

Article

Integrated Transcriptomic and Metabolomic Analysis Reveals Multi-Hormone Regulation of Seed Germination in *Paris polyphylla* var. *yunnanensis*

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Abstract

Seed germination of *Paris polyphylla* var. *yunnanensis* is intrinsically low, severely restricting its artificial propagation. Despite the known roles of ABA and GAs, the regulatory functions of other phytohormones in this process remain largely unexplored. In this study, we integrated transcriptomic and targeted metabolomic profiling to systematically investigate the dynamics of 34 phytohormones and global gene expression patterns across five germination stages of *P. polyphylla* seeds. Seven hormones showed significant changes during germination, including auxins, melatonin, cytokinins, and jasmonate, which exhibited distinct accumulation patterns. Integrative correlation analysis further uncovered candidate genes potentially involved in hormone biosynthesis and signaling. Collectively, our findings provide new insights into the hormonal regulation of seed germination in *P. polyphylla*, establish a foundational framework for understanding its germination mechanisms, and offer candidate molecular targets for improving seed propagation efficiency.

Keywords: *Paris polyphylla* var. *yunnanensis*; seed germination; transcriptomics; metabolomics; phytohormones

1. Introduction

Paris polyphylla var. *yunnanensis* (commonly known as Dian Chonglou) is a perennial herb in the family Melanthiaceae and is recognized as a valuable traditional Chinese medicinal plant. Its dried rhizome is traditionally used to reduce fever, eliminate toxins, relieve pain, calm the liver, and stop convulsions [1]. Modern pharmacological studies have revealed that the main bioactive constituents are steroidal saponins, which exhibit potent cytotoxic, anti-tumor [2], and anti-inflammatory activities [3]. These saponins are key active ingredients in well-known Chinese patent medicines such as “Yunnan Baiyao” and “Gongxuening” [4,5].

Due to the rapid development of the traditional Chinese medicine industry and increasing market demand, wild populations of *P. polyphylla* have been subjected to destructive overharvesting, leading to its classification as vulnerable (A4cd) on the IUCN Red List [6]. Cultivation represents an effective strategy to alleviate resource shortages. However, seeds of this species exhibit typical morphophysiological dormancy [7]. Under natural conditions, they require a prolonged after-ripening period lasting “two winters and one summer” to complete germination, resulting in low and uneven emergence, which severely restricts artificial propagation and large-scale cultivation [8].



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Seed germination is a critical turning point in the plant life cycle, precisely regulated by various endogenous hormones and environmental signals [9,10]. ABA and GA are the core hormones controlling seed dormancy and germination: ABA induces and maintains dormancy while inhibiting germination, whereas GA breaks dormancy and promotes germination. The balance between their contents and the antagonistic interplay between their signaling pathways determine the transition from dormancy to germination [11,12]. Several studies have measured hormonal changes during seed germination of *P. polyphylla*, consistently demonstrating that endogenous ABA and GA play important roles in dormancy and germination [13,14]. In addition, auxin, brassinosteroids, cytokinins, ethylene, and jasmonic acid have been identified as key endogenous regulators of seed germination [15–17]. In *P. polyphylla*, the contents of IAA, tZ, SA, and JA also play important roles in seed dormancy release, germination initiation, and seedling emergence [13]. Moreover, Fu et al. [18] found that during the seed germination of *P. polyphylla*, the IAA content peaked at S5.

With the advent of high-throughput sequencing technologies, several transcriptomic studies have analyzed gene expression profiles during seed dormancy and germination in *P. polyphylla*. Tang et al. [19] used RNA-seq to uncover potential regulatory factors, showing that differentially expressed genes were significantly enriched in carbohydrate metabolism, lipid metabolism, signal transduction, and translation. Liao et al. [20] performed transcriptome sequencing on seeds during warm stratification at the dormant and germinating stages and identified a series of genes involved in hormone metabolism and signaling. Additionally, transcriptome sequencing has led to the discovery of several key genes participating in seed stratification [21].

Despite these valuable insights, most transcriptomic studies have focused on identifying candidate genes and describing their expression patterns, without linking gene expression changes to downstream metabolite accumulation. Metabolomics enables comprehensive detection of dynamic changes in small-molecule metabolites, including plant hormones, thereby directly reflecting end-point physiological differences. Integrating metabolomics with transcriptomics provides a powerful multi-omics strategy to unravel the regulatory mechanisms of hormone metabolism and to screen for key hormones and core regulatory genes [22]. For example, Kang et al. [23] demonstrated significant overlap between DEGs and differentially accumulated metabolites in pathways such as phenylpropanoid biosynthesis in *Macadamia*. Xu et al. [24] combined transcriptomics and metabolomics to investigate saponin content and DEGs in three *Paris* species, identifying 17 candidate genes associated with saponin accumulation. Recently, Zheng et al. [25] employed a multi-omics approach to explore dormancy release mechanisms in *P. polyphylla* seeds. However, their study primarily focused on ABA and GA metabolites, with limited exploration of other hormones such as auxin, melatonin, cytokinins, and jasmonates that have been implicated in seed germination regulation.

Thus, current knowledge of hormonal changes during seed germination in *P. polyphylla* relies predominantly on single-omics data, with most studies concentrating on ABA and GA; the core regulatory genes governing the actions of these diverse hormones in *P. polyphylla* remain to be elucidated. We therefore performed integrated transcriptomic and targeted metabolomic analyses on seeds at five germination stages. We characterized the dynamic changes in gene expression and endogenous hormone contents to identify key hormone metabolic pathways and core regulatory genes governing seed germination in *P. polyphylla*. Our findings provide a theoretical basis for understanding the germination mechanisms of this important medicinal plant and offer molecular targets for shortening the germination cycle and improving the efficiency of large-scale cultivation.

2. Materials and Methods

2.1. Seed Germination Treatment

Seeds were harvested in November 2023 from Shizong County, Yunnan Province, China. After removing the red aril, Seed viability was assessed using the TTC (2,3,5-triphenyltetrazolium chloride) test. Five replicates of 10 seeds each were randomly selected from the seed lot. Seeds with completely stained red embryos were scored as viable (Figure S1). Approximately 1000 uniformly plump seeds were selected, soaked in 100 mg/L carbendazim for 2 h, rinsed five times with distilled water, and air-dried. The seeds were then mixed with five volumes of sand (dried at 80 °C) in Petri dishes, moistened with distilled water to achieve approximately 10% moisture content, and stratified at 20 °C in an artificial climate chamber for 150 days [20]. Based on morphological changes, five distinct developmental stages were identified (Figure 1). At each stage, 100 seeds were randomly collected and divided into two portions, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent transcriptomic and metabolomic analyses. Three biological replicates were included for each stage.

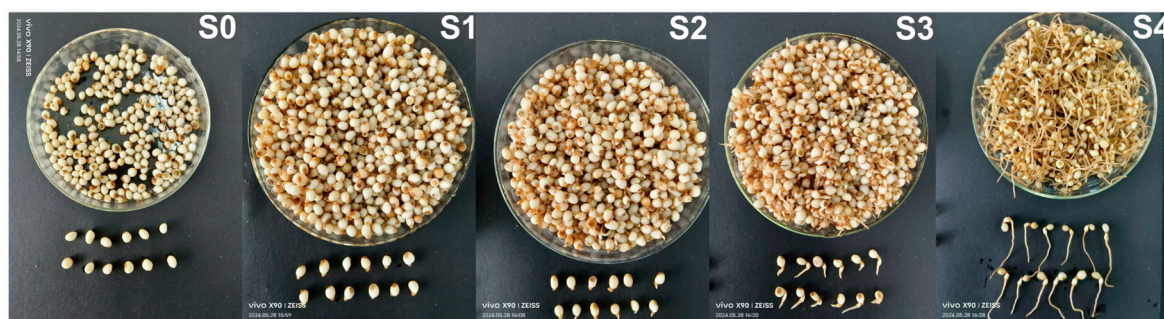


Figure 1. Five germination stages of *P. polyphylla* seeds. S0: pre-germination stage; S1: radicle swelling stage; S2: radicle protrusion through the seed coat; S3: post-protrusion radicle swelling stage; S4: root formation to epicotyl elongation stage.

