

Multionics strategies for decoding seed dormancy breakdown in *Paris polyphylla*

Guowei Zheng

Yunnan University of Chinese Medicine

Wenchun Li

Yunnan University of Chinese Medicine

Shunzhen Zhang

Yunnan University of Chinese Medicine

Qi Mi

Yunnan University of Chinese Medicine

Wenxiu Luo

Yunnan University of Chinese Medicine

Yanli Zhao

Yunnan University of Chinese Medicine

Xiangshi Qin

Chinese Academy of Sciences

Wei jiao Li

Yunnan University of Chinese Medicine

Shibiao Pu

Yunnan University of Chinese Medicine

Furong Xu (✉ xfrong99@163.com)

Yunnan University of Chinese Medicine

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Abstract

The disruption of seed dormancy is a complicated process and is controlled by various factors. Among these factors, membrane lipids and plant hormones are two of the most important ones. In this study, we integrated multiomics data to systematically investigate the global metabolic and transcriptomic profiles of *Paris polyphylla*, an important Chinese herbaceous species, during seed dormancy breaking. Untargeted metabolomics revealed that lysophospholipids (lysoPLs) were positively correlated with *P. polyphylla* seed dormancy breaking, and the increase in lysoPLs may be mediated by phospholipase A2 (PLA2). Absciscic acid (ABA) decreased dramatically during seed dormancy breaking of *P. polyphylla*, and gibberellins (GAs) increased. The catabolism of ABA may occur through conjugation rather than catalytic hydroxylation. The changes in the contents of different GA molecules varied during *P. polyphylla* seed germination: 13-OH GAs such as GA₁ and GA₃ were not detected and decreased, respectively, and 13-H GAs such as GA₄ and its precursors increased. The change in contents of 13-OH GAs may be attributed to the absence of GA 13-oxidase, and the upregulation of GA20ox may cause an increase in bioactive GA₄. *P. polyphylla* may selectively synthesize strong bioactive GA₄ to participate in the breaking of seed dormancy.

Background

Seed dormancy is an important adaptive trait that helps plants respond to unflavoured environments. However, the seeds of most medicinal plants undergo seed dormancy, which slows plant growth. *Paris polyphylla* var. *yunnanensis* is an important traditional Chinese medicinal herbaceous species in the family Melanthiaceae [1]. The demand for and industrial production of *P. polyphylla* is increasing due to the important medicinal qualities of this species. However, wild resources have been endangered in China because of overexploitation. Therefore, artificial cultivation in the field is an effective way to meet this demand. Seeds of *P. polyphylla* are morphophysiological dormant (MPD) and cannot germinate in a short period. The breaking of seed dormancy in *P. polyphylla* was the key factor that influenced its domestication and cultivation.

Gene expression profiles during *P. polyphylla* seed dormancy breaking are well studied. During this process, differentially expressed genes (DEGs) are enriched in the pathways of carbohydrate metabolism, hormone metabolism, lipid metabolism, and hormone signalling [2-4], which indicates that carbohydrate, lipid and hormone metabolism are important for the germination of *P. polyphylla* seeds. Among them, genes related to GA synthesis (*GA20ox2*, *GA20ox3*) and ABA catabolism (*CYP707A*) were upregulated, whereas dormancy-related genes (*NCED*, *PP2C*) were downregulated. However, evidence of metabolite changes is still lacking. Therefore, the relationships between gene expression and metabolism during *P. polyphylla* seed germination still need further investigation.

Hormones are the most important metabolites that regulate seed dormancy. The balance of absciscic acid (ABA) and gibberellin (GA) regulates seed dormancy [5], and a decrease in ABA and an increase in GA were also found in germinated *P. polyphylla* seeds [5]. ABA conjugation and catalytic hydroxylation are

two pathways of ABA catabolism [6], and both pathways are involved in the decrease in ABA content during seed germination [7, 8]. However, little is known about ABA catabolism during *P. polyphylla* seed germination. GA₁, GA₃, GA₄, and GA₇ are bioactive GA molecules in plants [9]. The bioactivity of GA₄ is stronger than that of GA₁ in both Arabidopsis and rice [10-13], and GA₄ may regulate dormancy breaking in *Syngonanthus verticillatus* seeds [14]. GA₃ treatment is usually used as an effective way to break seed dormancy [15, 16]. However, the effect of GA₃ on *P. polyphylla* seed germination is complex. In some studies, GA₃ was shown to promote the seed dormancy breaking of *P. polyphylla* seeds and rhizomes [1, 17, 18], while in other studies, GA₃ showed no effects on the seed germination of *P. polyphylla* [19, 20]. However, the metabolism of different GA molecules and their roles in the dormancy breaking of *P. polyphylla* seeds are not fully understood.

