

Article

Multi-Omics Analysis Sheds Light on the Relative Roles of Hormones and Nutrients in Regulating Secondary Flowering in *Prunus subhirtella* ‘Autumnalis’

Zichao Kan [†], Yanxia Xu [†], Guoshuai Li, Wenhui Wang, Pengyi Wang and Chunling Zhou ^{*}

College of Landscape Architecture and Forestry, Qingdao Agricultural University, Changcheng Road No. 700, Qingdao 266109, China

^{*} Correspondence: 199601032@qau.edu.cn

[†] These authors contributed equally to this work.

Abstract

Cherry blossom trees are iconic ornamental plants of the spring known for their vibrant colors and elegant forms. However, their short flowering period limits their ornamental value. *Prunus subhirtella* ‘Autumnalis’ is notable for its ability to flower a second time in autumn. Study of the secondary flowering of this variety may offer insights into the development of cherry blossoms. Here, we studied the secondary flowering of *Prunus subhirtella* ‘Autumnalis’ by collecting three types of flower buds: the terminal buds of long branches in autumn (LB), the basal buds of short branches in autumn (SB), and flower buds in spring (FB). Transcriptomic and metabolomic analyses were then conducted on autumn flower buds to identify key metabolic pathways associated with secondary flowering. These pathways were primarily involved in nutrient accumulation and plant hormone biosynthesis. We then quantified changes in indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), and gibberellic acid (GA₃), as well as levels of soluble protein, soluble sugar, and starch in flower buds. Correlation analysis indicated that IAA was necessary for flower bud development; ABA was weakly correlated with secondary flowering; and JA was significantly negatively correlated with secondary flowering. The GA₃ content was higher in LB than in SB and was significantly positively correlated with secondary flowering. Additionally, nutrient levels were higher in LB than in SB, suggesting that the accumulation of sufficient nutrients supports the second bloom. Correlation analysis revealed that ABA and GA₃ levels were positively correlated in flower buds, but GA₃ was negatively correlated with JA levels. This study provides a theoretical basis for understanding the molecular and physiological mechanisms underlying the secondary flowering phenomenon in *Prunus subhirtella* ‘Autumnalis’ and offers valuable insights for extending the ornamental period of cherry blossom trees.



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1. Introduction

Flowering is one of the most important adaptations in plants, as it not only ensures the reproduction and survival of species but also contributes to the regulation of material cycles and energy flow within ecosystems. The flowering of woody plants is an annual phenomenon that synchronizes the reproductive cycle with seasonal conditions. However, a small number of plant species or varieties are not confined to flowering only once during

the same year. A representative case is *Prunus subhirtella* ‘Autumnalis’ (Rosaceae), which exhibits an autumn flowering period. After its flower buds complete differentiation from late summer to early autumn, a portion bloom in October of the same year, while the remaining buds enter dormancy and await flowering the following March (FB). Preliminary observations indicate that there is a positional distribution pattern regarding the autumn flowering buds of this variety: the flowering rate of flower buds located at the tips of long branches in autumn is significantly higher than that of flower buds situated toward the base of short branches. In other words, autumn-flowering buds are mostly distributed at the tips of longer branches (LB), while non-flowering buds in autumn tend to be located toward the base of shorter branches (SB).

To understand this unconventional flowering phenomenon of *Prunus subhirtella* ‘Autumnalis’, it is first necessary to grasp the general principles governing the regulation of plant flowering. Molecular biology research has confirmed that flowering is controlled by a multi-pathway regulatory network. Gene-level variations underlie the evolution of species-specific traits and adaptations [1]. Multiomics research serves as a critical bridge connecting genotype and phenotype that has been widely applied to explore plant growth and development, and provides a powerful tool for deciphering complex traits such as flowering. In *Prunus* species, multiomics approaches have been utilized to study flower development, with particular emphasis placed on the regulation of flower bud development. For example, in *Prunus pseudocerasus*, the bud differentiation process has been investigated using transcriptomics and carbohydrate profiling [2]. In *Prunus persica*, transcriptomic studies have focused on the regulation of hormone signaling during flower bud development under low-temperature stress [3]. In contrast, systematic research on the regulation of the flowering phase remains relatively limited. However, in recent years, several studies have begun to focus on this area. For example, transcriptome sequencing of *Prunus yedoensis* ‘Somei-yoshino’ revealed key flowering-related genes and facilitated the development of a flowering prediction model [4]. Furthermore, transcriptomic studies on *Prunus mume* have investigated the role of the PmSBP transcription factor in regulating flowering time, revealing its influence on flowering [5]. Study of the metabolome can also provide insights into the biochemical basis of flowering through the identification of small metabolites involved in the flowering process [6–8]. Metabolomic analysis has clarified the role of proline metabolism in the regulation of flowering in *Prunus persica* [9]. Among these metabolites, plant hormones and nutrients, as key regulatory factors, have garnered particular attention. The integration of transcriptomic and metabolomic data provides a more comprehensive perspective on floral development from both the gene expression and metabolite accumulation levels [10].

Plant age, gibberellin signaling, and glycolides are particularly important for regulating the flowering process [11]. Among these, the hypothesis that plant hormones regulate flowering has gained broad consensus. For example, gibberellic acid (GA₃) is widely recognized as a key hormone promoting flowering in many plant species. Numerous studies have highlighted the central role of plant hormone signal transduction in the development of plant organs, and plant hormones are some of the most important endogenous signaling molecules [12,13]. However, hormone signaling is not regulated by a single hormone but rather through interactions among multiple hormones that alter the expression of genes [14]. GA₃, abscisic acid (ABA), and Indole-3-acetic acid (IAA) are particularly important regulatory hormones [15,16]. GA₃ influences flowering by altering the expression of genes involved in the GA₃ pathway, as well as by interacting with vernalization pathways and floral repressors such as the GAF1-TPR complex [17,18]. The role of ABA remains unclear. Some studies indicate that elevated ABA levels increase flower bud number, yet do not explicitly confirm its role in promoting flowering [19]. On the other hand, high ABA con-

tent delays flowering in *Prunus sibirica* [20]. Similarly, the influence of auxin on flowering varies [21]. For example, the increase in indole IAA content before flowering in *Prunus mume* suggests its promotive effect on flowering [22]. Other research has indicated that IAA exerts differential regulatory effects on flower buds across seasons [23]. Furthermore, other hormones, such as jasmonic acid (JA), have direct or indirect effects on the timing of flowering and bud development [24–26]. Therefore, what role these hormones play in the flowering process of *Prunus subhirtella* ‘Autumnalis’ cherry blossoms, thus leading to their secondary autumn blooming, is an urgent question that needs to be elucidated.

Nutrient accumulation is involved in the process of flower development [27,28]. Furthermore, due to its unique flowering habit, *Prunus subhirtella* ‘Autumnalis’ exhibits a pattern where a portion of its flower buds break dormancy and bloom in autumn, while the remaining buds continue dormancy through winter. This process may be influenced by nutrient availability. This association is indirectly supported by multiple studies. For example, Sangeeta et al. observed in a comparative study of two apple cultivars (‘Cripps Pink’ and ‘Honeycrisp’) with different flowering times that starch levels generally decrease during dormancy while soluble sugars increase. This pattern led to the hypothesis that differential nutrient availability could be a contributing factor to the observed flowering differences [28]. This finding suggests that the transformation of nutrient forms is closely linked to the dormancy status of buds and subsequent floral development. Furthermore, similar patterns of changes in nutrients have been observed during bud dormancy and its release in sweet cherry [29]. As dormancy release is a critical prerequisite for flowering, changes in nutrient availability also influence the subsequent initiation of flowering. Although these studies have not directly established a causal relationship between carbohydrate accumulation patterns and flowering time differences among cultivars, they collectively highlight the role of nutrients in flowering regulation, providing crucial evidence for their central function in floral development.

Unfortunately, current research in multiomics, hormones, and nutrients has predominantly focused on primary flowering, leaving systematic analysis of the distinct trait of secondary flowering limited. Although preliminary explorations have been conducted on plants in the Magnoliaceae family, such as a metabolomics study on *Magnolia* revealing the crucial role of sugar metabolism, their hormonal regulatory networks remain unclear [30]. Furthermore, secondary flowering in this plant relies on two independent cycles of floral bud differentiation, which fundamentally differ from the type of secondary flowering where the same cohort of buds blooms in stages. Secondary flowering of *Prunus dulcis* [Mill.] D. A. Webb is currently attributed only to resource competition at the physiological level, while its underlying mechanisms remain unelucidated. Furthermore, as a commercial crop, research has predominantly focused on its fruit-related traits [31].