2.2. Phytohormone Profiling by LC-MS/MS

2.2.1. Preparation of Standards

Stock solutions (1 mg/mL) for each of the 34 plant hormone standards were prepared in methanol. A standard curve stock solution was prepared by mixing appropriate aliquots of each individual stock solution, which was then serially diluted with methanol to generate a series of working solutions. All solutions were stored at −20 °C. For internal standards, 2 mg/mL stock solutions of IAA-D5 and ABA-D6 were prepared in methanol. A mixed internal standard solution containing 0.5 µg/mL IAA-D5 and 1 µg/mL ABA-D6 was subsequently prepared by dilution with methanol. Finally, 15 µL of each calibration working solution and 0.5 µL of the mixed internal standard solution were combined with 134.5 µL of the quality control (QC) sample to prepare the calibration curve solutions.

2.2.2. Hormone Extraction

Approximately 200 mg of frozen seed tissue from each of the five stages (three biological replicates per stage) was accurately weighed into a 2-mL brown centrifuge tube. Then, 1 mL of methanol and the mixed internal standard solution were added. The sample was sonicated for 10 min, followed by shaking extraction in a metal bath for 3 h. After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was collected. The remaining pellet was re-extracted with 0.5 mL of methanol for 1 h. The combined supernatants were filtered through a 0.22-µm membrane filter before LC-MS/MS analysis, following the method of Li et al. [26].

2.2.3. LC-MS/MS Analysis

Chromatographic separation was performed on an ExionLC UPLC system (AB Sciex, Framingham, MA, USA) equipped with an Acquity UPLC[®] CSH C18 column (1.7 μ m, 2.1 $\times$ 150 mm, Waters, Milford, MA, USA). The column temperature was maintained at 40 °C, and the injection volume was 2 μ L. The mobile phase consisted of (A) water containing 0.05% formic acid and 2 mM ammonium formate, and (B) methanol containing 0.05% formic acid. The flow rate was 0.25 mL/min, and the gradient elution program was as follows: 0–2 min, 10% B; 2–4 min, 10–30% B; 4–19 min, 30–95% B; 19–19.1 min, 95–10% B; 19.1–22 min, 10% B.

Mass spectrometry was conducted using an AB Sciex Triple Quadrupole 6500+ mass spectrometer (AB Sciex, Framingham, MA, USA) in multiple reaction monitoring (MRM) mode. Electrospray ionization (ESI) parameters in positive mode were: ion source voltage, 4500 V; source temperature, 400 °C; curtain gas, 40 psi; atomization gas, 40 psi; and auxiliary gas, 25 psi. Parameters were similar in negative mode, except the ion source voltage was −4500 V.

2.3. Metabolomic Data Analysis

Only metabolites detected in more than 50% of the samples were retained for further analysis. Prior to statistical comparisons, data normality and homogeneity of variances were assessed using Shapiro–Wilk and Levene’s tests, respectively. The results showed that the majority of metabolites met the assumptions of normality and homoscedasticity; therefore, one-way ANOVA was applied to identify differentially accumulated metabolites across the five germination stages. Metabolites with a *p*-value < 0.05 and a variable importance in projection (VIP) score > 1 were considered significantly different. Identified metabolites were mapped to KEGG pathways using the KEGG Mapper tool (v5.2) for biological interpretation.

2.4. Transcriptome Analysis

Total RNA was extracted from samples of the five developmental stages, with three biological replicates per stage. Libraries were constructed and sequenced on an Illumina HiSeq 2500 platform, generating 150 bp paired-end reads. Raw reads were filtered using FASTP [27] to remove low-quality reads (average quality < Q20), producing high-quality clean reads. De novo transcriptome assembly was performed using Trinity [28]. Gene expression levels were normalized as fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed genes (DEGs) were identified using the DESeq package in R, comparing S1, S2, S3, and S4 against the S0 control, with thresholds of nominal *p* < 0.05 and $|\log_2 \text{fold change}| \geq 1$. KEGG pathway enrichment analysis was subsequently performed to determine the biological functions and pathways associated with the DEGs.

2.5. KEGG Enrichment and Differential Expression Analysis

To capture phytohormone-related genes, we performed pathway mapping using the KEGG online database (<http://www.genome.jp/kegg/> (accessed on 5 July 2026)), supplemented with Swiss-Prot annotations. The pathways of interest included: tryptophan metabolism (auxin synthesis; map00380), zeatin biosynthesis (cytokinin synthesis; map00908), diterpenoid biosynthesis (gibberellin synthesis; map00904), carotenoid biosynthesis (abscisic acid synthesis; map00906), alpha-linolenic acid metabolism (jasmonic acid synthesis; map00592), and plant hormone signal transduction (map04075). Heatmaps of these DEGs were generated using R (v4.1.1) packages. Plant hormone synthesis pathways were curated based on methods described by Zhu et al. [29].

2.6. Correlation Analysis

Spearman's rank correlation coefficients were calculated between endogenous hormone content and gene expression levels using the *scipy.stats* library in Python (v3.12.4). Both metabolite and gene expression data were log-transformed to reduce bias. A correlation pair was considered statistically significant if it met the thresholds of $|r| \geq 0.7$ and $p \leq 0.05$.

3. Results

3.1. Seed Viability and Germination

The viability of the seed lot was assessed prior to stratification using the TTC test. Among the 50 randomly tested seeds (five replicates of 10 seeds each), the proportion of seeds with completely stained red embryos averaged $86.0\% \pm 5.5\%$, indicating that the seed population was of high quality and suitable for subsequent germination experiments (Figure S1). Approximately 1000 uniformly plump seeds were subjected to warm stratification at 20 °C for 150 days, during which they exhibited progressive morphological changes. Based on these observable changes, five representative developmental stages were identified for multi-omics sampling (Figure 1).

3.2. Differential Metabolite Analysis

To investigate the dynamic changes in endogenous hormones during seed germination of *P. polyphylla*, we performed targeted quantification of 34 hormones from nine classes across five key germination stages (S0–S4) using LC-MS/MS (Table S1). OPLS-DA results showed good inter-group clustering, indicating high data reliability (Figure 2a). A total of 15 hormones showed detectable changes (Table S2). Through differential metabolite screening, we identified 7 plant endogenous hormones that varied across the five stages, including auxins, jasmonates, cytokinins, and melatonin. Among the cytokinins, IPA content increased at S1, decreased at S2, then gradually rose again, peaking at S4 (Figure 2b). TZR content was highest at S0 and then gradually declined (Figure 2c). Among auxins, IAA content ranged from 2.71 to 5.07 ng/g during S0–S3, and increased to 18.24 ng/g at S4, representing an approximately 3.6 to 6.7 fold increase compared to earlier stages (Figure 2d). Its methylated form, Me-IAA, showed a similar accumulation trend. MT content gradually increased from S0 and peaked at S4. Among jasmonates, JA was not detected at S0, but fluctuated between 2.64 and 2.91 ng/g during S1–S4, while its derivative DHJA showed a gradual decline thereafter.