Transcriptomics and metabolomics are efficient techniques that can be used to study the gene regulation and metabolic profiles of plants at certain developmental stages. In this study, the seeds of *P. polyphylla* were collected and germinated under warm temperatures. Seed at four different germination stages, air-dried seeds, seeds that germinated, seeds whose radicles had emerged, and seeds whose roots were approximately 2 cm, were defined. The mechanism of seed dormancy was studied by combining transcriptomics and untargeted and targeted metabolomics techniques. The DEGs related to *P. polyphylla* seed dormancy disruption were confirmed via qRT-PCR. The present study was designed to clarify the metabolic profiles of ABA and different bioactive GAs and their roles in *P. polyphylla* seed dormancy.

Materials And Methods

Plant materials and treatments

Paris polyphylla var. *yunnanensis* plants were grown in Shizong (24.82822 N, 103.99084 E), Yunnan Province, China. Their seeds were harvested on 18 October 2021. After washing under tap water, the seeds were air dried for 2 days. The dry seeds were incubated in wet sand and allowed to germinate at 18/22 °C (day/night) in a growth chamber. Seeds at different germination stages were harvested and immediately placed into liquid nitrogen. Three biological replicates were included in the subsequent analysis.

Widely targeted metabolomics analysis

The freeze-dried seed samples were ground to a powder, and 100 mg of the powder was extracted with 0.6 mL of 70% aqueous methanol (4 °C) overnight. After centrifugation at 10000 × g for 10 min, the supernatant was filtered with a microporous membrane (0.22 µm pore size) and analysed using a UPLC-ESI-MS/MS system. The UPLC conditions and materials were as follows: column, Agilent SB-C18 column (1.8 µm, 4.6 × 100 mm); solvent system, water (0.1% formic acid):acetonitrile; gradient programme, 95:5 (v/v) at 0 min, 5:95 (v/v) at 9.0 min, 5:95 (v/v) at 10 min, 95:5 (v/v) at 11.1 min, and 95:5 (v/v) at 14 min; flow rate, 0.35 mL/min; temperature, 40 °C; and injection volume, 4 µL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS. An API 4500 Q Trap LC/MS/MS

system equipped with an ESI turbo ion spray interface was used to perform linear ion trap (LIT) and triple quadrupole (QQQ) scans as previously described[21].

Phytohormone analysis

For the analysis of phytohormones, seeds were immediately placed into liquid nitrogen and kept at -80 °C. The samples were ground to a powder, and 50 mg of powder was extracted by methanol:H₂O:formic acid (15:4:1, v:v:v). The extraction buffer was condensed and redissolved in 100 µL of 80% methanol and then filtered (PTFE, 0.22 µm) before LC-MS/MS analysis. The LC-ESI-MS/MS conditions were as follows: HPLC column, Waters ACQUITY UPLC HSS T3 C18 (1.8 µm, 2.1 mm × 100 mm); solvent system, water (0.05% acetic acid):acetonitrile (0.05% acetic acid); gradient programme, 95:5 (v/v) at 0 min, 95:5 (v/v) at 1 min, 5:95 (v/v) at 8 min, 5:95 (v/v) at 9 min, 95:5 (v/v) at 9.1 min, 95:5 (v/v) at 12 min; flow rate, 0.35 mL/min; temperature, 40 °C; and injection volume, 2 µL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS instrument.

An AB 6500 QTRAP LC/MS/MS system equipped with an ESI turbo ion-spray interface operating in both positive and negative ion modes and controlled by Analyst 1.6 software (AB SCIEX) was used. The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 500 °C; ion spray voltage (IS), 4500 V; curtain gas (CUR), 35.0 psi; and collision gas (CAD), medium. DP and CE for individual multiple reaction monitoring (MRM) transitions were performed with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the plant hormones eluted within the period.