In summary, despite the proven importance of nutrients and hormones in plant flowering, research on their interactions and the regulatory mechanisms of secondary flowering in cherry blossom trees remains limited. Based on the unique biological phenomenon of *Prunus subhirtella* ‘Autumnalis’, after all flower buds complete differentiation in autumn, some buds bloom directly in the same autumn, while others enter dormancy and delay flowering until the following spring. This phenomenon exhibits stable position-dependent characteristics. This study focuses on comparing flower buds with different developmental fates under the same physiological background, aiming to explore the factors influencing their developmental pathways. The findings are expected to provide novel insight into the recurring flowering phenomenon in woody plants and offer a theoretical foundation for extending the ornamental period of cherry blossoms and manipulating flowering time in woody species.

2. Results

2.1. Multi-Omics Analysis of Two Types of Flower Buds in Autumn

Transcriptomic and metabolomic analyses were performed on LB and SB during the critical transition period (S3) to explore their genetic and metabolic differences. Morphological states at different developmental stages are shown in Supplementary Figure S1 (Figure S1). Transcriptome sequencing yielded a total of 359,403,990 raw reads across all samples. After quality filtering, 352,670,110 clean reads were obtained, with Q30 values ranging from 95.33% to 95.63% and the GC content ranging from 45.41% to 45.49%. Non-targeted metabolomic analysis identified a total of 2599 metabolites.

Principal component analysis (PCA) revealed distinct patterns in the two datasets. In the transcriptomic PCA plot (Figure 1A), while LB and SB samples showed a separation trend along PC1, their confidence ellipses substantially overlapped, indicating that the transcriptomic differences between these groups were discernible but limited. In contrast, in the metabolomic PCA plot (Figure 1B), LB and SB samples were completely separated with no overlap, suggesting that these groups show substantial metabolic differences. Additionally, strong within-group clustering was observed for both datasets, indicating high intra-group consistency. In summary, the metabolic differences between LB and SB were pronounced and clear, whereas the transcriptomic differences were relatively modest.

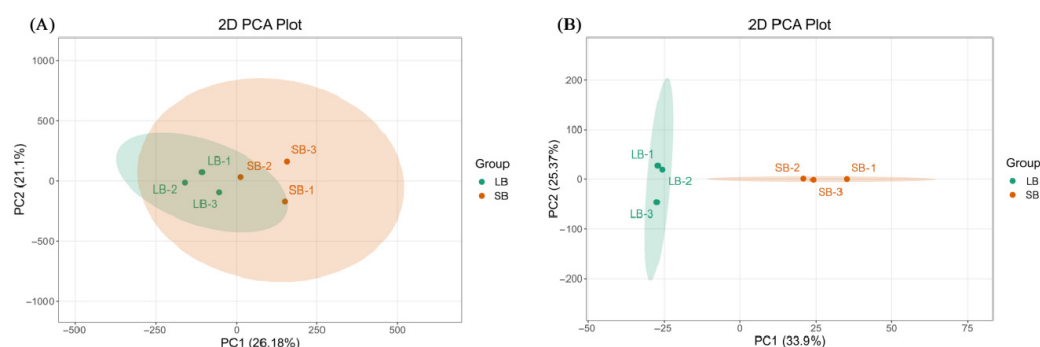


Figure 1. PCA plot of transcriptome samples (A); PCA plot of metabolome samples (B). Note: The horizontal coordinate PC1 represents the explained variance of the first principal component; the vertical coordinate PC2 represents the explained variance of the second principal component. The elliptical regions represent the 95% confidence intervals, with different ellipses corresponding to different groups. The degree of separation between ellipses reflects the extent of between-group differences—the greater the distance, the larger the difference. Dots represent samples, and the distance between dots indicates the degree of difference between samples—the greater the distance, the larger the difference.

2.1.1. Transcriptomic Analysis of Two Types of Flower Buds in Autumn

Differentially expressed genes (DEGs) between sample groups were identified using DESeq2 v1.22.2 and the following criteria: $|\log_2\text{Fold Change}| \geq 1$ and false discovery rate < 0.05 . A total of 214 DEGs were identified, including 82 up-regulated and 132 down-regulated genes (Figure 2). Additionally, functional annotation was performed for the genes without parameters, and the annotation results are provided in Supplementary Table S1 (Table S1). KEGG pathway annotation revealed that the DEGs were primarily involved in metabolic pathways, secondary metabolite biosynthesis, carbon metabolism, ABC transporters, MAPK signaling, plant hormone signal transduction, peroxisome, and glyoxylate and dicarboxylate metabolism (Figure 3). ABC transporters, MAPK signaling pathway, and peroxisome are primarily associated with plant stress responses. Carbon metabolism and glyoxylate and dicarboxylate metabolism are related to the distribution of nutrients, and the plant hormone signal transduction pathway plays a direct

role in regulating plant development through hormonal signaling. The differences in the resistance pathways stem from the fact that SB enters dormancy during the S3 period, but LB tends to flower during the same period. This phenological difference results in distinct resistance pathways between the two.

Analysis of plant hormone signal transduction pathways revealed three up-regulated DEGs and one down-regulated DEG in SB. Seven DEGs annotated to the carbon metabolism and glyoxylate and dicarboxylate metabolism pathways were detected. Functional annotations revealed that these DEGs are mainly involved in the synthesis of malic acid and glutamic acid, as well as in stress resistance and antioxidant responses. Broadly summarized, genes related to malic acid and glutamic acid synthesis were down-regulated in SB, while genes associated with stress resistance and antioxidant functions were up-regulated.

2.1.2. Non-Targeted Metabolomic Analysis of Two Types of Flower Buds in Autumn

Differentially Accumulated Metabolites (DAMs) were identified based on the following criteria: variable importance in projection (VIP) > 1 and fold change ≥ 2 or fold change ≤ 0.5 [32]. A total of 125 DAMs were detected, including 42 up-regulated and 83 down-regulated metabolites. Annotation of the DAMs to KEGG pathways showed that they were distributed across several specific pathways, including tryptophan metabolism, sphingolipid metabolism, nucleotide metabolism, nucleotide sugar biosynthesis, and amino acid biosynthesis, in addition to the broad categories of general metabolic pathways and secondary metabolite biosynthesis (Figure 4).

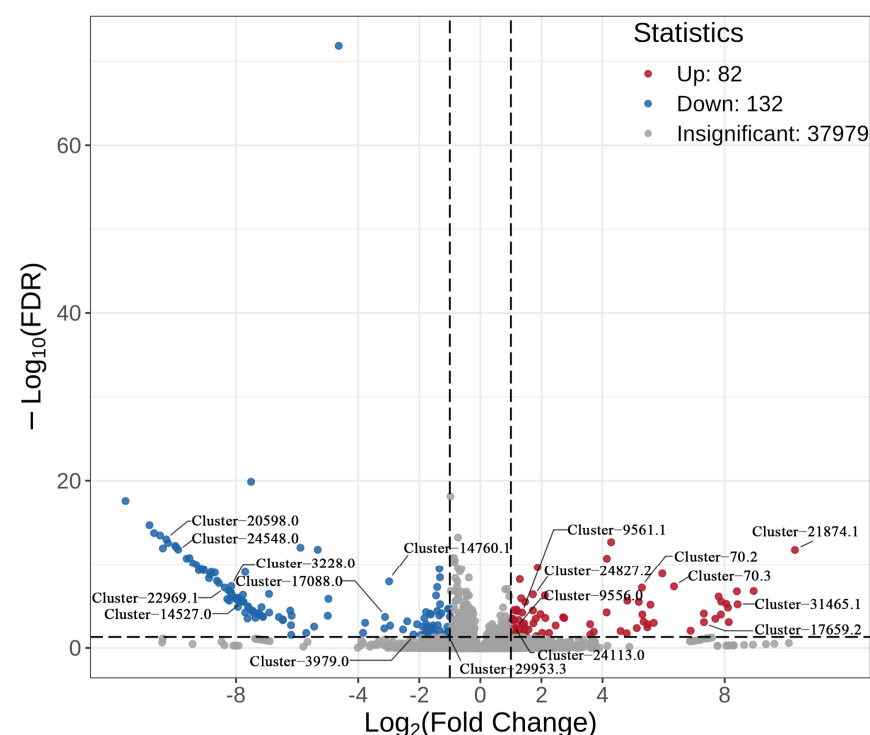


Figure 2. Volcano plot. Note: The horizontal axis represents $\log_2$ (fold change), which is the logarithmic representation of the fold change in gene expression levels. The vertical axis $-\log_{10}(\text{FDR})$ represents the significance level of differentially expressed genes (FDR is the false discovery rate). The red dots represent upregulated differentially expressed genes, the blue dots represent downregulated differentially expressed genes, and the gray dots represent genes that are not differentially expressed. The labeled genes are the candidate genes identified through screening in this study. The vertical dashed lines represent the thresholds for gene expression changes, set at $\log_2\text{FC} = 1$ and $\log_2\text{FC} = -1$, indicating a two-fold upregulation or downregulation. The horizontal dashed line represents the significance threshold, set at $-\log_{10}(\text{FDR}) = 1.3$ ($\text{FDR} = 0.05$).

