating that GA₃ profoundly influences strawberry fruit cell expansion.

3.2. Phytohormone Content of Strawberry Fruits Under GA₃

We employed HPLC to determine the endogenous auxin levels in the rosette shoots of WT and GA3-treated plants. The endogenous gibberellin GA3 was consistently higher than the control group at all time points. The content of GA7 increased significantly at 35 and 42 days (Figure 2A,B).

The endogenous auxin levels at 42 days were significantly higher compared to the control group. Among them, ICAId content increased progressively, while IAA and IAA-Asp showed significant increases at 14 and 21 days (Figure 2C–F, Supplemental Figure S2).

3.3. Regulatory Role of Exogenous GA₃ in the Transcriptional Profile

To identify differentially expressed genes (DEGs) associated with the regulation of strawberry fruit traits, we conducted an integrated transcriptomic comparison between WT and GA₃-treated plants. Thirty cDNA libraries were constructed from ten samples (with three biological replicates each) and subjected to high-throughput sequencing. This process yielded approximately 255.9 million high-quality reads, with all samples exhibiting a Q30 score $\geq 95.45\%$ (indicating a base call accuracy exceeding 99.9%) and GC contents ranging from 46.03% to 46.70% (Table S1). The alignment efficiency of reads to the reference genome varied between 92.94% and 97.95% across all libraries (Table S2). We assessed global gene expression patterns through a scatter plot and a heatmap, the latter illustrating expression variations among the thirty libraries (Figure 3A). Principal component analysis (PCA) revealed clear clustering of biological replicates, confirming high reproducibility of the RNA-seq data (Supplemental Figure S3).

To further investigate genes related to fruit swelling, we identified significant DEGs by applying thresholds of $|\text{fold change}| \geq 2.0$ and $\text{FDR} \leq 0.001$ when comparing WT and GA₃-treated plants (Table S3). The highest number of DEGs was detected at day 21, with a total of 10,254 genes differentially expressed between WT₂₁ and GA₃₂₁. Among these, 3677 were upregulated and 6577 were downregulated in WT₂₁ relative to GA₃₂₁. Overall, the number of downregulated DEGs exceeded that of upregulated ones, and 27 DEGs were consistently identified across all five comparative groups (Figure 3B). Analysis of the 27 overlapping DEGs identified *Fxa2Dg00829*, *Fxa1Cg01752*, and *Fxa4Dg03048* as being enriched in multiple GO terms (Table S4).