The phytohormone contents were measured by MetWare (<http://www.metware.cn/>) based on the AB SCIEX TRAP 6500 LC–MS/MS platform. Briefly, three replicates of each assay were assessed. Seven classes of phytohormones, including 26 kinds, were detected (Table S1).

Transcriptome sequencing and quantitative real-time PCR (qRT–PCR)

RNA extraction and quality analysis, library construction, sequencing and bioinformatics analysis were performed by staff at Wuhan Metaville Biotechnology Co., Ltd. (www.metavare.cn; Wuhan, China), as previously described [22]. The expression of 21 selected DEGs related to GA metabolism, ABA metabolism and seed dormancy was determined via qRT–qPCR. The primers used were designed based on sequence data of PacBio sequencing. *TUA* was used as an internal reference gene, and the genes and primers used are shown in Table S2. cDNA synthesis was performed with MonScript RTIII All-in-One Mix with dsDNase (Monad, MR05101) according to the manufacturer's instructions. qRT–PCR was performed with a QuantiNova SYBR Green PCR Kit (QIAGEN, 208054). The PCR initial heat activation was 95 °C for 2 min. The thermal cycling conditions were as follows: 40 cycles at 95 °C for 5 s for denaturation and 60 °C for 30 s for combined annealing and extension. All of the reactions were performed in triplicate. The relative expression levels of the DEGs were calculated using the comparative cycle threshold (Ct) method with normalization to the the expression level of internal reference gene.

Results

Four germination stages of *P. polyphylla* seeds

The germination of *P. polyphylla* seeds was divided into four different stages (Fig. 1). The air-dried seeds were ungerminated seeds and denoted as S0. In this stage, the embryo was not yet fully developed, and it was just an oval, primitive embryo. After stratification with warm temperature, the oval embryo fully developed; the seeds at this stage were designated as S1, and the seeds germinated at this stage. The seeds whose radicle had emerged were denoted as S2, and germinated seeds of which the roots were approximately 2 cm were denoted as S3.

Significant changed metabolites detected during the germination of *Paris polyphylla* seeds

To explore the changes in metabolite levels during seed dormancy break in *P. polyphylla*, a widely targeted metabolomics method based on ultra-performance liquid chromatography and tandem mass spectrometry was used. The metabolites were assigned to 13 classes: lipids, amino acids and derivatives, phenolic acids, organic acids, nucleotides and derivatives, alkaloids, flavonoids, steroids, lignans and coumarins, terpenoids, tannins, quinones, and others. A total of 585 metabolites were identified, and the main metabolites identified were amino acids and derivatives, organic acids, lipids, nucleotides and derivatives, and phenolic acids, of which there were 86, 62, 107, 42, and 83 metabolites detected, respectively. The content of these five classes in the dry seeds was more than 70% (Table S3). The levels of most metabolite classes, such as organic acids, nucleotides and derivatives, phenolic acids, steroids, terpenoids, flavonoids, lignans and coumarins, and tannins, decreased during the germination of *P. polyphylla* seeds. The levels of amino acids and derivatives and alkaloids showed a minor increase during seed germination, while the level of lipids increased dramatically during seed germination (Table S3).

The changes in the metabolome among the seeds at different germination stages were assessed using principal component analysis (PCA). The first two principal components explained 56.81% (PC1) and 20.14% (PC2) of the overall variance of all the samples (Fig. 2a). The PCA separated seeds of different germination stages efficiently. PC1 separated the S0 stage from the other three stages, whereas PC2 separated the S1 stage from the other three stages. The samples with three replicates clustered into each group and significantly varied among different groups during the different germination stages. These results indicated that the changes in most metabolites occurred during the initiation of germination in the S1 stage and that there was significant variation in the metabolism of these samples. Hierarchical cluster analysis of the metabolites showed that the most changed metabolites occurred in the S1 stage, and the metabolite profiles of S2 and S3 were similar, which indicates that no significant changes occurred from the seed germination stage of S2 to S3 (Fig. 2b).