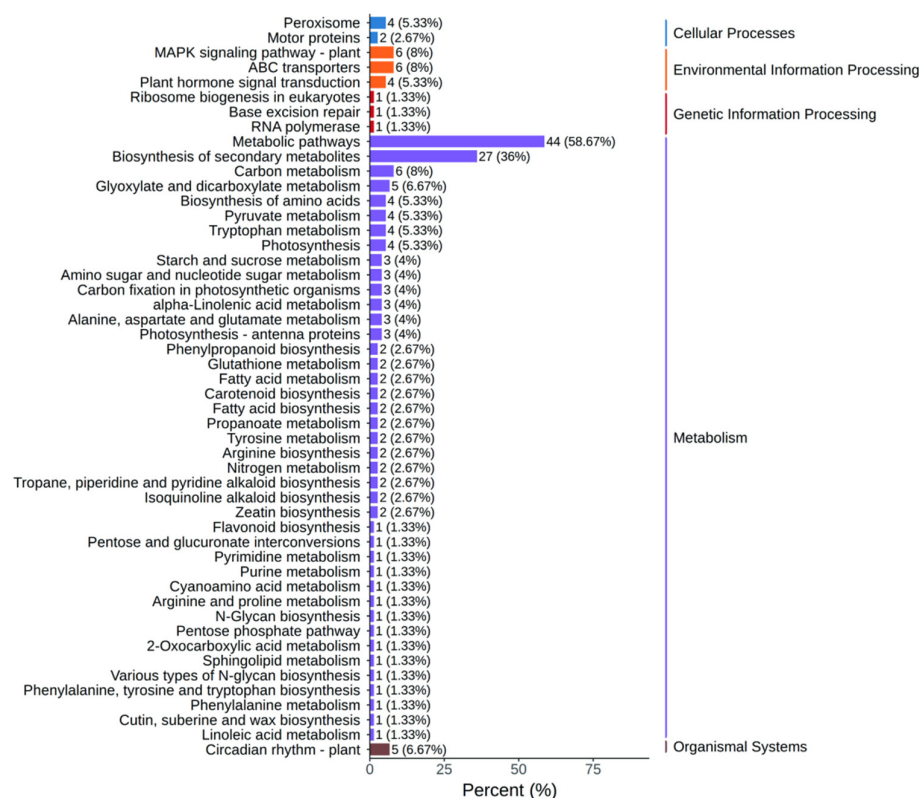


Figure 3. KEGG pathway annotation of differentially expressed genes. Note: The vertical axis lists the KEGG pathways to which the differentially expressed genes were annotated. The horizontal axis indicates the number of the differentially expressed genes (and their percentage) in each pathway. The rightmost column corresponds to the KEGG pathway classification (Level 1), with each major category distinguished by a different color.

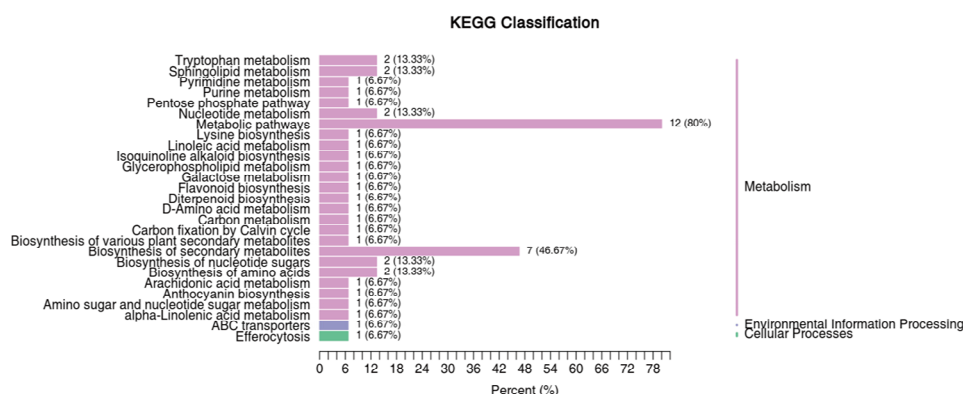


Figure 4. KEGG pathway annotation of differential metabolites. Note: The vertical axis lists the KEGG pathways annotated with differentially accumulated metabolites. The horizontal axis shows the number (and percentage) of differentially accumulated metabolites in each pathway. The rightmost column corresponds to the KEGG pathway classification (Level 1), with each major category distinguished by a different color.

2.1.3. Multi-Omics Analysis of Two Types of Flower Buds in Autumn

To more comprehensively identify the hormones and nutrients involved in secondary flowering, KEGG pathway annotation was performed using an integrated multi-omics approach (Figure 5). A total of six core pathways related to flowering were identified: the glyoxylate and dicarboxylate metabolism pathway, carbon metabolism pathway, amino acid biosynthesis pathway, plant hormone signal transduction pathway, tryptophan metabolism pathway, and alpha-linolenic acid metabolism pathway.

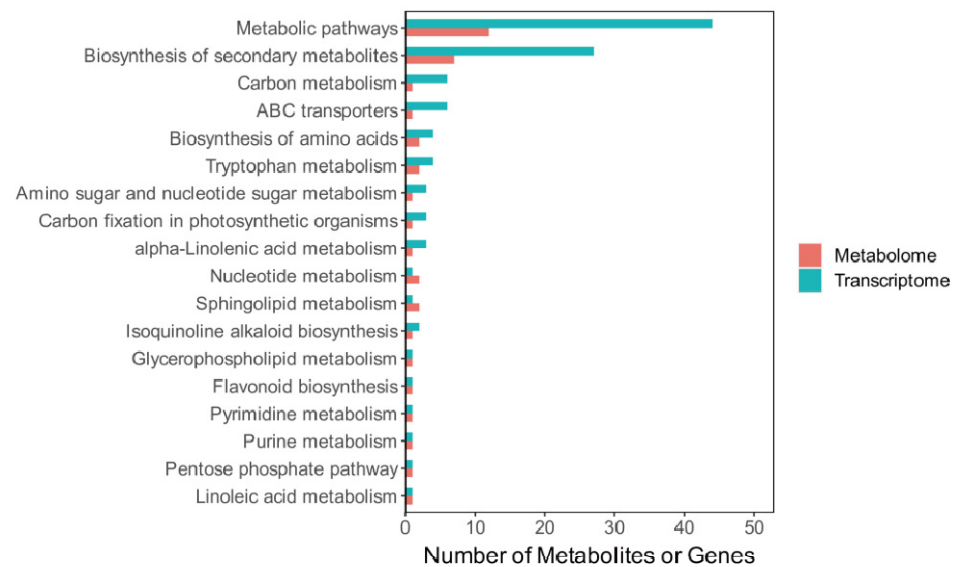


Figure 5. Bar chart of KEGG pathway annotation. Note: The horizontal bars represent individual pathways, with their length corresponding to the number of mapped items (differentially expressed genes or metabolites). The vertical axis lists the pathway names in descending order based on this count.

In the tryptophan metabolism pathway, notable differences were observed in the levels of indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM) between SB and LB samples (Figure 6). IAM levels were significantly higher in LB than in SB. As tryptophan is a key precursor for IAA biosynthesis, variation in its downstream metabolites may contribute to differences in IAA levels. Additionally, in the GA₃ module of the plant hormone signal transduction pathway, genes encoding DELLA proteins showed higher expression in SB than in LB. In SB, the upregulated expression of a DELLA protein-related gene (Cluster-24827.2) may indirectly inhibit the gibberellin signaling pathway. Additionally, within the ABA module of the plant hormone signaling pathway, a gene associated with protein phosphatase 2C (PP2C) (Cluster-29953.3) showed lower expression in SB compared to LB (Figure 2).