3.3. Transcriptome Assembly and Annotation

To investigate transcriptional changes during seed germination of *P. polyphylla*, a total of 15 cDNA libraries were constructed for RNA sequencing. Transcriptome sequencing generated 631,760,140 raw reads. After quality control, 626,627,046 clean reads were retained for in-depth transcriptome analysis. The average Q20 and Q30 values of the clean reads were 97.71% and 93.54%, respectively, indicating high sequencing quality and highly reliable results (Table S3). Owing to the lack of a reference genome for *P. polyphylla*, the data were subjected to de novo assembly and redundancy removal, ultimately yielding 254,988 non-redundant unigenes with an average length of 790.05 bp and an N50 of 997 bp. The sequences were aligned to the NR, Swiss-Prot, eggNOG, Pfam, GO, and KEGG databases for annotation. A total of 98,860 unigenes (38.77%) were successfully annotated (Table S4), of which 12,478 non-redundant genes were annotated in all seven databases. Principal component analysis (PCA) based on the expression levels of all genes revealed good within-group clustering, with the exception of S0_1 and S3_1. PCA results showed that S0_1 clustered more closely with the S1 samples on both PC1 and PC2 (Figure S2a). Consistently, correlation analysis revealed that S0_1 shared relatively high correlations

with samples from both S0 and S1; however, all correlation coefficients remained below 0.8 (Figure S3), suggesting a possible transitional status of this sample between S0 and S1. For S3_1, it clustered closely with S2 samples, with correlation coefficients greater than 0.9, indicating a greater similarity to the S2 (Figures S2a and S3). Following the exclusion of these two samples, PCA was reperformed and showed improved clustering (Figure S2b), confirming the robustness of the dataset.

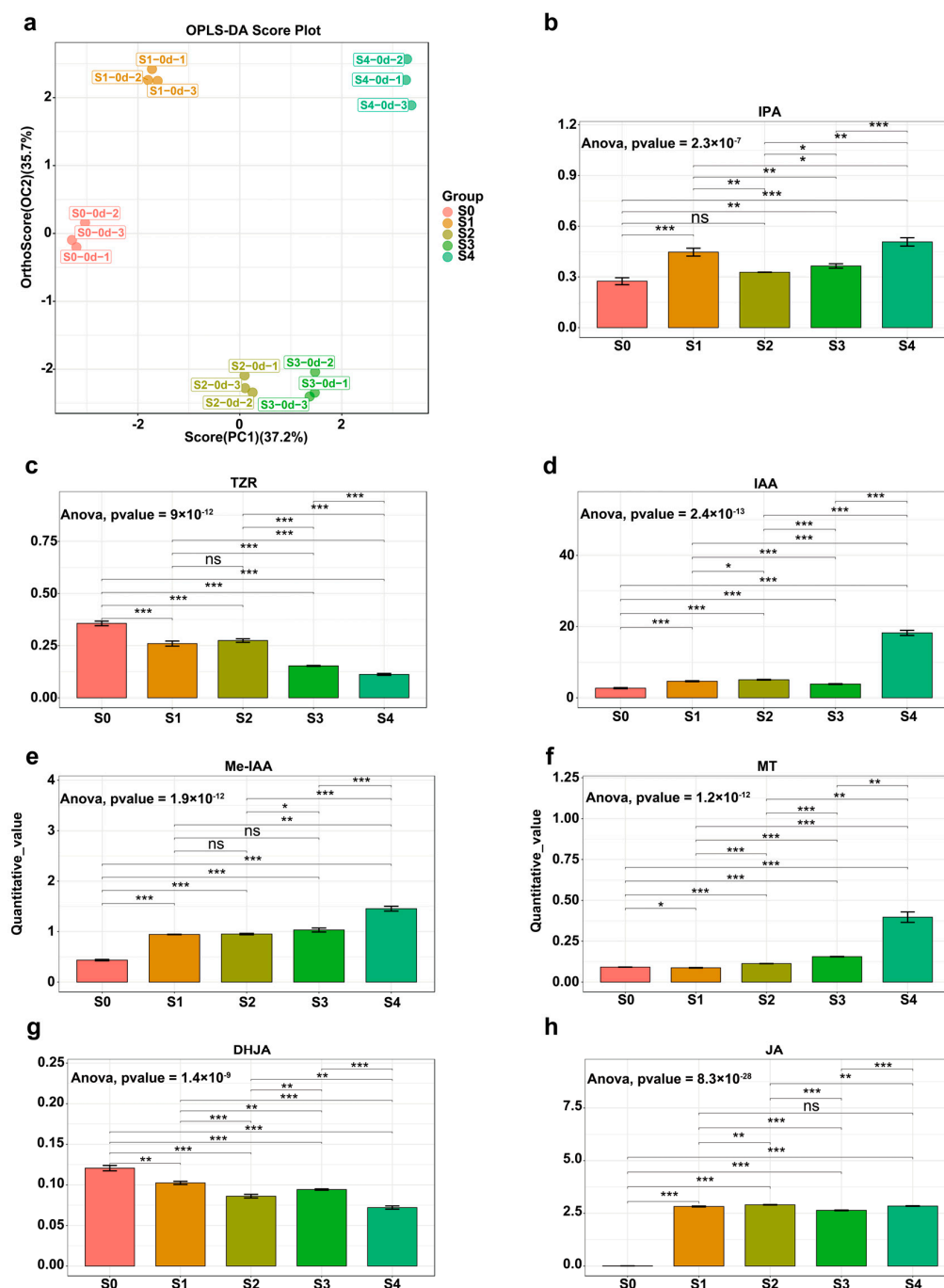


Figure 2. OPLS-DA score plot and content of seven differentially accumulated endogenous hormones. (a) OPLS-DA score plot showing clustering of samples from five developmental stages (S0–S4). (b–h) Content of endogenous hormones across the five stages. (b) IPA (isopentenyl adenosine), (c) TZR (trans-zeatin riboside), (d) IAA (indole-3-acetic acid), (e) Me-IAA (methyl indole-3-acetate), (f) MT (melatonin), (g) DHJA (dihydrojasmonic acid), (h) JA (jasmonic acid). Data are presented as mean $\pm$ SD ($n = 3$). Asterisks indicate significant differences compared with S0: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.4. Differential Expression Gene Analysis

To evaluate the differential changes in gene expression during seed germination of *P. polyphylla*, we identified DEGs across different germination stages (Figure 3). A total of 12,990 DEGs were commonly identified in all four pairwise comparisons, while 52,176 DEGs were detected in at least one comparison (Figure 3a). We then statistically analyzed the DEGs in each comparison. In S0 vs. S1, a total of 17,217 DEGs were detected, of which 3805 were upregulated and 13,412 downregulated. In S0 vs. S2, 19,445 DEGs were identified, comprising 5441 upregulated and 14,004 downregulated genes. In S0 vs. S3, 19,172 DEGs were found, with 5771 upregulated and 13,401 downregulated. In S0 vs. S4, 23,996 DEGs were detected, including 7634 upregulated and 16,362 downregulated genes (Figure 3b). We further quantified the number of transcription factors (TFs) among the DEGs, identifying a total of 722 TFs (Figure 3c). The top five TF families were bHLH, MYB, NAC, C2H2, and bZIP, suggesting that these TF families play important roles during seed germination of *P. polyphylla* (Figure 3c).