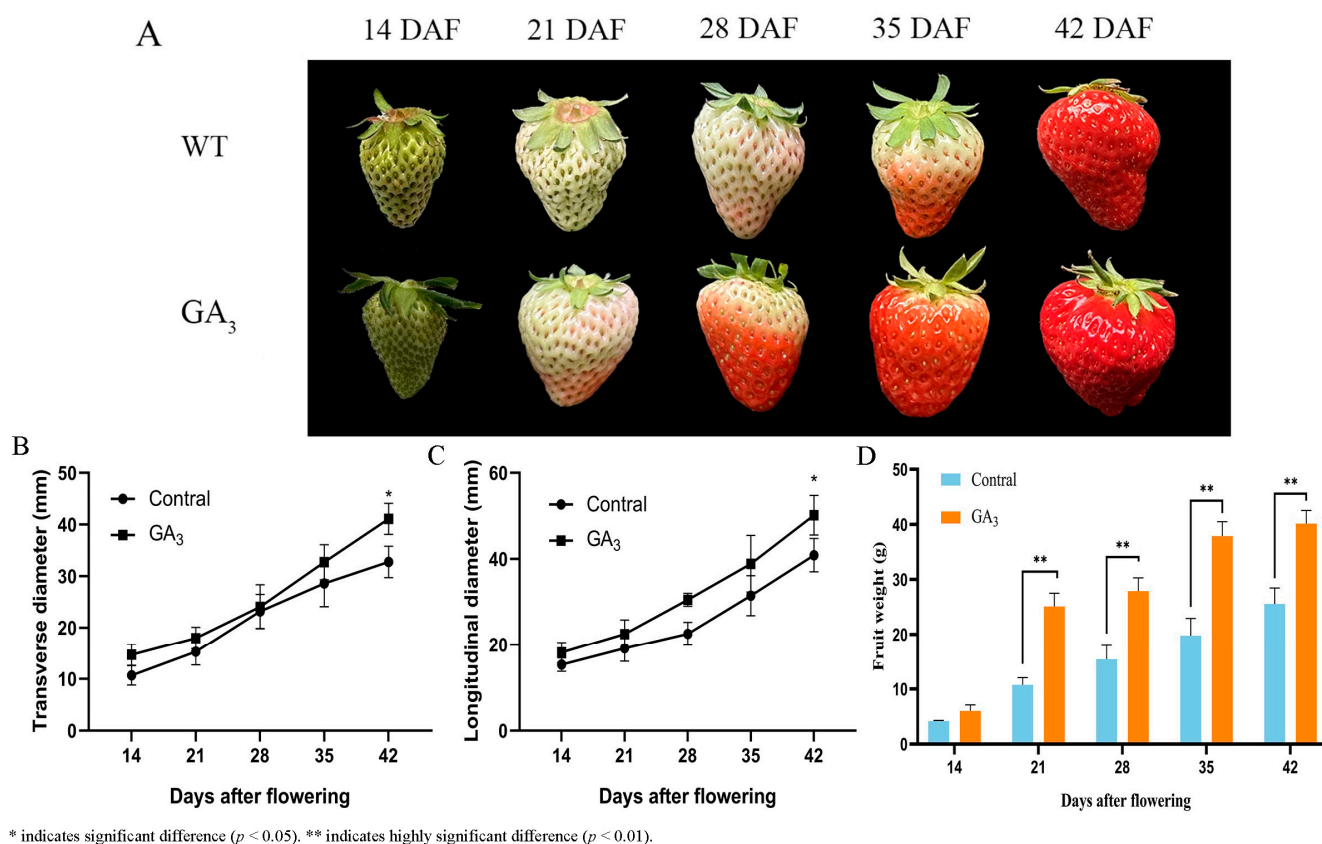


Figure 1. Effect of GA₃ treatment on the shape and size in early fruit development of 'Benihoppe'. (A) Representative images of untreated control (WT) and GA₃-treated fruits collected at 14, 21, 28, 35, and 42 days after flowering (DAF). Developmental dynamics of fruit transverse diameter (B), Developmental dynamics of fruit longitudinal diameter (C), Developmental dynamics of fruit weight (D). Data are presented as mean $\pm$ SE ($n = 3$).