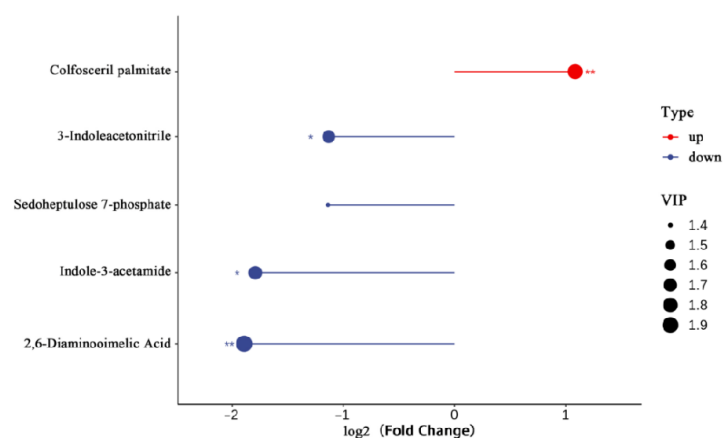


Figure 6. VIP lollipop plot of differentially expressed metabolites in key pathways. Note: Each dot in the figure represents a differential metabolite that meets the screening criteria (VIP > 1 and fold change ≥ 2 or fold change ≤ 0.5). The vertical axis shows metabolite names. The horizontal axis represents log₂ (fold change), which is the logarithmic representation of the fold change in gene expression levels. Dot color indicates the direction of change: red represents upregulation in the SB vs. LB group, while blue represents downregulation. Dot size is proportional to the VIP value of the metabolite. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$).

The differential expression of both DEGs and DAMs was observed in the α -linolenic acid metabolism pathway. Colifoscerl palmitate (PC) can be converted into α -linolenic acid (ALA), which undergoes a series of enzymatic reactions to produce (-)-JA. JA can then be further methylated to jasmonate (MeJA) by jasmonic acid methyltransferase (JMT). There are differences in the PC content between the LB and SB strains, and genes related to allene oxide cyclase (AOC) and JMT exhibit differential expression (Figure 2). The differential expression of the AOC gene (Cluster-9561.1) influences the synthesis level of JA, while the differential expression of the JMT gene (Cluster-14527.0) is involved in regulating the conversion of JA to MeJA.

In addition, the pathways of carbon metabolism, glyoxylate and dicarboxylate metabolism, and amino acid biosynthesis are closely related to nutrient synthesis. Among these, the changes in the carbon metabolism pathway are the most significant. Integration of these pathways revealed that glucose is converted into pyruvate via glycolysis in the carbon metabolism pathway. Pyruvate is then transformed into acetyl-CoA by specific enzymes, which is a necessary substrate for the tricarboxylic acid (TCA) cycle.

The expression levels of genes related to malate synthase and aspartate aminotransferase (Cluster-22969.1, Cluster-3979.0) in the SB samples were lower than those in the LB samples (Figure 2). Malate synthase catalyzes the conversion of glyoxylate to malate, which is a key intermediate metabolite in the tricarboxylic acid cycle and participates in cellular energy production. The downregulation of expression of malate synthase-related genes (Cluster-22969.1) indirectly affected the efficiency of this metabolic flux (Figure 2). Additionally, the aspartate aminotransferase gene (Cluster-3979.0), which is involved in amino acid synthesis, was also expressed at low levels in SB. This enzyme catalyzes the conversion of oxaloacetate to aspartate while simultaneously converting 2-ketoglutarate to glutamate. Both glutamate and aspartate serve as fundamental building blocks for protein synthesis. 2,6-diaminooimelic acid (DAP) in the amino acid biosynthesis pathway can also affect the synthesis of lysine and thereby the content of soluble protein.

2.1.4. Validation of Key Differentially Expressed Genes by RT-qPCR

To evaluate the accuracy and reliability of gene expression levels obtained from transcriptome sequencing, this study re-extracted RNA from the two types of floral bud samples and employed RT-qPCR technology to validate the expression of 12 DEGs selected from key metabolic pathways. The results showed that the expression trends of these genes across the samples were consistent with the transcriptome sequencing data, with only slight variations in expression levels observed for a few genes (Figure 7). Further Pearson correlation coefficient analysis revealed that the correlation coefficient (r) between the two datasets ranged from 0.744 to 0.768, confirming the positive correlation and thereby supporting the reliability of the transcriptome data.

2.2. Changes in the Content of Hormones Before Flowering in Spring and Autumn

2.2.1. Changes in the IAA Content

The concentration of IAA in flower buds initially increased and then decreased over time (Figure 8A). IAA levels in both LB and FB samples peaked during the S4 stage, with values of $0.67 \text{ ng} \cdot \text{g}^{-1}$ and $0.73 \text{ ng} \cdot \text{g}^{-1}$, respectively. In contrast, SB samples peaked earlier (during the S3 stage), after which the IAA content declined. Moreover, the peak IAA content was significantly lower in SB than in LB and FB, suggesting that IAA levels in SB were not sufficient for supporting further development. Significant differences in the IAA content were observed between the two types of autumn flower buds (LB and SB) at the same developmental stages. From S1 to S3, IAA levels were consistently higher in

SB than in LB. However, during S4, the IAA content in SB declined, and LB continued to accumulate IAA; IAA levels were significantly higher in LB than in SB at this stage.

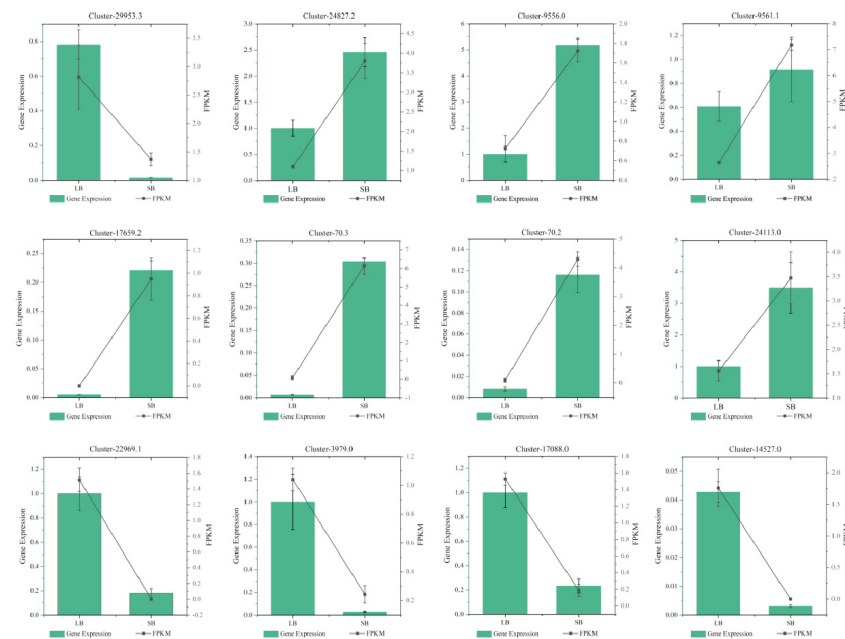


Figure 7. Changes in the relative expression levels of differentially expressed genes. Note: Validation by RNA-seq and RT-qPCR. The left vertical axis represents the relative gene expression level measured by RT-qPCR (bar chart); the right vertical axis represents the FPKM values from RNA-seq (line chart). The data were expressed as mean $\pm$ standard deviation ($n = 3$).