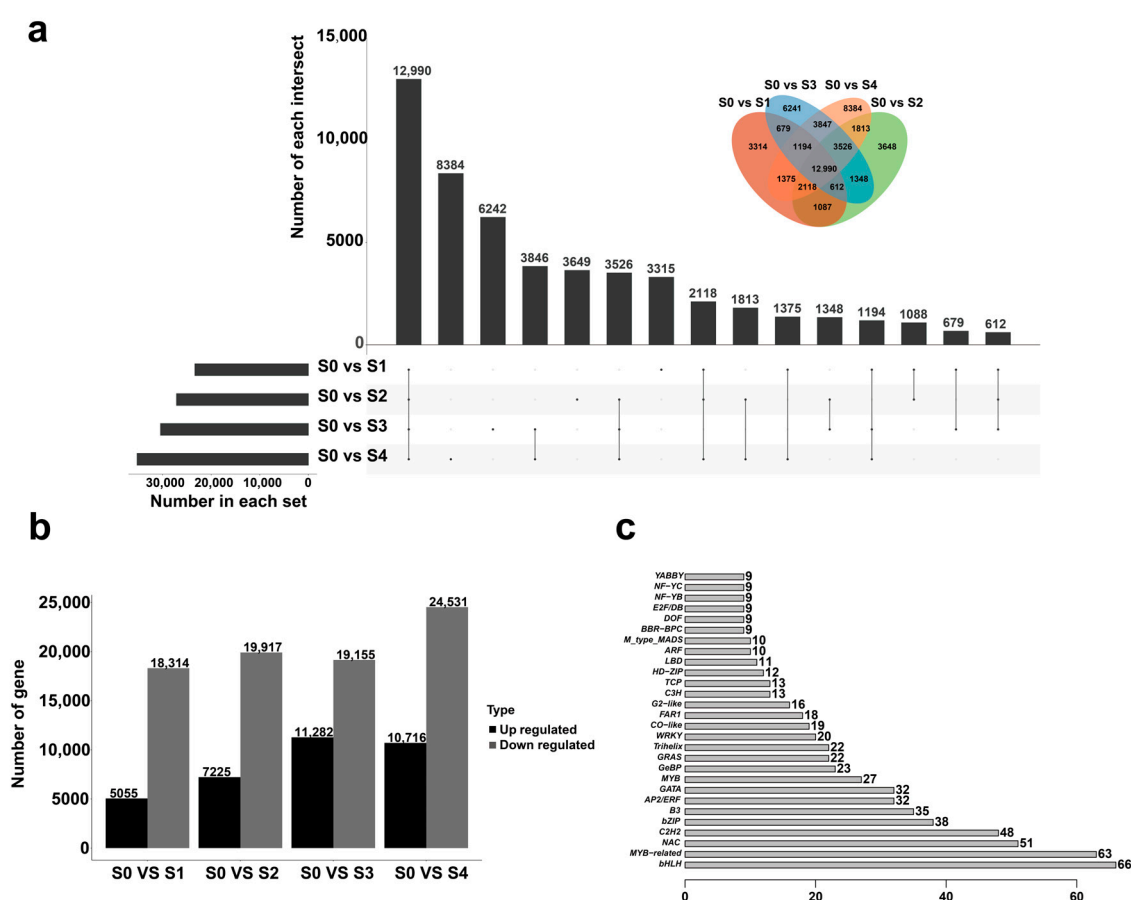


Figure 3. DEGs and TFs in *P. polyphylla* seed germination. (a) Number of DEGs per comparison. (b) Upregulated vs. downregulated DEGs per comparison. (c) Number of TFs identified among DEGs.

KEGG enrichment analysis (Figure 4) revealed that multiple hormone biosynthesis-related pathways were significantly activated at various stages of seed germination, with dynamic changes across comparisons. In the S0 vs. S1 comparison, zeatin biosynthesis, tryptophan metabolism, and phenylpropanoid biosynthesis pathways were significantly enriched. In S0 vs. S2, in addition to the above pathways, steroid biosynthesis was enriched. In S0 vs. S3, carotenoid biosynthesis, plant hormone signal transduction, and α -linolenic acid metabolism were further enriched. By the S0 vs. S4, zeatin biosynthesis, tryptophan metabolism, phenylpropanoid biosynthesis, and the MAPK signaling pathway

were enriched. Notably, pathways such as zeatin biosynthesis and tryptophan metabolism were continuously enriched throughout the entire germination process, suggesting that they play sustained roles in regulating seed germination.

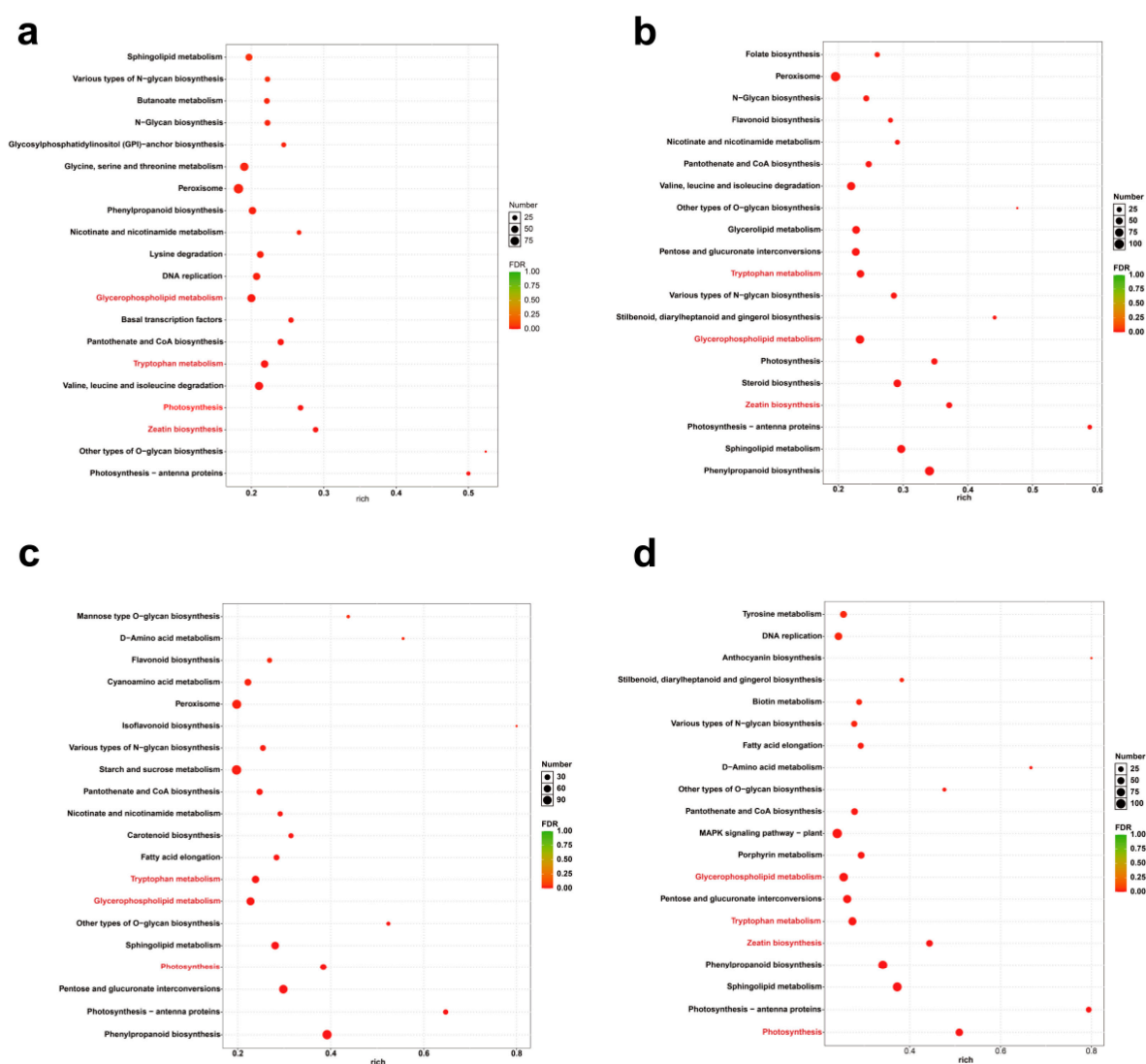


Figure 4. KEGG enrichment analysis of DEGs during seed germination of *P. polyphylla*. (a) S0 vs. S1. (b) S0 vs. S2. (c) S0 vs. S3. (d) S0 vs. S4.