Metabolites for which there was a fold change ≥ 2 or ≤ 0.5 between two samples were considered significantly changing metabolites (SCMs). A total of 262 SCMs (190 increased, 72 decreased) were detected between S0 and S1 seeds, 56 SCMs (34 increased, 22 decreased) were

identified between S1 and S2 seeds, and only 19 SCMs (6 increased, 13 decreased) were detected between S2 and S3 seeds (Fig. 2b, 2d, 2e, 2f). Among these SCMs, 8 metabolites, namely, ethylmalonic acid, 1-methylpiperidine-2-carboxylic acid, 3-hydroxyphenylacetic acid methyl ester, 3-(3-hydroxyphenyl)-propionate acid, thymidine, 6-hydroxy-5,7,4'-trimethoxyflavone, diosgenin, and LysoPC 12:0, were differentially expressed across all the germination stages (Fig. 2b, Table S4). A total of 222 SCMs were specifically detected in S0/S1, 21 SCMs were specifically detected in S1/S2, and only 2 SCMs were specifically detected in S2/S3 (Fig. 2b).

During the primary germination stage (from S0 to S1), the most changed SCMs were lipids, of which 85 lipids increased in abundance in this process, whereas only 1 lipid (13S-hydroxy-9Z,11E,15Z-octadecatrienoic acid) decreased in abundance (Fig. 3). The decreased lipids were free fatty acids, which may be precursors of other lipids. Four coumarins, 6,7-dimethoxy-4-methylcoumarin, o-feruloyl 4-hydroxycoumarin, 1-methoxyphaseollin, and 4-hydroxycoumarin di-glucoside, increased during this stage, and 1 coumarin, namely, 5,7-dimethoxycoumarin, decreased (Table S4). In the stage of radicle emergence (from S1 to S2), lipids were also the most significantly changed metabolites; however, 11 lipids decreased in abundance in this process, and 4 increased, which was lower than that at the primary germination stage. One coumarin, 1-methoxyphaseollin, decreased in this process (Fig. 3). During the process of radicle elongation (from S2 to S3), the abundance of few metabolites changed. LysoPC 12:0 increased in all germination stages (S1, S2, S3), which may indicate that LysoPC may participate in the germination of *P. polyphylla*.

Hormones of different classes changed variably during the seed germination of *P. polyphylla*

Hormones play important roles in seed germination, and the roles of different plant hormones may vary during the process of *P. polyphylla* seed dormancy breaking. To investigate the role of plant hormones during the breaking of seed dormancy in *P. polyphylla*, hormones of 7 classes, ABA, gibberellin A (GA), auxin, jasmonic acid (JA), cytokinin (CK), salicylic acid (SA) and ethylene, were tested. These 7 hormone classes included 26 hormone molecules (Table S1). The content of most hormones, except ethylene, showed significant changes during the seed germination of *P. polyphylla* (table 1). Significant changes in most hormones occurred at the stage of primary germination (S0 to S1). The total contents of ABA, CK, JA and SA decreased significantly at this stage, which decreased by 98.36%, 29.33%, 97.91%, and 51.38%, respectively (table 1). However, the contents of GA and auxin increased by 147.31% and 91.68%, respectively, at this stage (table 1). Only hormones in 3 classes changed from stage S1 to S2; JA and SA increased by 46.87% and 52.48%, respectively, while auxin decreased by 34.55% (Table 1). No significant change was observed in the total content of hormones from stage S2 to S3.

Table 1. Total content of each hormone class during the germination of *P. polyphylla* seeds.

Hormone class	S0	S1	S2	S3	RC (%)
	Hormones content (ng/g)				S0 to S1
abscisic acid	42.70 ± 3.28 ^a	0.70 ± 0.12 ^b	0.63 ± 0.05 ^b	0.62 ± 0.12 ^b	-98.36
gibberellin A	5.20 ± 0.39 ^b	12.86 ± 1.65 ^a	12.89 ± 1.10 ^a	12.06 ± 2.00 ^a	147.31
auxin	31.62 ± 1.55 ^b	60.61 ± 8.15 ^a	39.67 ± 3.43 ^b	32.41 ± 4.29 ^b	91.68
jasmonic acid	11.08 ± 2.01 ^a	7.83 ± 0.53 ^b	11.50 ± 0.52 ^a	9.30 ± 1.323 ^{ab}	-29.33
cytokine	1.91 ± 0.29 ^a	0.04 ± 0.00 ^b	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	-97.91
salic acid	31.47 ± 3.35 ^a	15.30 ± 1.60 ^c	23.33 ± 1.37 ^b	22.33 ± 1.88 ^b	-51.38
ethylene	53.63 ± 9.03 ^a	51.93 ± 0.86 ^a	49.63 ± 8.13 ^a	60.83 ± 1.18 ^a	-3.17

The relative change (RC) in hormones from S0 to S1 is the percentage values for the difference between the values at S0 and S1 over the values at S0. Values in the same row with different letters are significantly different ($P < 0.05$). Values are means ± standard deviation (n=3).