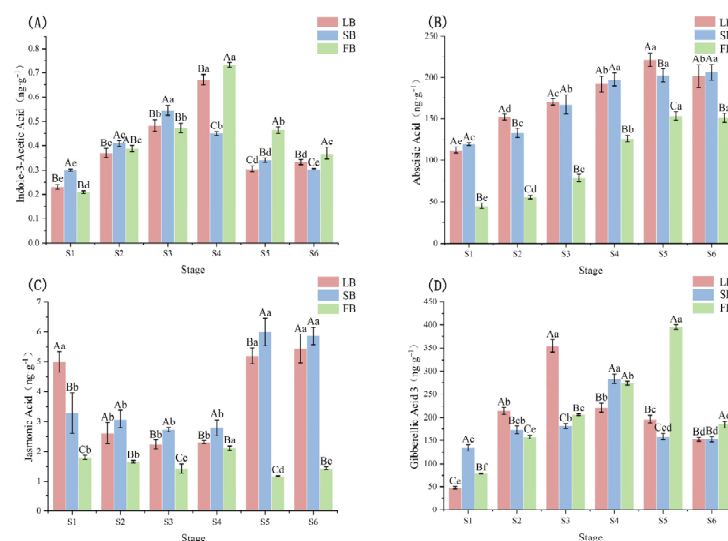


Figure 8. Changes in the IAA content (A), ABA content (B), JA content (C), and GA₃ content (D). Note: The abscissa represents the six sampling periods corresponding to different stages of flower development, while the ordinate represents the content. LB refers to flower buds formed at the tips of long branches in autumn; SB refers to flower buds formed at the base of short branches in autumn; FB refers to flower buds that bloom in spring. This study used six consecutive flower developmental stages of normal flowering individuals as the sampling time reference; namely, S1 (flower bud pre-swelling stage), S2 (flower bud swelling stage), S3 (bud scale cracking stage), S4 (calyx emergence stage), S5 (flower bud stage), and S6 (initial blooming stage). The data were expressed as mean $\pm$ standard deviation ($n = 3$). Different capital letters above the numerical values indicate significant differences within groups, and different lowercase letters indicate significant differences between groups ($p < 0.05$).

2.2.2. Changes in the ABA Content

The overall content of ABA initially increased and then decreased (Figure 8B). In LB, ABA levels rose steadily after the S1 stage, peaking at $221.65 \text{ ng}\cdot\text{g}^{-1}$ at S5, and this peak was significantly higher than that observed in SB. SB displayed a similar trend as LB but peaked later; ABA levels plateaued at S6. Although significant differences in the ABA content were observed among the flower buds during the same developmental periods, the pattern of variation among flower bud types was inconsistent. Specifically, ABA levels were significantly higher in LB than in SB during S2, S3, and S5, but they were significantly lower in LB than in SB during S1, S4, and S6. These findings suggest that ABA levels are not strongly associated with autumn flowering and that fluctuations in ABA likely stem from the combined influence of multiple physiological and environmental factors.

2.2.3. Changes in the JA Content

Measurement of the JA content revealed that LB had significantly higher JA levels than SB during S1. However, the JA content in LB declined sharply thereafter; from S2 to S5, the JA content was significantly lower in LB than in SB (Figure 8C). The dynamics of JA content during floral development differed between spring and autumn. In both autumn types (LB and SB), JA levels exhibited a pattern of initial decrease followed by an increase. The rate of decline was significantly faster in LB than in SB. The lowest JA levels in both LB and SB were observed in S3 ($2.23 \text{ ng}\cdot\text{g}^{-1}$ and $2.73 \text{ ng}\cdot\text{g}^{-1}$, respectively). Subsequently, JA levels rose rapidly from S4 to S6. The JA content peaked earlier in SB (during S5) than in LB. By S6, there was no significant difference in the JA content between LB and SB. In contrast, JA levels in FB remained largely stable throughout its development. Furthermore, the JA concentration in FB was consistently lower than that observed during corresponding stages of autumn floral development.

2.2.4. Changes in the GA₃ Content

The GA₃ content initially increased and then decreased (Figure 8D). The lowest GA₃ levels were observed in the S1 stage among all types. At this point, the GA₃ content was significantly lower in LB than in SB. However, during the development phase (S1–S3), GA₃ levels in LB rose sharply, peaking at $354.60 \text{ ng}\cdot\text{g}^{-1}$, which was significantly higher than the corresponding value in SB. In contrast, GA₃ levels in SB increased more gradually and peaked at S4 ($283.52 \text{ ng}\cdot\text{g}^{-1}$), and they remained significantly lower in SB than in LB. Following the development phase, the GA₃ content in LB declined markedly, and it was significantly lower in LB than in SB during S4. From S4 onward, GA₃ levels in both types of flower buds gradually decreased, and this was consistent with the transition from active development to a more quiescent stage. By S6, GA₃ concentrations had returned to baseline levels in all types.

2.3. Changes in the Content of Nutrients Before Flowering in Spring and Autumn

2.3.1. Changes in the Soluble Protein Content

Changes in the soluble protein content are illustrated in Figure 9A. Overall, FB and SB exhibited relatively stable soluble protein levels throughout the developmental stages, whereas LB showed a pronounced trend of initial decline followed by a marked increase. During the flower bud expansion stage (S1–S2), the soluble protein content in LB decreased rapidly; it then increased during the later stages (S5–S6). In contrast, soluble protein levels in SB and FB did not significantly fluctuate. This pattern suggests that, compared with the other two types, LB underwent a clear phase of protein consumption, followed by compensatory accumulation at a later stage. Comparison of LB and SB in autumn revealed significant differences in the soluble protein content across the sampling period.

LB consistently had higher soluble protein levels than SB, which is consistent with the latter's extremely low development rate during autumn.

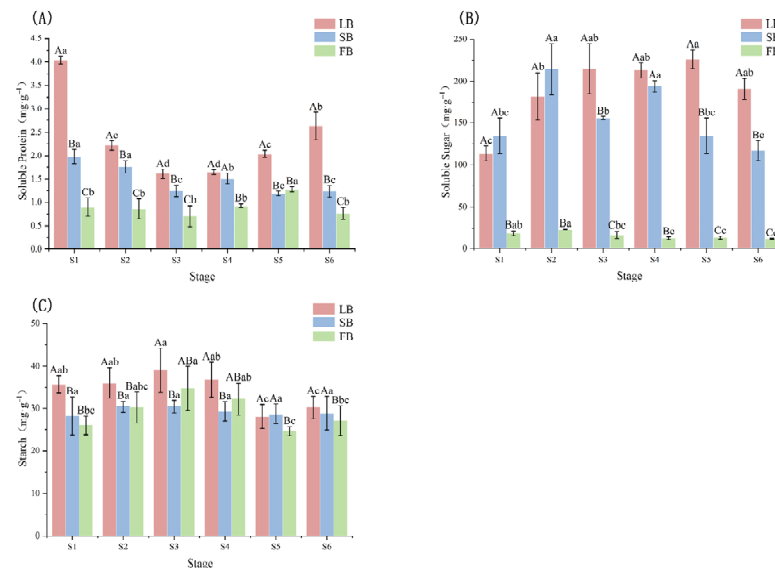


Figure 9. Changes in the soluble protein content (A), changes in the soluble sugar content (B), and changes in the starch content (C). Note: The abscissa represents the six sampling periods corresponding to different stages of flower development, while the ordinate represents the content. LB refers to flower buds formed at the tips of long branches in autumn; SB refers to flower buds formed at the base of short branches in autumn; FB refers to flower buds that bloom in spring. This study used six consecutive flower developmental stages of normal flowering individuals as the sampling time reference; namely, S1 (flower bud pre-swelling stage), S2 (flower bud swelling stage), S3 (bud scale cracking stage), S4 (calyx emergence stage), S5 (flower bud stage), and S6 (initial blooming stage). The data were expressed as mean $\pm$ standard deviation ($n = 3$). Different capital letters above the numerical value indicate significant differences within groups, and different lowercase letters indicate significant differences between groups ($p < 0.05$).

2.3.2. Changes in the Soluble Sugar Content

A comparison of seasonal trends in development (Figure 9B) revealed that soluble sugar content initially rose before declining in both LB and SB, while remaining relatively constant in FB. The soluble sugar content was significantly lower in FB than that in LB and SB. In autumn, flower buds require soluble sugars not only to support secondary flowering but also to withstand low-temperature stress. In contrast, spring buds experience a warmer environment, which reduces the demand for soluble sugar and results in a lower overall soluble sugar content.

In the later stages (S4–S6), SB experienced a rapid decline in the soluble sugar content, whereas the change in the soluble sugar content in LB was more gradual. From S3 to S6, soluble sugar levels were consistently and significantly higher in LB than in SB. High levels of soluble sugar provide an adequate energy supply in flower buds, which promotes floral development. No significant difference in the soluble sugar content between LB and SB was observed before S2. However, following floral development (S3–S6), the soluble sugar content was higher in LB than in SB; this suggests that the energy reserves of LB were greater than those of SB, which contributed to its greater secondary flowering capacity.

2.3.3. Changes in the Starch Content

As shown in Figure 9C, the starch content in LB and FB increased prior to development (S1–S3) and declined thereafter; the starch content in SB remained stable throughout all stages. The starch content was significantly higher in LB than in SB during the S1–S4 stages.