3.5. Cytokinin Biosynthesis and Signal Transduction Pathways

During seed germination of *P. polyphylla*, two nucleoside forms of cytokinins, namely IPA and TZR, were predominantly detected, and they showed a negative correlation with each other (Figure S4). In contrast, the levels of free-base forms IP and TZ were very low. IP was detected only at S3, and TZ only at S0 (Table S1). A total of 52 genes involved in cytokinin biosynthesis pathways were identified (Figure 5, Table S5). Among the IP-type cytokinins, the expression level of the *UGT4* gene was negatively correlated with IPA content (Table S5). For TZ-type cytokinins, seven *UGT* genes showed positive correlations with TZR content (Table S5). The expression levels of *IPT*, *CYP735A*, and *CKX* genes did not show clear correlations with IPA or TZR content. A total of 19 genes involved in cytokinin signal transduction pathways were identified (Figure S5, Table S5). Among them, *b-ARR3* positively correlated with IPA content, whereas *b-ARR6*, *a-ARR7*, and *CRE1-4* negatively correlated with IPA content. *b-ARR5*, *a-ARR7*, *AHP1*, and *CRE1-4* positively correlated with TZR content, while *b-ARR2*, *b-ARR3*, and *b-ARR4* negatively correlated with TZR content.

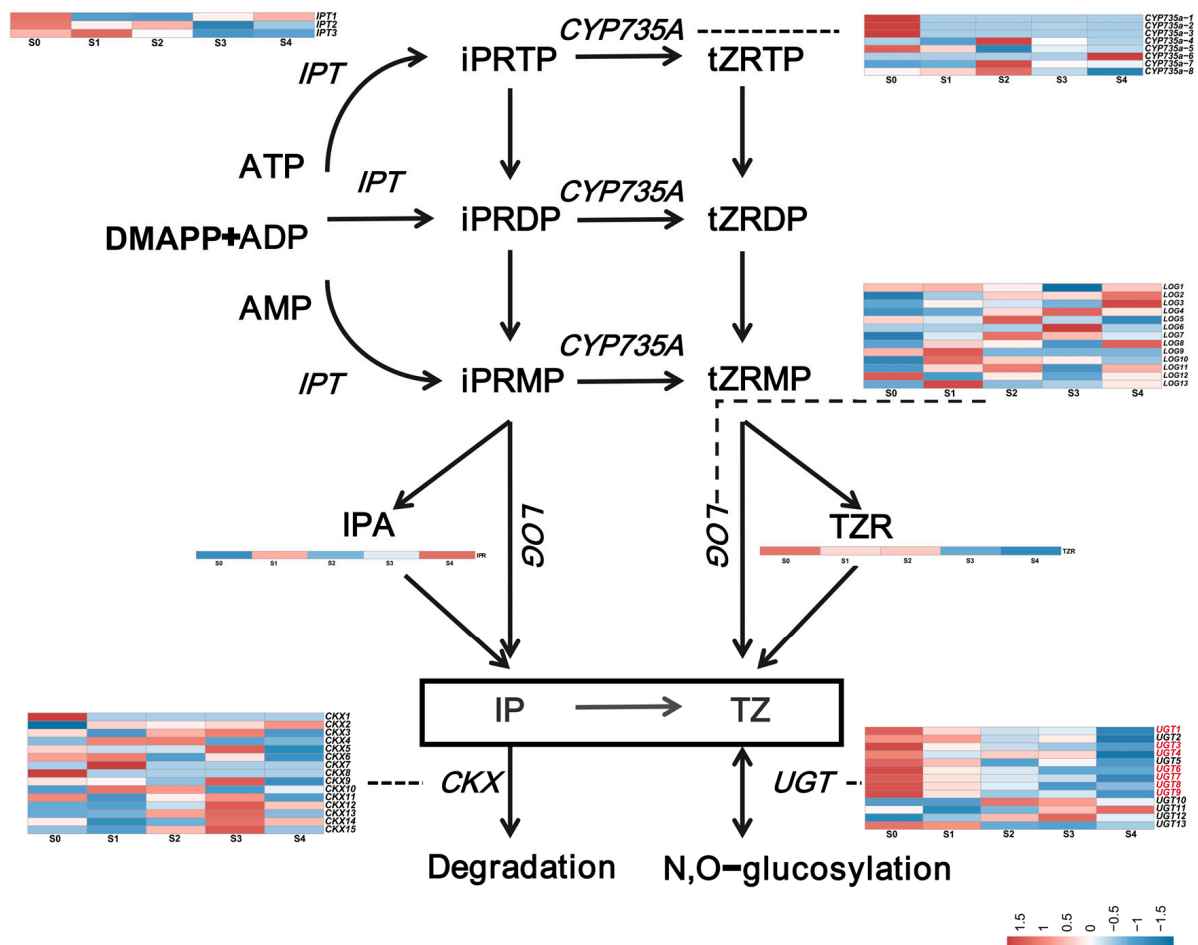


Figure 5. Schematic diagram of the cytokinin biosynthesis pathway. DMAPP, dimethylallyldiphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IPT, isopentenyl transferase; iPRTP, isopentenyl adenosine-5'-triphosphate; iPRDP, isopentenyladenosine-5'-diphosphate; iPRMP, isopentenyl adenosine-5-monophosphate; CYP735A, cytokinin trans-hydroxylase; tZRTP, trans-Zeatin riboside triphosphate; tZRDP, trans-Zeatin riboside diphosphate; tZRMP, trans-Zeatin riboside monophosphate; LOG, cytokinin-activating enzyme LONELY GUY; IPA, isopentenyl adenosine; TZR, trans-zeatin riboside; IP, isopentenyl adenine; TZ, trans-zeatin. Genes whose expression showed significant correlations with corresponding metabolites are highlighted in red.

3.6. IAA and MT Biosynthesis and Signal Transduction Pathways

Both IAA and melatonin biosynthesis require tryptophan as a starting substrate. In the IAA biosynthesis pathway, a total of 40 genes were identified (Figure 6, Table S6). Three TAA genes were identified in the IPA pathway, among which the expression level of TAA3 showed a significant positive correlation with IAA content. 19 YUCCA genes were identified, with YUCCA15 and YUCCA18 positively correlated with IAA content. A total of 18 ALDH genes were identified, five of which were negatively correlated with IAA content. In the IAA signal transduction pathway (Figure S5, Table S6), a total of 54 related genes were identified. Among them, AUX/IAA5 and AUX/IAA14 were negatively correlated with IAA content, whereas AUX/IAA9 and AUX/IAA15 were positively correlated with IAA content. SAUR1, SAUR3, and SAUR5 were positively correlated with IAA, while SAUR15 was negatively correlated with IAA. ARF6 and ARF9 were positively correlated with IAA. In the melatonin biosynthesis pathway (Figure 6), a total of six ASMT/COMT candidate genes were identified, among which the expression level of ASMT3 was positively correlated with MT content (Table S6).