Among the five types of auxin we detected, the content of indole-3-acetic acid (IAA) decreased in the S1 stage and increased in the S2 stage, whereas the contents of methyl indole-3-acetate (ME-IAA), indole-3-carboxylic acid (ICA) and indole-3-carboxaldehyde (ICAld) increased in the S1 stage and decreased in the last two stages of S2 and S3, and we did not detect 3-indolebutyric acid (IBA) in *P. polyphylla* seeds (Fig. 4, Table S1). The metabolism of the three types of JA showed differences during different seed germination stages. JA showed little changes in content, but dihydrojasmonic acid (H2JA) increased significantly during the germination stage (S1, S2 and S3), while the content of jasmonoyl-L-isoleucine (JA-ILE) decreased greatly at the S1 stage and recovered to the level of the S0 stage in the last two stages of S2 and S3 (Fig. 1). Cytokinins such as trans-zeatin (tZ) and N6-isopentenyladenine (IP) were negatively correlated with the germination of *P. polyphylla* seeds and decreased to undetectable levels during the germination stage (S1, S2 and S3), whereas cis-zeatin (cZ) and dihydrozeatin (DZ) were not detected in *P. polyphylla* seeds throughout the whole germination stage (Fig. 2, Table S1). Taken together, these results may indicate that hormones of different classes and different hormone molecules play different roles during the seed germination of *P. polyphylla*: ABA, CK, and SA were negatively correlated with *P. polyphylla* seed germination, whereas GA and auxin were positively correlated with *P. polyphylla* seed germination.

Non-13-hydroxylated GAs play important roles in the germination of *Paris polyphylla*

GA was the most important hormone that was positively correlated with seed germination. GA₁, GA₃, GA₄, and GA₇ are the major bioactive GAs and are synthesized through two parallel pathways, namely, the 13-hydroxylation (13-OH) and non-13-hydroxylation (13-H) pathways. GA₁ and GA₃ are synthesized through

the 13-OH pathway, while GA₄ and GA₇ are synthesized through the 13-H pathway [9]. The content of 13-OH GAs, GA₁ and its precursors GA₅₃, GA₁₉, and GA₂₀ were not detected, and the content of GA₃ decreased dramatically to the level of the detection limit during the seed germination process (Fig. 4, Table S1). The content of 13-H GA, GA₄ and its precursors GA₁₅ and GA₂₄ increased significantly during the germination process (Fig. 4). However, GA₉, the precursor of GA₄, was under the detection level limit in all the germination stages of *P. polyphylla* seeds (Table S1). Taken together, these results indicated that 13-H GAs played a positive role in *P. polyphylla* seed germination and that 13-OH GAs may play a negative role in *P. polyphylla* seed germination.

DEGs identified during seed dormancy breaking of *Paris polyphylla*

To evaluate the difference in gene expression during the seed dormancy breakdown of *P. polyphylla*, RNA sequencing (RNA-seq) was performed based on the Illumina HiSeq platform. A total of 110.19 Gb of clean bases were obtained, and the total amount of clean bases of all samples constituted more than 8 Gb. The sequencing error rate of each sample did not exceed 0.03%, and the Q30 value was greater than 93% (Table S5). The quality of the sequencing data was high enough for subsequent analysis. A total of 22084 transcripts, with a mean length and N50 length of 1660 and 1867 nt, respectively, were assembled. The transcripts included those of 18984 unigenes, and the mean length and N50 length of the unigenes were 1665 and 1880 nt, respectively. The square of the Pearson correlation coefficient (R^2) between biological replicates was greater than 0.99, except for S0-1 with S0-3, the R^2 of which was 0.98 (Fig. S1).