By S5, the starch in LB was hydrolyzed into soluble sugars, which resulted in a marked decline in starch levels. Between S5 and S6, no significant difference in the starch content was observed between LB and SB. However, due to the low demand for starch to support vegetative growth in SB, its starch content remained relatively constant over time.

2.4. Correlation Analyses

Distinct patterns were observed in the relationships among the content of hormones in the three types of flower buds (Figure 10). GA₃ was positively correlated with ABA but negatively correlated with JA. Soluble sugar and soluble protein were negatively correlated in LB and FB, with correlation coefficients of −0.94 and −0.24, respectively, but they were positively correlated in SB (correlation coefficient: 0.36).

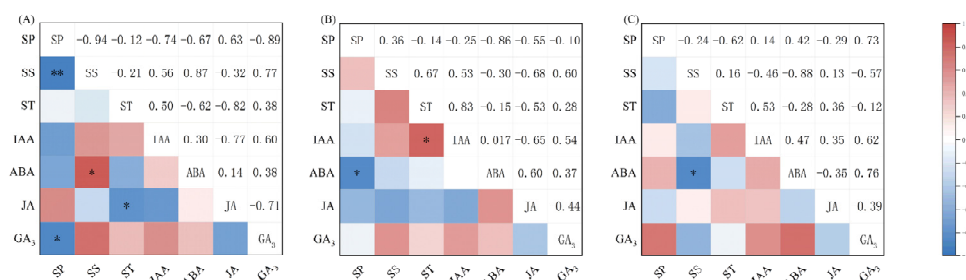


Figure 10. Correlation heatmap for LB (A), correlation heatmap for SB (B), and correlation heatmap for FB (C). Note: SP stands for soluble protein; SS stands for soluble sugar; ST stands for starch; IAA stands for indole-3-acetic acid; ABA stands for abscisic acid; JA stands for jasmonic acid; GA₃ stands for gibberellic acid 3. The color intensity indicates the strength of correlation: shades of red denote positive correlations (+1 indicates a perfect positive correlation), while shades of blue denote negative correlations (−1 indicates a perfect negative correlation). Darker colors represent stronger correlations. All correlation coefficients have been tested for significance (* $p < 0.05$, ** $p < 0.01$).

3. Discussion

3.1. Analysis of Multi-Omics Data to Screen Key Metabolic Pathways

Based on the phenotypic changes observed in our previous studies and the current research, both LB and SB buds can ultimately complete the processes of differentiation and enlargement. Therefore, this study conducted a multi-omics analysis at the key time point (S3) when the differences in floral bud morphological differentiation between the two types first appeared. These early differences most likely reflect the upstream signals that induce the divergent fate of flower buds. The multi-omics analysis results indicate that the DEGs or pathways are primarily involved in regulatory networks related to nutrients, and plant hormone signal transduction, rather than directly involving downstream execution pathways such as “petal development” or “pollen tube growth.” This finding suggests that the factors on which this study centers are potential inductive elements influencing the differences between LB and SB flower buds.

To focus on the direct influencing factors of secondary flowering, we excluded pathways primarily associated with environmental stress resistance and conducted an integrated analysis of hormone and nutrient-related pathways from transcriptomic and metabolomic data. The results revealed six key metabolic pathways, primarily involving two aspects: nutrient supply and metabolism, and hormone synthesis and signaling. This preliminary finding indicates that the allocation of hormones and nutrients may play a regulatory role in the secondary flowering process of *Prunus subhirtella* ‘Autumnalis’.

The hormone signal transduction pathway is for understanding plant growth, development, environmental adaptation, and trait improvement. Transcriptomic studies of *Prunus mume* have revealed marked activation of the hormone signal transduction pathway

during flowering. DEGs include those associated with DELLA proteins involved in GA₃ signaling pathway, PP2C proteins in the ABA signaling pathway, as well as genes participating in the JA and IAA signaling pathways [33], which agrees with the findings of this experiment. DELLA proteins are key negative regulators in the GA₃ signaling pathway. In this experiment, the higher expression level of the DELLA protein-encoding gene in SB than LB may have partially inhibited GA₃ signaling activity and contributed to delayed flowering. A similar mechanism has been reported in flowering Chinese cabbage, where DELLA proteins affect bolting and flowering by suppressing GA₃ signal transduction [34]. As a key negative regulator in the ABA signaling pathway, PP2C inhibits ABA signal transduction upon activation, thereby promoting release from dormancy and the transition to growth [35]. In *Chimonanthus*, PP2C gene expression exhibits stage-specific patterns, being higher during the flowering stage and lower during the bud stage [36]. PP2C expression was significantly higher in LB than in SB, suggesting the release of dormancy in LB, preparing it for the transition to the flowering stage. The biosynthesis of IAA is dependent on tryptophan [37], with IAM serving as a key intermediate in this pathway [38]. We detected a significant upregulation of IAM content in LB, which contrasts with findings in plants, such as *Arabidopsis*. In *Arabidopsis*, IAM accumulation inhibits plant development, whereas this experiment suggests that IAM may facilitate secondary flowering of *Prunus subhirtella* ‘Autumnalis’ [39]. We speculate that this discrepancy may stem from species-specific differences or variations in developmental stages. Differences in metabolites and genes within the α -linolenic acid metabolic pathway may have contributed to the variation in JA content between LB and SB. Notably, JMT, an enzyme that catalyzes the conversion between JA and MeJA [40], was expressed at lower levels in SB, which may impede this conversion process, leading to the accumulation of JA, which was not observed in LB. Therefore, the lower JA levels in LB may be associated with flowering, a conclusion consistent with findings in *Jatropha*, where lower JA levels correlate with more flowering [41].

Hormones mediate plant growth, development, and environmental responses [42–45]. Nutrients support the transition from vegetative growth to reproductive growth, thereby promoting flowering [44,46,47]. Studies on the transition from dormancy to flowering in *Prunus mume* Sieb. et Zucc. have revealed the marked prominence of nutrient-related pathways, such as the glyoxylate and dicarboxylate metabolic pathways, carbon metabolism, and amino acid biosynthesis [48], which agrees with the findings of the metabolomics analysis in this study. Moreover, similar findings have been experimentally validated in studies of flower development across multiple species, such as grapevines [49] and *Prunus pseudocerasus* L. [2]. In *Prunus subhirtella* ‘Autumnalis’, the differential expression of enzyme-related genes in autumn floral buds may influence nutrient production.

KEGG analysis revealed that the expression of MAS-related genes was lower in SB, which potentially reduced TCA cycle activity and carbon flow. The lower expression of AST- and DAP-related genes may lead to a reduction in glutamate, lysine, and aspartate, thereby affecting protein synthesis. The attenuation of these metabolic pathways may collectively contribute to the relative insufficiency of nutrient reserves in non-secondary flowering plants.

3.2. Effect of Changes in the Content of Hormones and Nutrients on Secondary Flowering

In this study, multi-omics analysis was used to identify key hormones (IAA, ABA, JA, and GA₃) and nutrients (soluble protein, soluble sugar, and starch) potentially associated with the secondary flowering of *Prunus subhirtella* ‘Autumnalis’. The changes in the levels of IAA, JA, and GA₃ are closely associated with flowering, while the effects of ABA are relatively limited. Additionally, the higher nutrient levels in autumn floral buds provide the material and energy foundation necessary for flowering.

IAA typically plays a dual role in floral bud differentiation and flowering initiation. In this study, IAA levels increased initially, followed by a decline during flowering, with the peak requiring a certain threshold to promote flowering. This observation suggests that the accumulation of IAA may provide the necessary growth conditions for secondary flowering of *Prunus subhirtella* 'Autumnalis'. These findings are supported by earlier studies on the 'Yametsu-Hime' rose [50].