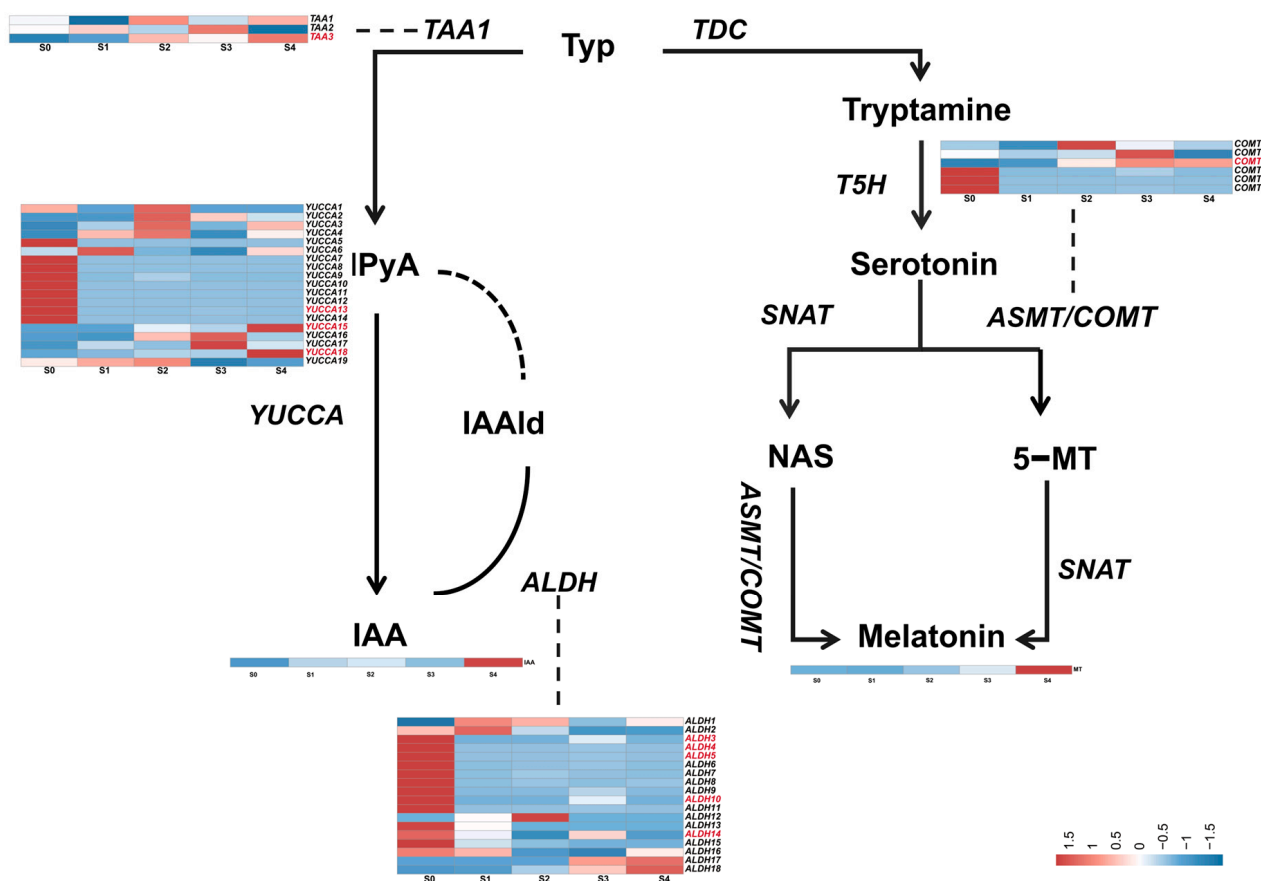


Figure 6. Schematic diagram of the auxin and melatonin biosynthesis pathways. TAA, tryptophan aminotransferase; IPyA, indole-3-pyruvate; YUCCA, indole-3-pyruvate monooxygenase; IAAld, Indole-3-acetaldehyde; ALDH, aldehyde dehydrogenase; IAA, Indole-3-acetate; TDC, Tryptophan decarboxylase; T5H, tryptamine-5-hydroxylase; SNAT, serotonin N-acetyltransferase; NAS, N-acetylserotonin; ASMT/COMT, ASMT, N-acetylserotonin methyltransferase (also known as COMT); 5MT, 5-methoxytryptamine. Genes whose expression showed significant correlations with corresponding metabolites are highlighted in red.

3.7. JA Biosynthesis and Signal Pathways

During JA biosynthesis, JA was not detected at S0 (Table S2). A total of 52 genes involved in the JA biosynthesis pathway were identified (Figure 7, Table S7). Five *PLA* genes were expressed almost exclusively at S0, two of which showed a negative correlation with JA content. Two *LOX* genes, *LOX2* and *LOX3*, were negatively correlated with JA. *OPR10* showed a significant positive correlation with JA content. Additionally, we detected the JA biosynthesis intermediate OPDA. OPDA was present at all stages, and its content was higher than that of JA (Figure S6). OPDA content decreased to its lowest level at S2, then began to increase at S3; In contrast, JA content peaked exactly at S2, and the expression level of *OPR10* also reached its highest level at S2.

3.8. ABA and GA Biosynthesis and Signal Transduction Pathways

ABA did not reach the significance threshold in our differential metabolite screening (Table S2); as a core hormone regulating seed dormancy and germination, we still analyzed the genes involved in its biosynthesis and signal transduction pathways (Figure 8a, Table S8). ABA content decreased during the early germination stages and then began to increase at S3 (Figure S7, Table S2). A total of 18 genes involved in ABA biosynthesis were identified, but none showed a significant correlation with ABA content (Table S8). Notably, some genes exhibited distinct stage-specific expression patterns: *ZEP1*, *ZEP2*, *ZEP3*, *ABA2*-

2, *ABA2-3*, and *NCED2* were highly expressed at stage S0; whereas *ZEP4*, *ABA2-1*, *NCED3*, and most *AAO* genes peaked at S3 (Figure 8c), consistent with the re-accumulation of ABA content at S3, suggesting that these genes may be involved in ABA re-accumulation. A total of 35 genes involved in ABA signal transduction were identified (Figure S5). Most of these signaling genes were actively expressed during S0–S2, with a marked decrease in expression at S3. In contrast, several *PP2C* genes were highly expressed at S3.

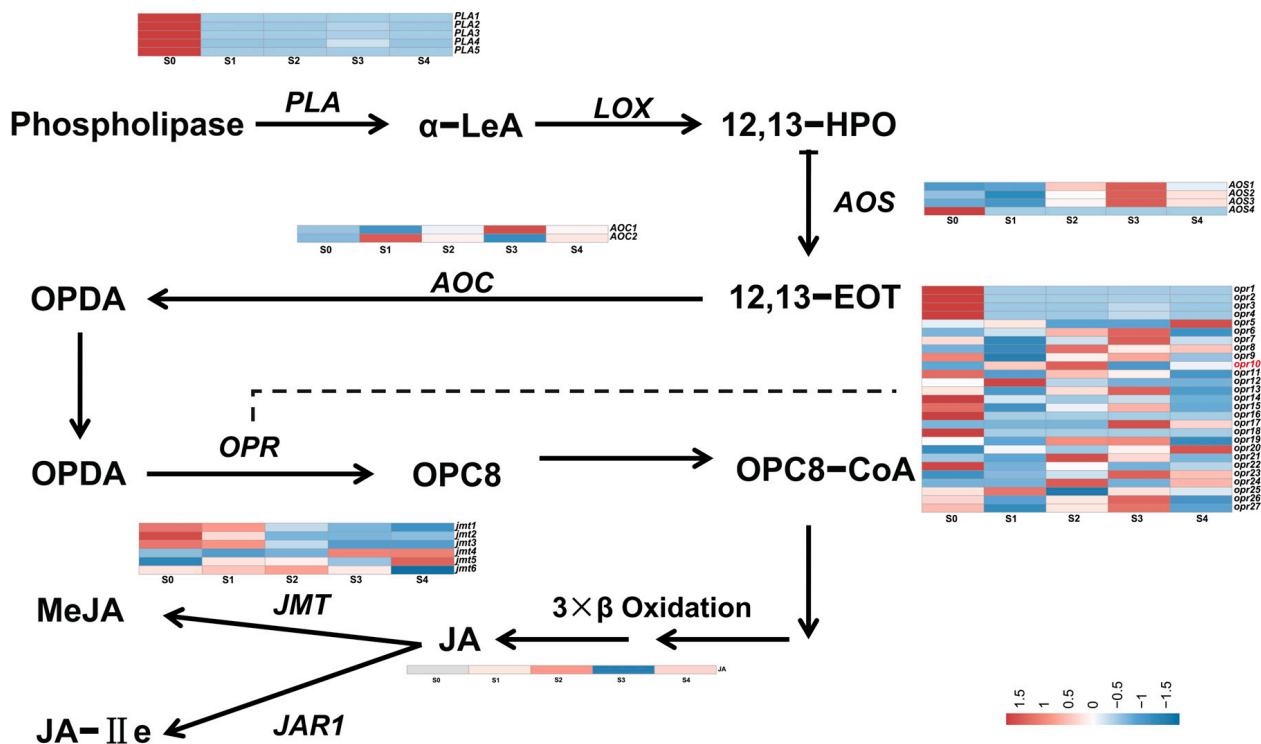


Figure 7. Schematic diagram of the jasmonic acid biosynthesis pathway. PLA, phospholipase A1; α -LeA, alpha-Linolenic acid; LOX, lipoxygenase; 12,13-HPO, 12,13-hydroperoxylinoleic acid; AOS, allene oxide synthase; 12,13-EOT, 12,13-epoxyoctadecatrienoic acid; AOC, allene oxide cyclase; OPDA, oxophytodienoic acid; OPR, oxophytodienoic acid reductase; OPC8, 3-oxo-2-(2'-(Z)-pentenyl)-cyclopentane-1-octanoic acid; OPC8-CoA, 3-oxo-2-(2'-(Z)-pentenyl)-cyclopentane-1-octanoic acid-CoA; JA, jasmonic acid; JMT, jasmonic acid carboxyl methyltransferase; JAR1, jasmonic acid resistant 1; MeJA, Methyl jasmonate; JA-Ile, Jasmonoyl-L-isoleucine; Genes whose expression showed significant correlations with corresponding metabolites are highlighted in red.