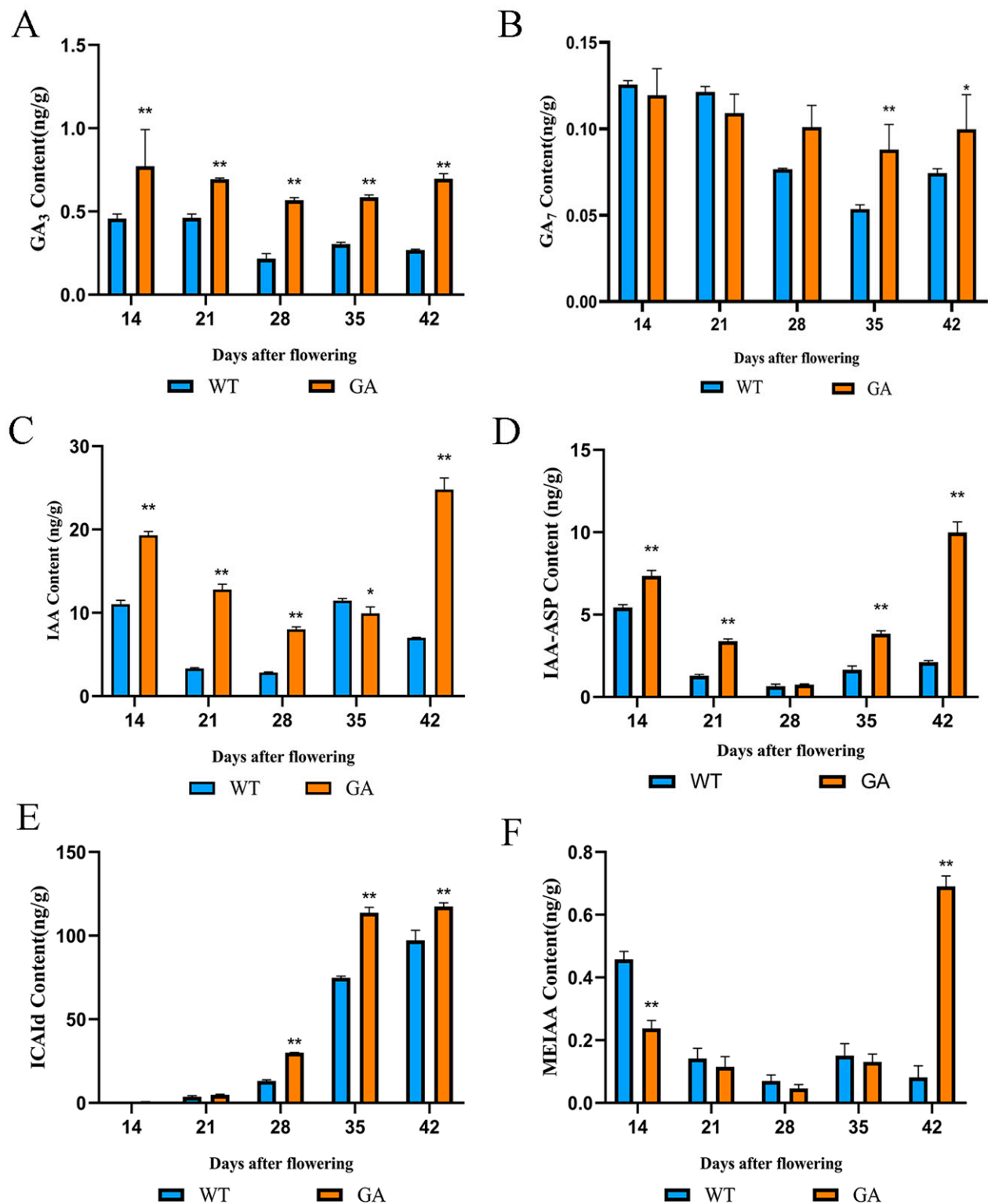


Figure 2. Determination of the content of auxin and gibberellins in WT and GA₃. (A) the GA₃ content in WT and GA₃ fruits from 14 to 42 DAF; (B) the GA₇ content in WT and GA₃ fruits from 14 to 42 DAF; (C) the IAA content in WT and GA₃ fruits from 14 to 42 DAF; (D) the IAA-ASP content in WT and GA₃ fruits from 14 to 42 DAF; (E) the ICAId content in WT and GA₃ fruits from 14 to 42 DAF; (F) the MEIAA content in WT and GA₃ fruits from 14 to 42 DAF. * $p < 0.05$, ** $p < 0.01$ determined by Student's t -test.