The DEGs were identified after transcriptome assembly and functionally annotated. To analyse the function of the assembled unigenes, the sequences of the unigenes were subjected to BLAST searches of seven public databases (i.e., Pfam, KEGG, NR, SwissProt, GO, KOG, and Trembl), and more than 90% of the genes were annotated in at least one database (Table S6). A total of 10804 DEGs were identified in at least one of the three pairwise comparisons (i.e., S0 vs. S1, S1 vs. S2 and S2 vs. S3). The largest number of DEGs was found in S0 vs. S1 (6427 upregulated, 2445 downregulated). However, the smallest number of DEGs was found in S2 vs. S3, with only 203 and 536 genes up- and downregulated, respectively, and 614 DEGs were present in all three comparisons (Table S7, Fig. S2).

According to the GO annotation and classification results, the top 50 GO enriched terms were classified into three groups, biological processes, cellular components, and molecular functions, and plotted, the results of which are shown in Fig. 5. According to the GO enrichment results, 5909 DEGs were enriched in the primary germination stage (S0 vs. S1), 4430 DEGs were enriched in the radicle emergence stage (S1 vs. S2), and only 909 DEGs were enriched in the radicle elongation stage (S2 vs. S3). DEGs involved in biological processes were the most abundant in S0 vs. S1, and these DEGs were mainly associated with cellular carbohydrate metabolic processes, carbohydrate catabolic processes, polysaccharide metabolic processes, and cell wall organization or biogenesis (Fig. 5). The DEGs involved in cellular components were mainly associated with chromosomes, the apoplast, chromosomal parts, chromatin, and DNA packaging complexes in S0 vs. S1. Hydrolase activity, acting on glycosyl bonds and hydrolase activity, and hydrolysing O-glycosyl compounds were the main molecular functions of the enriched DEGs in S0 vs.

S1. In the S1 vs. S2 and S2 vs. S3 comparisons, most DEGs were involved in molecular functions (Fig. 5), which is different from that in S0 vs. S1.

Key genes related to *P. polyphylla* seed dormancy

The balance of GA and ABA is the main factor that controls the germination of seeds. According to the annotation results of the 7 public databases, we analysed GA-, ABA- and seed dormancy-related DEGs during different germination stages of *P. polyphylla*. Among the enzymes regulating GA synthesis, only one transcript of ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS) was detected in *P. polyphylla* seeds, and these two genes were upregulated during seed germination (Fig. 6 and S3). Therefore, CPS and KS are encoded by a single gene in *P. polyphylla* seeds. Five *ent-kaurene oxidase* (KO) and one *ent-kaurenoic acid oxidase* (KAO) genes were found in *P. polyphylla* seeds, and these genes were upregulated during the primary germination S1 stage (Fig. 6 and S3). Gibberellin 13-oxidase is the key enzyme catalysing the synthesis of 13-OH GAs, and the transcript of the gene encoding gibberellin 13-oxidase was not detected in *P. polyphylla* seeds. Genes that are involved in the regulation of the synthesis of 13-H GA synthesis, such as *gibberellin 20-oxidase* and *gibberellin 3-oxidase*, were upregulated during the germination of *P. polyphylla* seeds. Fourteen transcripts of *gibberellin 2-oxidase*, a gibberellin deactivation-related gene, were detected in *P. polyphylla* seeds, 9 of which were upregulated, 2 of which were downregulated, and 3 of whose expression was not significantly changed. Nine transcripts were found to encode the gibberellin receptor GID1; these transcripts were upregulated during seed germination, and 2 of these transcripts were downregulated during this process (Fig. 6). Taken together, these results indicate that 13-H GAs may act in the dormancy breaking of *P. polyphylla* seeds.

ABA catalytic hydroxylation and conjugation are two methods of ABA catabolism. The expression of *CYP707A5*, which encodes abscisic acid 8'-hydroxylase 1, did not show significant changes at the S1 stage (Fig. 6 and S3). Most genes encoding UDP-glucosyltransferase (UGT), which can glucosylate ABA to its inactive form of glucose ester AGA-GE, were significantly upregulated, and some of them showed downregulation (Fig. 6 and S3). *ABA insensitive 3* and *5* (*ABI3*, *ABI5*), the key transcription factors that positively regulate seed dormancy, showed significant downregulation (Fig. 6 and S3). However, the expression of ABA receptor genes, such as *PYL4*, *PYL8* and *PYR1*, increased significantly during the germination of *P. polyphylla* seeds..