GAs are key endogenous hormones that promote seed germination. No GAs were detected in this study (Table S1). Nevertheless, we analyzed the expression levels of GA biosynthesis genes (Figure 8b, Table S9). The results showed that four *CPS* genes began to be expressed from S2 onward; *KO* genes were highly expressed across all five stages, peaking at S2; *KAO3* and *KAO4* were highly expressed throughout all stages, with peaks at S3; *GA2ox1* was highly expressed at S3, whereas *GA2ox4* and others were mainly highly expressed during S0–S2. Overall, the expression levels of GA biosynthesis-related genes exhibited a progressive increase from S0 onward, peaking at S3 (Figure 8c). Additionally, the GA signaling genes *GID1* and *TF2* were also highly expressed across all stages (Figure S5).

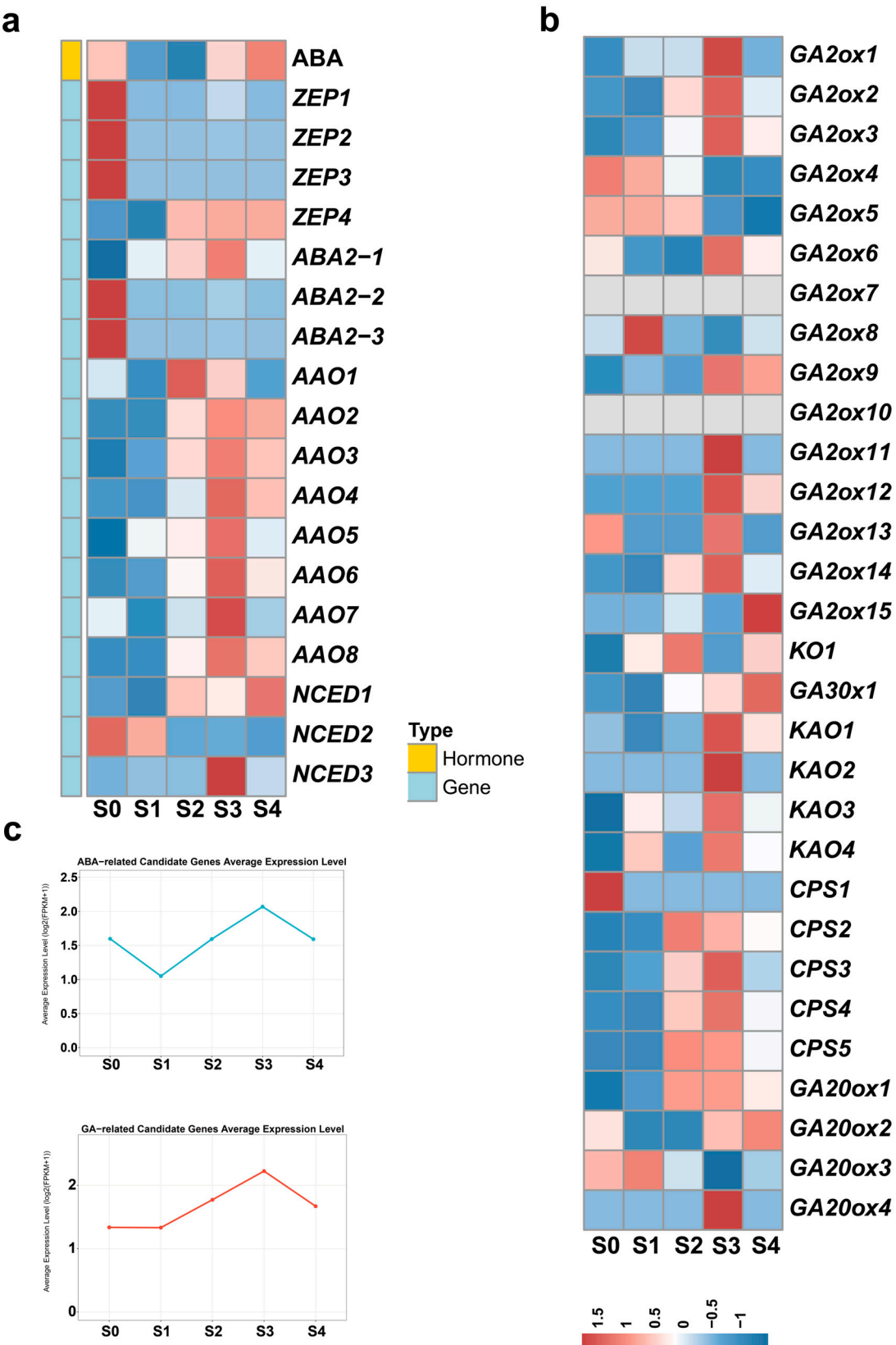


Figure 8. Expression levels of genes involved in the ABA and GA biosynthesis pathways. (a) Heatmap showing expression levels of ABA biosynthesis-related genes across the five stages. (b) Heatmap showing expression levels of GA biosynthesis-related genes across the five stages. (c) Average expression profiles of the relevant genes at the five developmental stages. The upper panel shows ABA-related genes, and the lower panel shows GA-related genes.

4. Discussion

Hormonal regulation is a core biological process controlling seed dormancy release and germination initiation. In this study, we performed integrated transcriptomic and metabolomic analyses to characterize the dynamic changes of 34 hormones and their associated gene expression regulatory networks across five germination stages of *P. polyphylla* seeds, thereby establishing a comprehensive hormonal profile. Our results demonstrate that seed germination in *P. polyphylla* is governed by multiple hormones.

Cytokinins regulate cell division and act as dose-dependent and species-specific modulators of seed dormancy and germination [30]. In this study, the predominant forms of cytokinins in *P. polyphylla* seeds were the nucleosides IPA and TZR, whereas the levels of free-base IP and TZ were extremely low. Consistent with our findings, a previous study reported that IP and TZ levels decreased to undetectable levels during seed germination in *P. polyphylla* [25]. Therefore, we speculate that in *P. polyphylla*, IP and TZ may be rapidly converted, and their functions rely more on the dynamic balance of nucleoside forms and their glycosylation modifications. TZR content continuously declined during germination and showed a positive correlation with the expression of seven *UGT* genes. *UGT*-catalyzed glycosylation is an important pathway for cytokinin inactivation and storage [31]. We hypothesize that the high TZR content at S0 may act as a substrate inducing the high expression of *UGT* genes to accelerate TZR glycosylation; as TZR content decreases, substrate induction weakens, leading to reduced *UGT* expression levels. Such a dynamic interplay may constitute a homeostatic regulatory mechanism that protects germinating seeds from the detrimental effects of cytokinin overaccumulation. Regarding signal transduction, the expression of *a-ARR7* gradually decreased from S0 to S4, while IPA content gradually increased, showing a negative correlation. As a negative regulator of cytokinin signaling, the decreased expression of *a-ARR7* may relieve the inhibition of cytokinin synthesis or accumulation. We speculate that the reduced expression level of *a-ARR7* during the later stages of seed germination in *P. polyphylla* creates conditions for IPA accumulation. This is consistent with the function of *OsRR9*, the rice homolog of *a-ARR7*, in regulating cytokinin content [32].