JA and GA₃ exhibit opposite trends in their roles. As a stress-related hormone, JA demonstrates a negative regulatory relationship with flowering. Specifically, elevated JA levels are associated with inhibited flowering, which agrees with recent research trends [25]. JA levels declined rapidly during flowering in LB, a pattern consistent with observations in *Michelia crassipes* [51]. GA₃, a classic flowering-promoting hormone, was confirmed in this study to exhibit a positive regulatory relationship with flowering. GA₃ levels increased sharply in LB, likely promoting floral bud burst in *Prunus subhirtella* 'Autumnalis'. Similar flowering-promoting effects of GA₃ have been reported in plants, such as 'Red-haven' peach (*Prunus persica*) [52]. Growing evidence indicates an antagonistic interaction between JA and GA₃, which balances plant growth and defense [53–55]. Therefore, the opposite trends observed for JA and GA₃ during secondary flowering of *Prunus subhirtella* 'Autumnalis' align with this regulatory mechanism. Additionally, JA signaling is often upregulated under stress and may delay flowering to prioritize plant defense and survival under adverse conditions [56,57]. GA₃ not only promotes stem elongation and floral bud differentiation but also accelerates flowering by downregulating the expression of DELLA proteins, thereby relieving the inhibition on flowering-related genes [24,58,59]. Indeed, differential expression of DELLA protein-related genes was observed in the transcriptome.

The role of ABA in flowering is complex. In this study, ABA levels fluctuated, but their trends were inconsistent and showed no significant correlation with secondary flowering, suggesting a limited influence of ABA on secondary flowering under the experimental conditions. This finding indicates that ABA may not be a primary regulator for secondary flowering of *Prunus subhirtella* 'Autumnalis' in this context. Previous studies have demonstrated that the effect of ABA on flowering is species-specific: in some plants, ABA may inhibit flowering [60], while under other conditions, it may indirectly influence floral development through interactions with other hormones [12].

In addition to hormonal regulation, nutrient supply also provides a key basis for explaining secondary flowering. In the comparative analysis, the nutrient content in LB was consistently higher than in SB. This sustained and adequate nutritional status accumulated energy reserves for the plant's secondary flowering. Sugars, aside from serving as primary energy sources, also function as signaling molecules in plants [61]. After starch hydrolysis, the resulting soluble sugars support plant development through enzyme-mediated pathways [62]. A similar phenomenon has been observed in *Hylocereus polyrhizus*, where the later stages of flower induction are also accompanied by a significant increase in nutrient levels [63]. In summary, adequate nutrient supply serves as both a material foundation and a regulatory basis for secondary flowering.

3.3. Correlation Analyses of Hormones and Nutrients

Based on hormone assays and correlation heatmaps, the data revealed that ABA and GA₃ levels were consistently positively correlated across all flower bud types. This interaction between ABA and GA₃ may be indirectly influenced by ABA pathway regulators and DELLA proteins. Previous studies have shown that the two collaboratively regulate development in *Arabidopsis* under stressed conditions [64]. Therefore, this widely observed correlation suggests that this mechanism likely plays a coordinating role in floral bud development. The antagonistic interaction between GA₃ and JA reallocates resources from

growth to defense under stress conditions. Specifically, JA promotes the degradation of JAZ proteins, releasing DELLA proteins that bind to PIF, thereby suppressing GA₃ activity, inhibiting cell division and elongation, and ultimately limiting growth [65]. These findings are consistent with experimental observations in *Prunus subhirtella* 'Autumnalis' indicating that JA suppresses secondary flowering while GA₃ promotes it; levels of JA and GA₃ were negatively correlated.

In addition, the soluble protein content and the soluble sugar content were negatively correlated in LB and FB but positively correlated in SB, which likely reflects distinct changes in their accumulation patterns. The model suggested that nutrients are consumed and hormone levels increase during the transition of flower buds into the flowering stage. This pattern is especially evident at stage S6, when nutrients are redistributed within the plant. As the flowering rate increases, the content of nutrients declines, which results in a negative regulatory relationship between flower buds and nutrients, whereas hormones have a positive regulatory effect. A similar nutrient–hormone cascade has been reported to enhance the uptake of nutrients by the root system [66]. However, this study suggests that its functional context may also be applicable to the regulation of floral bud development and flowering.

3.4. Shortcomings of Our Study

Due to the rapid development of flower buds and the limited flowering period of *Prunus subhirtella* 'Autumnalis', sampling after precise stage identification via paraffin sectioning would fail to yield comparable samples from the same timeframe. Preliminary attempts also indicated that large-scale sampling followed by stage identification compromises the integrity of metabolites and RNA in preserved samples due to high metabolic activity. Therefore, to ensure sample freshness and experimental comparability, sampling in this study was conducted based on external morphological characteristics of normally flowering individuals, combined with phenological observations. It should be noted that, although operable, this method does not allow for precise cytological stage delineation, which constitutes a technical limitation of this study.

When screening for differential metabolic pathways related to flowering traits using the multiomics data, we did not fully explore resistance-related pathways, including the melatonin synthesis branch of the tryptophan metabolic pathway. Although some of these pathways were identified as key differential pathways, the current literature predominantly focuses on their classic roles, such as in plant hormones and nutrients. Therefore, our analysis intentionally concentrated on hormone and nutrient pathways directly associated with developmental regulation to construct a clear model of flowering control.

However, we acknowledge that stress-related pathways may also participate in the regulation of flowering. Tryptophan serves as a key metabolic node, with its downstream products exhibiting functional diversity. It not only acts as a precursor for auxin synthesis but also for melatonin synthesis. Melatonin is not only an important stress-response hormone [67,68] but is also involved in the regulation of flowering time [63]. Therefore, overlooking this branch may mean missing a regulatory factor that explains how tryptophan metabolism influences flowering traits. Thus, future research should delve deeper into this direction to address this gap. Exploring whether stress pathways fine-tune the precise execution of developmental programs, such as flowering, will help compensate for this limitation.

4. Materials and Methods

4.1. Experimental Materials

We collected flower buds from *Prunus subhirtella* ‘Autumnalis’ at three distinct stages and from distinct positional categories: spring flower buds in full bloom (FB), autumn non-flowering buds located at the posterior ends of short branches (SB), and autumn flowering buds located at the anterior ends of long branches (LB). The experimental site was located at Dongjia Xiaozhuang Nursery in Qingdao City, Shandong Province, China (36°32′ N, 120°39′ E), which experiences a temperate monsoon climate. Six healthy and similarly sized *Prunus subhirtella* ‘Autumnalis’ were selected as the sample source. Using the mixed sampling method, flower buds at a uniform developmental stage and in good health were collected randomly from the plants.

4.2. Multi-Omics Analysis

Preliminary studies have identified the third sampling period as a crucial turning point in determining whether flower buds can bloom in autumn. We selected flower buds from the third sampling period and conducted transcriptomic and metabolomic analyses on them. Healthy branches with similar growth conditions were selected from six *Prunus subhirtella* ‘Autumnalis’ plants as the sample source. From these branches, nine replicates each of LB and SB flower bud samples were randomly collected. Each tube contained at least 0.6 g of material; three biological replicates were performed in the transcriptomic analysis, and six biological replicates were performed in the metabolomic analysis. Immediately after collection, the samples were rapidly frozen in liquid nitrogen and transported to the laboratory, where they were stored at −80 °C. The samples were then sent to Metware Metabolic Biotechnology Co., Ltd. (Wuhan, China) for transcriptomic and metabolomic analysis.

4.2.1. Transcriptomics Analysis

The RNA was extracted from samples using the CTAB-PBI0Z0L method, dissolved in DEPC water, and then subjected to quality control (QC) by Qubit and Qsep400. For library construction, mRNA was enriched using oligo(dT), fragmented, and processed through double-stranded cDNA synthesis, end repair, adapter ligation, and size selection (250–350 bp), followed by PCR amplification and another round of QC. Qualified libraries were sequenced on the Illumina platform with 150 bp paired-end reads. Raw data were processed by fastp to remove adapter sequences, reads with a high N content (>10%), and low-quality reads ($Q \leq 20$ bases >50%), yielding clean data. Transcripts were assembled using Trinity, and fragments per kilobase of transcript per million mapped fragments (FPKM) values were calculated by RSEM. Differential expression analysis was performed using DESeq according to adjusted p -values and $|\log_2\text{FoldChange}|$. DEGs were screened based on the criteria of $|\log_2\text{FoldChange}| \geq 1$ and an False Discovery Rate (FDR) < 0.05.

The functional annotation pipeline for non-reference genes is as follows: First, DIAMOND BLASTX is used to align Unigene sequences against databases such as KEGG. Subsequently, based on the predicted amino acid sequences, HMMER is employed to align against the Pfam database to obtain corresponding gene annotation information. Detailed annotation information is provided in Supplementary Table S1 (Table S1).