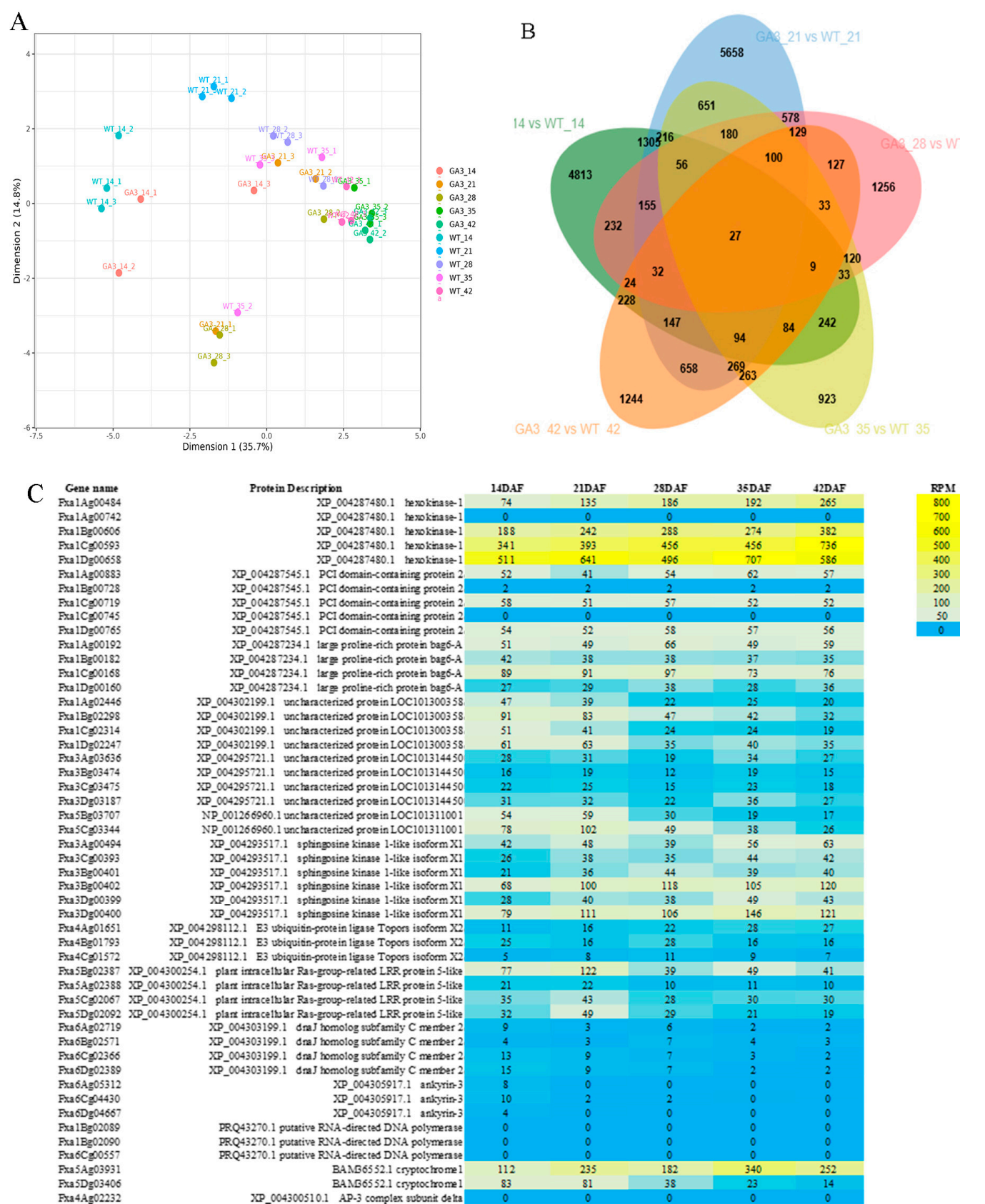


Figure 3. Expression profiling of Differentially Expressed Genes (DEGs) across thirty segments of GA and WT plants. Hierarchical cluster diagram of gene expression level in samples. Multi-dimension scaling (MDS) of the differentially expressed genes between WT and GA₃ (**A**); Venn diagram of DAMs in different comparison groups (**B**); The top 50 DEGs of 14DAF to 42DAF were visualized in a heatmap, indicating the digital gene expression index, measured in Reads Per Million (RPM) (**C**).

The results of the characteristically expressed genes in 14DAF, 21DAF, 28DAF, 35DAF and 42DAF are presented, highlighting a pronounced peak in expression at 21DAF and 35DAF (Figure 3C).

3.4. Functional Enrichment Analysis of DEGs in GO and KEGG Pathways

To further characterize the biological functions of fruit ripening-related genes, a functional enrichment analysis was performed. DEGs were categorized using Gene Ontology (GO) with a significance threshold of $p \leq 0.05$. Charts were subsequently generated to display the top 15 enriched GO terms and the top five enriched KEGG pathways.

Significant GO enrichment was observed for DEGs between WT_14 and GA₃_14. In Biological Process, terms included cell wall organization or biogenesis (GO:0071554), cell wall biogenesis (GO:0042546), and plant-type cell wall biogenesis (GO:0009832). Cellular Component enrichment featured the microtubule-associated complex (GO:0005875) and kinesin complex (GO:0005871). Molecular Function enrichment involved obsolete transcription factor activity, protein binding (GO:0000988) and obsolete transcription factor activity, transcription factor binding (GO:0000989). KEGG analysis indicated primary enrichment in Plant hormone signal transduction, Phenylpropanoid biosynthesis, Starch and sucrose metabolism, Pentose and glucuronate interconversions, and DNA replication, with the first two pathways being the most significantly populated with DEGs (Figure 4A).

In WT_21 and GA₃_21, the most enriched biological processes included response to water deprivation (GO:0009414), photosynthesis (GO:0015979), and rhythmic process (GO:0048511). The predominant cellular components were thylakoid (GO:0009579), chloroplast thylakoid (GO:0009534), and plastid thylakoid (GO:0031976). Molecular function was characterized by hydroxymethylglutaryl-CoA reductase (NADPH) activity (GO:0004420). KEGG pathway analysis further indicated significant enrichment in plant hormone signal transduction and starch and sucrose metabolism (Figure 4B).

In WT_28 and GA₃_28, the top biological processes comprised response to alcohol (GO:0097305), response to abscisic acid (GO:0009737), and developmental maturation (GO:0021700). Cellular components were predominantly enriched in nuclear chromatin (GO:0000790), aleurone grain (GO:0033095), and nuclear nucleosome (GO:0000788), while molecular function was represented by nutrient reservoir activity (GO:0045735). KEGG analysis revealed notable enrichment in plant hormone signal transduction and protein processing in the endoplasmic reticulum (Figure 4C).

In WT_35 and GA₃_35, the key biological processes were response to wounding (GO:0009611), secondary metabolic process (GO:0019748), and response to karrikin (GO:0080167). The primary cellular components included the intrinsic component of the plasma membrane (GO:0031226) and protein–DNA complex (GO:0032993). Molecular functions were related to inorganic molecular entity transmembrane transporter activity (GO:0015318) and transmembrane transporter activity (GO:0015075). KEGG pathway analysis highlighted significant enrichment in plant hormone signal transduction and the MAPK signaling pathway in plants (Figure 4D).

In WT_42 and GA₃_42, the top enriched biological processes were cellular response to extracellular stimulus (GO:0031668), response to cytokinin (GO:0009735), and the phospho-relay signal transduction system (GO:0000160). Cellular components were mainly localized to the amyloplast (GO:0009501) and aleurone grain (GO:0033095). Molecular functions involved glucosyltransferase activity (GO:0046527) and quercetin 3-O-glucosyltransferase activity (GO:0080043). KEGG analysis showed that plant hormone signal transduction and starch and sucrose metabolism were again significantly enriched (Figure 4E).