Auxin is an important signaling molecule in plants and functions throughout plant growth and development [33]. In seeds, it controls embryogenesis, endosperm and seed coat morphogenesis, as well as the transition from dormancy to germination. In our study, IAA content remained low from S0 to S3 but showed a burst of accumulation at stage S4, which is generally consistent with previous findings [13]. The Indole-3-pyruvic acid (IPyA) pathway is the main IAA biosynthesis pathway in higher plants, and the expression levels of three genes in this pathway were closely related to IAA content. *TAA3* is homologous to *OsTAA1*, which has been shown to play an important role in auxin synthesis [34]. *YUCCA15* and *YUCCA18* are homologous to *Arabidopsis YUC6*, the overexpression of which causes a typical auxin overproduction phenotype and increases free IAA levels [35,36]. Given the well-established roles of these homologous genes in auxin synthesis, we suggest that *TAA3*, *YUCCA15*, and *YUCCA18* represent three candidate genes potentially involved in auxin biosynthesis during seed germination of *P. polyphylla*. Auxin regulates plant growth and development by coordinating signal transduction, biosynthesis, and polar transport [37]. The *Aux/IAA* and *SAUR* gene families are auxin early response genes and play roles in regulating root formation, development, and hypocotyl elongation [38]. *Aux/IAA* proteins, encoded by *Aux/IAA* genes, are negative regulators of the auxin signaling pathway. When auxin levels rise, *Aux/IAA* proteins bind to TIR1/AFB receptors and are ubiquitinated, followed by degradation via the 26S proteasome pathway, thereby relieving their inhibition of ARF transcription factors and initiating downstream gene expression. In this study, the expression levels of two *Aux/IAA* genes (*IAA9* and *IAA15*) were positively correlated with

IAA content. *IAA9* is homologous to rice *OsIAA10*, whose protein stability is regulated by auxin signaling; high auxin levels promote its ubiquitination by *OsTIR1*, thereby releasing specific auxin response factors and affecting traits such as grain size [39]. *IAA15* is homologous to rice *OsIAA1* and participates in regulating morphogenesis [40]. The positive correlation between *Aux*/*IAA* expression and IAA content observed in this study may reflect a feedback mechanism in which auxin induces the expression of negative regulators at the transcriptional level to finely tune signaling homeostasis. The auxin transcriptional response is governed by sequence-specific transcription factors of the ARF family, which bind to auxin response elements (AuxREs) [41]. In this study, the expression levels of two *ARF* genes were positively correlated with IAA content. Notably, these two *ARF* genes are homologous to rice *OsARF7*, which positively regulates root development through the auxin signaling pathway [42]. Therefore, we speculate that during the later stages of seed germination in *P. polyphylla*, IAA accumulation may induce the expression of these two *ARF* genes, thereby activating downstream genes involved in root development and promoting radicle formation and elongation. Melatonin is structurally similar to auxin and shares the same precursor, tryptophan; its functions in promoting plant growth and development are also highly similar to those of auxin, as it can induce the growth of aboveground parts and roots [43]. In our study, melatonin content also continuously increased during the germination process. The significant positive correlation between *ASMT3* and MT content suggests that this gene may encode a key rate-limiting enzyme in melatonin biosynthesis. Furthermore, this study reveals the synergistic accumulation of IAA and MT during the late stages of seed germination in *P. polyphylla*, suggesting that both hormones play important roles in this stage.

The critical roles of ABA and GAs in seed germination have been demonstrated by numerous studies. However, we did not detect accumulation of any GAs in our study, which contrasts with previous studies. Zheng et al. [25] detected that GA3 content declined rapidly to undetectable levels during germination, while the contents of GA4, GA15, and GA24 significantly increased during germination. Meanwhile, both Su et al. [13] and Fu et al. [18] detected changes in GA3 content during germination. These discrepancies suggest a lack of consistency across studies regarding the major GA types and their dynamic trends, which may reflect differences in detection platforms, sample processing methods, stratification conditions, or seed materials. Despite the absence of detectable GAs, we observed that numerous GA biosynthesis genes were significantly activated during S1–S4, with the GA inactivation gene *GA2ox* also highly expressed at S3. We therefore speculate that GA biosynthesis may have been initiated at the transcriptional level, even though the corresponding metabolites were not captured.

In plants, endogenous hormone concentrations can span up to five to six orders of magnitude [44], which places high demands on detection sensitivity and dynamic range. Moreover, plant hormones typically accumulate at very low intracellular levels, requiring sample enrichment to reach detectable concentrations. Although the targeted metabolomic platform used in this study was designed to simultaneously cover 34 hormones, this broad-spectrum strategy may have compromised extraction efficiency and ionization optimization for specific hormone classes, thereby leading to the failure to detect GAs.

We acknowledge several limitations in this study. First, the candidate genes implicated in hormone metabolism and signaling were identified primarily through correlation-based analyses, and their proposed functions rely on homology to characterized genes from other species; therefore, direct experimental validation remains to be conducted. Second, the undetectability of certain hormones that are closely associated with seed germination, including GAs, constrains our ability to fully interpret the regulatory landscape. Future work will focus on functional validation of the candidate genes identified herein, along

with refinement of extraction and detection methods to improve sensitivity and coverage, thereby enabling a more comprehensive and accurate assessment of hormonal regulation during seed germination in *P. polyphylla*.

5. Conclusions

In this study, by integrating transcriptomic and targeted metabolomic data, we dissected the dynamic changes and regulatory mechanisms of endogenous hormones during seed germination of *P. polyphylla*. Our results demonstrate that seed germination in *P. polyphylla* is associated with the dynamic regulation of multiple hormones. Notably, IAA and MT exhibited coordinated accumulation during the later stages of germination, whereas TZR and IPA displayed inverse accumulation patterns throughout the germination process. We further identified a set of candidate genes involved in hormone biosynthesis and signal transduction, including *TAA3*, *YUCCA15*, and *YUCCA18* for auxin; *ASMT3* for melatonin; *OPR10* for jasmonic acid; and multiple *UGT* genes for cytokinins. These findings elucidate the molecular regulatory mechanisms underlying changes in endogenous hormone levels during seed germination of *P. polyphylla*, and provide a theoretical basis and potential targets for optimizing artificial propagation techniques. In future studies, we aim to explore the synergistic regulatory mechanisms of multiple phytohormones during seed germination in *P. polyphylla*, and to functionally characterize the core candidate genes, thereby clarifying their precise roles within the hormonal regulatory network.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070888/s1>, Figure S1: TTC staining of *P. polyphylla* seeds. Seeds with completely stained red embryos were scored as viable, while seeds with unstained or partially stained embryos were scored as non-viable; Figure S2: PCA plot of gene expression levels. (a) PCA plot of 15 samples. (b) PCA plot of 13 samples; Figure S3: Correlation matrix between samples; Figure S4: Correlation analysis of 15 endogenous hormones in *P. polyphylla*. The heatmap shows Spearman correlation coefficients among the detected hormones; Figure S5: Signal transduction pathways of endogenous hormones; Figure S6: Contents of jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA); Figure S7: Contents of abscisic acid (ABA); Table S1: Quantitative analysis results of 34 endogenous hormones; Table S2: Analysis results of differential metabolites; Table S3: Summary of RNA-Seq data; Table S4: Overview of *P. polyphylla* transcriptome assembly and functional annotation; Table S5: Association analysis between cytokinin content and gene expression levels; Table S6: Association analysis between auxin content and gene expression levels; Table S7: Association analysis between jasmonic acid content and gene expression levels; Table S8: Association analysis between abscisic acid content and gene expression levels; Table S9: Expression levels of GA biosynthesis-related genes.

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