4.2.2. Non-Targeted Metabolomics Analysis

The biological samples were freeze-dried (Scientz-100F, Ningbo, China) and ground (MM 400, 30 Hz, 1.5 min), and 50 mg was mixed with 1200 µL of cold 70% methanol (with internal standard). After vortexing (30 s each time, with 30 min intervals, repeated 6 times) and centrifugation (12,000 rpm, 3 min), the supernatant was filtered (0.22 µm) for

UPLC-MS/MS analysis. HPLC conditions were as follows: Waters ALBUITY UPLC HSS T3 column (1.8 μ m, 2.1mm $\times$ 100 mm; Waters, Milford, MA, USA); column temperature 40 $^{\circ}$ C; flow rate 0.40 mL/min and 4 μ L injection volume. The mobile phase was (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The MS conditions on the Sciex TripleTOF (Sciex, Framingham, MA, USA) were set as follows: the ion source was electrospray ionization (ESI) in negative mode. The gas settings included GAS1 (nebulizer) at 50 psi, GAS2 (heater) at 60 psi, CUR (curtain gas) at 35 psi. The temperature was maintained at 550 $^{\circ}$ C. The declustering potential (DP) was set at 80 V in positive mode or -80 V in negative mode; the ion spray voltage floating (ISVF) was 5500 V in positive mode or -4500 V in negative mode. The TOF MS scan parameters were set as follows: mass range, 50–1250 Da; accumulation time, 200 ms. The product ion scan parameters were set as follows: mass range, 50–1250 Da; accumulation time, 40 ms; collision energy, 30 V or -30 V in positive mode, and 30 V or -30 V in negative mode, respectively. The VIP value quantifies the contribution of each metabolite to intergroup discrimination in the model. Metabolites with a VIP score > 1 and a fold change ≥ 2 or ≤ 0.5 were considered significantly differential.

4.2.3. RT-qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a reverse transcription kit (Vazyme R233-01, Nanjing, China). qPCR was performed with SYBR Green master mix (Vazyme, Q713-02) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences are listed in Table 1. Gene expression levels were normalized to β -actin and calculated using the $2^{-\Delta\Delta C_t}$ method.

Table 1. qRT-PCR primers for key differentially expressed genes in the SB and LB comparison groups.

ID	Upstream Primer (5'-3')	Downstream Primer (5'-3')
Cluster-29953.3	GCACCAGAGATGAAGATGGC	GCACCAGAGATGAAGATGGC
Cluster-24827.2	TCTCACTTCACGTCCAACCA	GAGGATCCGAACCCGGTTAT
Cluster-9556.0	GGAAGTCCAAGGATGGTGGA	AGGTGACCCATGCAATCGTA
Cluster-9561.1	ATCGAACACGTCCCAGAGAA	AAGATTCCAGCACCACCAGT
Cluster-17659.2	GAAGCGATTCCACAAGCAGT	GCCAGATTGCACCAAGTCAT
Cluster-70.3	CCTCCTGAAGCAGATCGAGT	GTCCAGTACCTGCCATCGTA
Cluster-70.2	TATCGTACCTGCCACCACTC	ACATGGTCCAATACCTGCCA
Cluster-24113.0	TACAGGCACATGGAGGGTTT	CCAACAACCTTGGCCTCTTC
Cluster-22969.1	GTATTTGGCAGCGTGGCTTA	ACTCCAAGCCCATCTCCATC
Cluster-3979.0	AGTCGCAAGACCCTAATCCC	CTGGGCTGGGATCCTTGTTA
Cluster-17088.0	TAGCTGCCTATGGAGAAGGC	AGTAACCCTTGCCAGCTCTT
Cluster-14527.0	TGTGTAGCAGACTTGGGTTG	GGAGACCTGGTCGTTGAAGA
actin7	ACCTCATGCCATCCTTCGTCT	CCTGCCCATCTGGTAACTCG

Note: The ID corresponds to the Unigene number of the target gene. Upstream primer and Downstream primer are the forward and reverse primers used to amplify the gene fragment, respectively, with all sequences oriented from the 5' end to the 3' end (5'-3').

4.3. Determination of Hormone and Nutrient Content

4.3.1. Sampling Time

Starting from September 1, we conducted regular sampling of the developmental process of autumn flower buds (SB and LB) at a frequency of every five days. The sampling began during the non-swelling stage, which all flower buds undergo, and continued until the flowers were in full bloom. A total of six key time points were established, sequentially named S1 to S6. This study used six consecutive flower developmental stages of normal flowering individuals as the sampling time reference, namely S1 (flower bud pre-swelling

stage), S2 (flower bud swelling stage), S3 (bud scale cracking stage), S4 (calyx emergence stage), S5 (flower bud stage), and S6 (initial blooming stage). In autumn, when normally flowering individuals reached each developmental stage, corresponding short branch basal bud samples were synchronously collected. Sampling of the spring floral developmental process (FB) began on March 1, following the same frequency and also comprising six time points (S1–S6). All collected samples were immediately flash-frozen in liquid nitrogen and stored at -80°C .

4.3.2. Sampling Method

Hormone Extraction Procedure from Floral Buds: Samples collected at stages S1 to S6 were processed under low-light conditions and at $<4^{\circ}\text{C}$. Briefly, 0.5 g of floral bud tissue was ground to a fine powder in liquid nitrogen. Then, 2 mL of 80% methanol was added, and the mixture was incubated at 4°C for 4 h. After centrifugation at 1000 g for 15 min, the supernatant was collected. The pellet was re-extracted with 1 mL of methanol for 1 h, followed by another centrifugation step. The resulting supernatant was combined with the first extract for subsequent analysis.

4.3.3. Hormone Content Determination

Weighed 5 mg standard and prepared stock solution with methanol, diluted before use. Analysis used ACQUITY UPLC CSH C18 column (2.1 mm $\times$ 150 mm, 1.7 μm ; Waters, Milford, MA, USA) with mobile phases: (A) 2 mM ammonium formate with 0.05% formic acid in water, (B) methanol with 0.05% formic acid. Gradient: 0–22 min, B increased from 10% to 95% and returned; flow rate 0.25 mL/min, column temperature 40°C , injection volume 5 μL . MS detection: positive ESI mode, 4500 V, 400°C ; curtain, nebulizer, auxiliary gas pressures 40, 40, 25 psi [69–71].

4.3.4. Soluble Sugar, Soluble Protein, and Starch Content Determination

Samples for nutrient analyses were collected using the same procedure as that used for hormone analyses. The soluble sugar content, soluble protein content, and starch content were measured using the phenol-sulfuric acid method, the Coomassie Brilliant Blue method, and Fehling's solution colorimetric assay, respectively. Absorbance values were recorded, and the concentrations of the corresponding indicators were calculated using a dual-beam spectrophotometer (UH5300, Hitachi, Tokyo, Japan).

4.4. Data Analysis

The experimental data were first organized using Excel, then statistically analyzed with SPSS 26 and Origin 2021. Results are expressed as mean $\pm$ standard deviation (or standard error) ($n = 3$). Inter-group comparisons were performed using one-way analysis of variance (ANOVA), and the correlation was conducted with Pearson's correlation coefficient analysis. A p -value < 0.05 was considered significant. Figures were generated using Origin and GraphPad, and annotated with Photoshop [72].

5. Conclusions

Based on the multiomics data, we preliminarily elucidated the roles and potential regulatory factors of hormone signaling and nutrient metabolism during secondary flowering in *Prunus subhirtella* 'Autumnalis'. The results indicate that both plant hormone-related metabolic pathways and nutrient metabolic pathways are involved in this process. Specifically, accumulation of IAA may induce secondary flowering of *Prunus subhirtella* 'Autumnalis' in autumn, with GA_3 showing a positive correlation and JA exhibiting a negative correlation with the progression of secondary flowering. Concurrently, floral bud development is accompanied by dynamic changes in nutrient levels, with their accumula-

tion levels positively correlated with flowering. These findings provide new insight into the relationship between hormones and nutrients in the secondary flowering of woody plants.

We have preliminarily identified early candidate factors associated with flowering induction. At the same time, we recognize that integrating a temporal dynamic analysis of spring-flowering buds with buds from various parts of the plant will make the research more systematic and comprehensive, and subsequent studies will delve deeper into this aspect. Furthermore, to explore whether the apical mechanism is involved in the secondary flowering process, we plan to investigate the apical mechanism of *Prunus subhirtella* ‘Autumnalis’ to assess its relevance and refine the regulatory network of secondary flowering.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants15050812/s1>, Figure S1. Schematic diagram of the developmental period (S1–S6); Table S1. Annotation results of differential genes.

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