Figure 4. Twenty most significantly enriched GO metabolic pathways and five most significantly enriched KEGG metabolic pathways. (A) GO enrichment analysis for WT₁₄ vs. GA_{3_14}; KEGG enrichment analysis for WT₁₄ vs. GA_{3_14}; (B) GO enrichment analysis for WT₂₁ vs. GA_{3_21}; KEGG enrichment analysis for WT₂₁ vs. GA_{3_21}; (C) GO enrichment analysis for WT₂₈ vs. GA_{3_28}; KEGG enrichment analysis for WT₂₈ vs. GA_{3_28}; (D) GO enrichment analysis for WT₃₅ vs. GA_{3_35}; KEGG enrichment analysis for WT₃₅ vs. GA_{3_35}; (E) GO enrichment analysis for WT₄₂ vs. GA_{3_42}; KEGG enrichment analysis for WT₄₂ vs. GA_{3_42}.

These results indicate that the co-enrichment of gibberellin signaling components and maturation-related genes highlights a coordinated regulatory network controlling fruit development.

3.5. DEGs Associated with Plant Hormones in Fruit Traits

The most prominent finding from the KEGG enrichment analysis was the significant enrichment of the plant hormone signaling pathway, which comprised the largest number of DEGs. This prompted a focused study on plant hormone-related genes, leading to the identification of 38 DEGs enriched in the IAA signal transduction pathway—a result that implies a functional link between auxin and GA. Core components of the auxin signaling machinery were represented among these DEGs, including auxin1 (*AUX1*), auxin/indole-3-acetic acid (*AUX/IAA*), and auxin response factor (*ARF*), *SMALL AUXIN-UPREGULATED RNA* (*SAUR*) and *GH3* involved in IAA biosynthesis. Four *AUX1* gene (*Fxa2Ag02913*, *Fxa2Dg02549*, *Fxa6Dg01005*, *Fxa7Cg02133*). *AUX/IAA* genes (*Fxa2Ag02490*, *Fxa2Ag03703*, *Fxa4Cg01996*, *Fxa6Ag00286*, *Fxa6Ag03168*, *Fxa6Bg00251*, *Fxa6Cg02773*, *Fxa6Dg00231*, *Fxa6Ag03037*, *Fxa1Dg00585*, *Fxa6Bg02858*, *Fxa6Bg03018*, *Fxa6Cg02716*) were annotated of which 16 DEGs may be associated with early fruit ripening. Six *ARF* gene (*Fxa4Bg02258*, *Fxa5Dg03125*, *Fxa2Bg03756*, *Fxa5Ag01012*, *Fxa5Ag01246*, *Fxa7Ag01712*). Six *SAUR* genes (*Fxa2Bg03757*, *Fxa2Dg03539*, *Fxa2Ag01135*, *Fxa5Cg02155*, *Fxa5Ag02504*, *Fxa5Cg02155*) were downregulated in GA₁₄ to GA₄₂. The *GH3* upregulated genes showed log₂ ratios of −1.423, −3.375, −1.156, −1.317, −0.508 and −1.317, respectively. Six *GH3* genes were annotated, of which five genes (*Fxa2Ag00460*, *Fxa2Ag02676*, *Fxa2Bg00492*, *Fxa2Dg00398*, *Fxa4Ag02121*, *Fxa4Dg01837*) were upregulated in GA₁₄ to GA₄₂ and one gene (*Fxa2Ag02676*) were downregulated in GA₁₄ to GA₄₂ (Figure 5A).

Cytokinin is often used as the second messenger of auxins to regulate branching development [29]. Four expressed genes were identified in *AHK2_3_4* (a signal transduction protein kinase). Among these, the genes *Fxa4Ag03357*, *Fxa4Bg02338*, and *Fxa4Dg02852* exhibited up-regulated expression during the 21–42 DAF period, whereas *Fxa6Bg03863* showed down-regulated expression (Figure 5B).

Gibberellin has an important effect not only on internode elongation but also on early fruit ripening. In the gibberellin (GA) signaling pathway, five differentially expressed genes were detected in *GID1* (gibberellin receptor protein). Among these, *Fxa6Dg00378* and *Fxa6Ag00460* exhibited upregulation during the 14–21 DAF, while *Fxa2Ag02693*, *Fxa2Cg03888*, and *Fxa2Bg00910* showed downregulation. In DELLA proteins, a single gene (*Fxa1Ag00466*) was identified with downregulated expression. Additionally, within TFs (phytochrome-interacting factors), the gene *Fxa4Bg02622* displayed an upregulated expression (Figure 5C).