Genes positively related to dormancy, such as *DOG1*, *NCED6*, *DREB2C*, and *MARD1*, were downregulated, while genes negatively related to dormancy during the seed germination process, such as *SLY1*, were upregulated (Fig. 6 and S3). Two NCED genes, *NCED5* and *NCED6*, showed upregulation during this process. We identified 3 transcripts of *RGL1* in *P. polyphylla* seeds, whereas none of these 3 transcripts showed significant changes during seed germination. These results may indicate that part of the gene regulatory process of *P. polyphylla* seed dormancy may be the same as that of other plant species.

Discussion

Breaking seed dormancy is a complicated process that involves remarkable reprogramming of genes expression, proteins, and metabolites [23–26]. Integrating transcriptomics and metabolomics is an effective way to study biological processes in plants [21, 27, 28]. Investigating the mechanism of seed dormancy at the molecular level is important for the breeding and cultivation of medicinal plants. In this study, using transcriptomics and metabolomics methods, we investigated different stages of seed dormancy breaking in *Paris polyphylla*, an important medicinal herbaceous species in China.

Changes in lipid metabolism-related genes were strongly correlated with *P. polyphylla* seed germination [3]. In our experiment, the most changed metabolites in the primary germination stage (S0) were lipids such as free fatty acids, glycerol ester, lysophosphatidylcholine (LPC), and lysophosphatidylethanolamine (LPE) (Table S4). The increase in free fatty acids may occur through the metabolism of storage lipids, and lipids can act as precursors of other lipids [29]. Lysophospholipids (lysoPLs) such as LPC and LPE are the products of phospholipid deacylation catalysed by phospholipid:diacylglycerol acyltransferase (PDAT) or phospholipase A (PLA) [30]. The expression of *PDAT* showed no significant difference among the four germination stages of *P. polyphylla*, while the expression of *PLA2* increased dramatically in the S1 stage (Fig. S3). The increase in LPC and LPE in *P. polyphylla* may be attributed to the PLA2-mediated deacylation of PC and PE. PLA2-induced generation of lysoPLs plays an important role in plant growth. LPC and LPE levels were shown to increased several times when plants are under stress [31]. lysoPLs are negatively correlated with the seed viability of soybean, a dicotyledonous species [32], whereas they are positively correlated with the seed viability of rice, a monocotyledonous species [33]. These results may indicate that PLA2-mediated lysoPL generation in the monocotyledonous *P. polyphylla* may have a positive effect on seed viability and may hasten seed germination.

The catabolism and signal transduction of ABA is important for seed germination. ABA catabolism occurs through two pathways: conversion to phaseic acid by cytochrome P₄₅₀ monooxygenase (P₄₅₀) encoded by *CYP707As* [6] or glycosylation by UGT to ABA-glucose ester (ABA-GE) [7]. *CYP707A* may participate in the germination of *P. polyphylla* [2]. However, in our study, the expression of *CYP707A* was not synchronous with the change in ABA content during *P. polyphylla* seed germination (Figs. 4, 6 and S3). The expression of most UGTs increased during germination, and the expression of some USTs decreased during germination (Fig. 6 and S3). It is suspected that the decrease in ABA during the germination of *P. polyphylla* seeds may be attributed to the glycosylation process to form ABA-GE. *ABI3* and *ABI5* are two key transcription factors that play a role ABA signal transduction; the two transcripts of *ABI3* were downregulated, and 17 of 20 transcripts of *ABI5* were downregulated. The downstream transcription factor of *ABI3*, *NCED6*, was also downregulated. *ABI3* may regulate the expression of *NCED6* to control the dormancy of *P. polyphylla* seeds (Figs. 6 and S3). Therefore, ABA signal transduction during *P. polyphylla* seed germination may share the same pathways with those in other plants species [25].