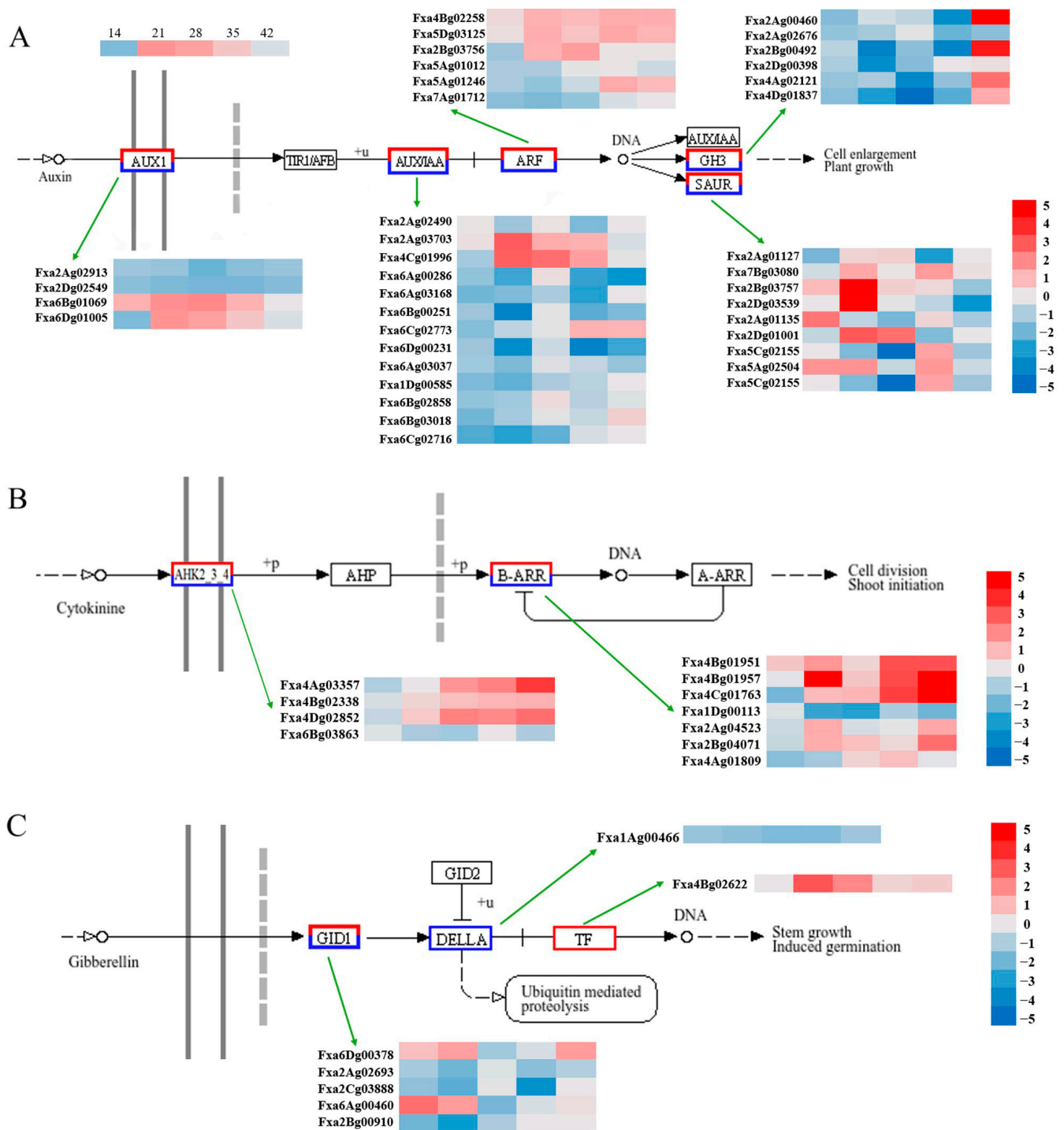


Figure 5. Expression Patterns of Differentially Expressed Genes in Plant Hormone Pathways. (A) Auxin signal transduction pathway. (B) Cytokinin signal transduction pathway. (C) Gibberellin signal transduction pathway. Note: The color bar indicates relative expression levels. Genes are highlighted with red (upregulated), blue (downregulated), or gray (unchanged) borders.

4. Discussion

4.1. Regulatory Cascade Linking GA₃ and Auxin-Mediated Cell Expansion

Plant hormones mediate responses to external and endogenous stimuli through complex regulatory networks [30]. Auxin and gibberellin interact in processes such as root elongation and stem growth. At the signaling level, auxin promotes the degradation of DELLA proteins in roots, which is essential for GA-induced root elongation. At the

biosynthesis level, auxin positively regulates GA biosynthetic genes (*GA20ox*) via ARF transcription factors [29]. Previous research involving transient overexpression in grape demonstrated that *VvARR* upregulates endogenous levels of GA₃, IAA, and 6-BA, thereby increasing both the number of cells per unit area and cell dimension parameters, collectively contributing to grape phenotype regulation [31]. Our experimental data suggest a potential regulatory cascade initiated by exogenous GA₃. The upregulation of the gibberellin receptor gene *GID1* likely enhances GA signal perception. Concurrently, we observed a significant increase in endogenous IAA levels alongside dynamic expression changes in key auxin signaling components, including *AUX/IAA*, *ARF*, and *SAUR* genes. As a result, we postulate a hypothetical model wherein GA₃ signaling, potentially via the downregulation of a DELLA gene, modulates the activity of transcription factors controlling the expression of auxin-responsive genes. For instance, specific *ARFs* may be activated, subsequently inducing the expression of *SAUR* genes, which are known to promote cell expansion. The concurrent upregulation of several *GH3* genes suggests the existence of a feedback mechanism to fine-tune IAA levels. Although our data implies this sequential relationship, further investigation is required to determine whether the regulation of *ARF*, *GH3*, or *SAUR* by the gibberellin signaling pathway is direct or indirect.

4.2. Differential Gene Expression Analysis via Transcriptomics and Metabolomics Across Strawberry Fruit Developmental Stages

In this study, RNA-seq analysis identified a total of 27,809 Differentially Expressed Genes (DEGs), including both up- and downregulated genes, between the 'Benihoppe' strawberry control and GA-treated groups. The highest number of DEGs was identified at 21 days, indicating that the early stage of strawberry fruit development involves the most significant transcriptional changes. This coincides with the initiation of key developmental and morphogenetic processes likely requiring extensive gene activation and repression. In contrast, the comparison at 35 days yielded the fewest upregulated genes, suggesting that gene expression becomes more stabilized at later stages, possibly reflecting the completion of developmental programs and the transition to fruit maturation [32]. Notably, the fewest downregulated genes were found in the 42-day comparison, indicating a more gradual transcriptional repression as the fruit approaches full maturity. This downregulation may be associated with the cessation of cellular processes no longer required or the activation of programmed cell death pathways [33]. To gain deeper insights into the biological processes influenced by GA during strawberry fruit development, we performed functional analysis of DEGs at various stages. GO enrichment analysis revealed that DEGs were enriched in biological processes related to cell wall composition and biochemical synthesis, photosynthesis, abscisic acid response, and secondary metabolism. Regarding cellular components, DEGs were enriched in microtubule-associated complexes, thylakoids, and intrinsic protein components of membranes; these genes are closely linked to fruit development and cellular size [34]. In molecular function, DEGs were enriched in transcription factor activity and protein binding, *HMG-CoA* reductase activity, and glycosyltransferase activity. A substantial number of genes were associated with DNA-binding transcription factor activity, including members of the *NAC* and *MYB* families, which are known regulators of structural genes promoting fruit coloration and ripening [35]. KEGG pathway analysis further revealed enrichments in Plant hormone signal transduction, Starch and sucrose metabolism, and the *MAPK* signaling pathway, highlighting the importance of controlled cell proliferation and sugar accumulation during strawberry fruit development under exogenous GA induction [36]. The enrichment of auxin and gibberellin-related signaling components aligns with previous studies, indicating the significant roles these hormones play throughout fruit development. This study demonstrates that exogenous GA₃ application significantly promotes strawberry fruit enlargement and accelerates ripen-

ing. Our integrated transcriptomic and metabolomic analysis provides novel evidence that GA₃-induced fruit development is orchestrated through a synergistic interaction with auxin signaling, rather than by GA action alone.

4.3. The Role of GA in Interaction with Other Hormone Pathways

GA interacts with all major plant hormones, predominantly in a bidirectional regulatory manner [37]. KEGG enrichment analysis indicated significant alterations in the Plant hormone signal transduction pathway, encompassing abscisic acid (ABA), cytokinins (CKs), and jasmonic acid (JA). Previous research has shown that during seed germination, ABA inhibits GA-induced gene expression downstream of DELLA proteins by inducing the protein kinase *PKABA1* and *WRKY* transcription factors [38]. In root growth, ABA inhibits GA's growth-promoting effects by stabilizing DELLA proteins. GA and cytokinins often act antagonistically; in the shoot apical meristem, *KNOX* transcription factors promote CK synthesis while directly inhibiting GA synthesis and promoting GA deactivation. Under low GA conditions, the *SPY* protein inhibits GA signaling and promotes CK response, whereas high GA signaling suppresses the *SPY* protein, thereby inhibiting the CK response [39]. Recent studies found that the anthocyanin suppressor *MdbHLH162* integrates GA and JA signals to negatively regulate anthocyanin biosynthesis, and regarding disease resistance in cassava, research indicates that gibberellin and jasmonic acid act antagonistically, underscored by the interaction between the key repressors *GAI1* and *JAZ2.2* [40]. Therefore, we hypothesize that the GA₃-accelerated ripening might be linked to a temporal shift in ABA accumulation, a hormone known to promote ripening in non-climacteric fruits. Interactions with cytokinin and JA pathways might fine-tune the balance between cell division and stress responses during fruit development. This hypothesis underscores the complexity of gibberellin-mediated fruit developmental processes.

4.4. The Role of DELLA Proteins in the GA Hormone Pathway

As key components of the GA signaling pathway, DELLA proteins function as crucial plant growth repressors. They primarily suppress plant growth by interacting with growth-promoting factors (such as *PIFs* and *ARFs*), thereby inhibiting their activation of downstream genes. Research on *Arabidopsis thaliana* fruit development found that auxin acts upstream of GA signaling. The auxin signal produced by fertilized seeds ultimately drives fruit growth by inducing GA biosynthesis, which subsequently promotes the degradation of DELLA proteins [41]. Furthermore, this study demonstrated that among the five DELLA members (*GAI*, *RGA*, *RGL1*, *RGL2*, *RGL3*), *RGL1* plays the predominant role in inhibiting fruit growth, while the functions of other members appear somewhat redundant [42]. Additionally, DELLA proteins have been found to influence auxin transport, further emphasizing their pivotal role in the cross-regulation of hormonal pathways.

Upon binding to its receptor *GID1*, GA initiates a protein–protein interaction between *GID1* and DELLA proteins, ultimately leading to DELLA hydrolysis and subsequent initiation of plant elongation growth. The GA-*GID1* complex forms a dimer, which further associates with DELLA proteins to create a GA-*GID1*-DELLA trimeric complex [43]. This ternary complex plays a crucial role in GA signal transduction, regulating molecular signaling throughout the GA pathway. Subsequent studies in fruit trees have revealed *GID1* gene functions—for instance, *MdGID1* was identified in columnar apple cultivars [44]. Sequence analysis shows it contains two exons and one intron, with cis-regulatory elements in its upstream regulatory sequence responsive to phytohormones, light, and temperature signals.

These insights deepen our understanding of how exogenous GA₃ coordinates hormonal crosstalk to modulate strawberry fruit development and quality traits. Our study

observed the downregulation of a DELLA gene transcript. Consequently, we infer that DELLA proteins act as negative regulators of GA biosynthesis and signal transduction. However, future research quantifying DELLA protein levels is required to validate this inference.

4.5. Linking GA to Agronomic Traits

Studies on hormone levels have shown that multiple factors contribute to differences in fruit size, such as genetic factors, endogenous hormone levels, solute content, and external environmental influences [45,46]. Current research indicates that the application of exogenous GA₃ leads to morphological variations in fruits by promoting both cell elongation and division, while also modulating the levels of endogenous hormones [47]. The molecular changes identified in our experiments provide a direct link to the observed phenotypes of fruit enlargement and accelerated maturation. The core of this connection lies in the interaction between cell expansion and plant hormones. Research in pear indicated that ABA drives the depolymerization of pectic substances by upregulating a series of cell wall degradation enzyme genes (*PcPG1*, *PcPG2*, *PcPL*, *PcPME3*, and *PcGAL1*) and enhancing their enzymatic activities, forming a positive feedback loop to sustain the softening signal [48]. Studies in peach found that suppressing the expression of key genes *PpBGAL10/16* significantly reduced the activities of polygalacturonase (PG) and pectin methylesterase (PME), thereby delaying cell wall degradation and reducing ethylene production, suggesting the existence of a positive feedback circuit [49]. Notably, our transcriptome data revealed the differential expression of genes encoding cell wall-modifying enzymes, although we have not yet deeply investigated the specific relevant genes. Therefore, we hypothesize that the coordinated GA-IAA signaling cascade ultimately leads to the transcriptional activation of these cell wall remodeling genes, directly promoting cell expansion, fruit softening, increased fruit weight, and an accelerated maturation process. This highlights the novelty of the integrated analysis of GA₃-IAA regulation in strawberry fruit development, elucidates the mechanistic connections and limitations, and links molecular changes to the final agronomic traits.

5. Conclusions

Through exogenous GA₃ treatment and multi-omics analysis, this study systematically elucidates the molecular mechanism by which GA₃ promotes strawberry fruit enlargement via synergistic regulation of the GA-IAA signaling axis. Our findings demonstrate that GA₃ signaling likely downregulates DELLA proteins, thereby modulating auxin-responsive transcription factors and inducing the expression of cell expansion-related genes, ultimately leading to significantly enlarged fruits and approximately 7-day advanced maturation. This discovery not only reveals a novel hormonal interplay mechanism regulating fruit development but also provides both theoretical foundations and practical strategies for precision cultivation in strawberry production.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy15112645/s1>, Table S1. Transcriptome sequencing data. Table S2. Transcriptome sequencing reads alignment with reference genome data. Table S3. Differentially expressed genes. Table S4. The 27 overlapping DEGs protein description. Figure S1. Experimental Site and Materials. Figure S2. Representative UPLC-MS chromatograms for the quantification of phytohormones. Figure S3. Principal component analysis (PCA) of the differentially expressed genes between WT and GA₃.

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and H.G. (Hongsheng Gao); Resources, Y.C., W.W. and Q.C.; Software, W.W. and Q.C.; Validation, S.C.; Visualization, H.G. (Han Gao); Writing—original draft, H.G. (Han Gao); Writing—review and editing, S.C. All authors have read and agreed to the published version of the manuscript.

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