The phytohormone GA is one of the most important hormones involved in seed germination. Both the increase in endogenous GAs and the application of exogenous GAs can break seed dormancy and accelerate seed germination. Among 136 fully characterized GA molecules, only GA₁, GA₃, GA₄, and GA₇

exhibit biological activity in plants [9]. GA₁ and GA₃ are synthesized through the 13-OH pathway, while GA₄ and GA₇ are synthesized through the 13-H pathway [9]. GA₁ and GA₇ were not detected in the *P. polyphylla* seeds. GA₃ was detected only in the S0 stage but not in the other germination stages, while GA₄ increased during *P. polyphylla* seed germination. GA 13-oxidase (GA13ox), which catalyses the synthesis of GA₁₂ to GA₅₃, is the key enzyme for the synthesis of 13-OH GAs, and the *CYP714* gene family encodes GA13ox [34]. We did not detect transcripts of *CYP714* in *P. polyphylla* seeds, which may cause the absence of GA13ox in seeds and, further, the absence of most 13-OH GAs, such as GA₅₃, GA₁₉, GA₂₀, and GA₁ (Table S1). GA₃ was the only 13-OH GA that was present in ungerminated dry *P. polyphylla* seeds (S0 stage). GA₃ may be synthesized in other tissues and transported into seeds during seed maturation of *P. polyphylla*. The synthesis of GA₄ follows the order of GA₁₂→GA₁₅→GA₂₄→GA₉→GA₄; GA20ox catalyses the metabolism of GA₁₂ to GA₉, and GA3ox catalyses GA₉ to GA₄ [11]. GA₁₅ and GA₂₄ increased during *P. polyphylla* seed germination, while GA₉ was not detected (Table S1). Overexpression of *GA20ox* induced an increase in GA₄ [8], and the increase in GA₄ during *P. polyphylla* seed germination may be attributed to the significant upregulation of *GA20ox* (Fig. S3). The synthesis of GA₄ in *P. polyphylla* seeds may be different from that in other plant species, the synthesis of which does not occur through the catalysis of GA₉ to GA₄. To further confirm the different roles of exogenous GA₃ and GA₄ on *P. polyphylla* seed germination, we treated *P. polyphylla* seeds with 150 mg/mL GA₃ and GA₄ and found that after incubation for 3 months, only 15% of the GA₃-treated seeds germinated, 30% of seeds with no treatment germinated, and 52% of GA₄-treated seeds germinated. However, another study showed that the application of GA₃ can also break seed dormancy in *P. polyphylla* [17]; the *P. polyphylla* seeds in our experiment were obtained from eastern Yunnan Province, while the seeds used by Zhou et al. were derived from central Yunnan Province. The different roles of GA₃ in *P. polyphylla* seed germination may be attributed to the different growth environments of *P. polyphylla*. Thus, for *P. polyphylla* from eastern Yunnan Province, GA₃ was negatively correlated with *P. polyphylla* seed germination, while GA₄ was positively correlated with *P. polyphylla* seed germination.

The bioactivity of the various bioactive GAs is different; the bioactivity of GA₄ is stronger than that of other GAs [9]. Magome et al. suggested that weakly active GAs may be involved in small growth change processes, whereas strongly active GAs would be useful for strong GA signal output, and that plants can dynamically adjust their use of strongly and weakly bioactive GAs [11]. The seed dormancy breaking process is an intense growth change that requires a strong GA signal, so GA₄ with strong bioactivity is involved in the seed dormancy breaking of *P. polyphylla*; this same activity also occurs when *Syngonanthus verticillatus* seed dormancy is broken [14]. However, GA₁ and GA₃ increased dramatically, while GA₄ and GA₇ were not detected in polyaerial shoot rhizomes during *P. polyphylla* rhizome dormancy breaking, and GA₃ application can also break rhizome dormancy in *P. polyphylla* [1]. Therefore, *P. polyphylla* has the ability to alter its bioactive GA synthesis to match GA-dependent growth.

Conclusions

Through a combined analysis of metabolomics and transcriptomics data, this study investigated the dormancy mechanism of eastern Yunnan *P. polyphylla* seeds. A decrease in ABA and increase in GA regulate the dormancy breaking of *P. polyphylla* seeds. The decrease in ABA was mainly attributed to the conversion of ABA to ABA-GE. *P. polyphylla* tends to block the synthesis of low-bioactivity 13-OH GAs (GA₁ and GA₃) and accelerate the synthesis of strong-bioactivity 13-H GA (GA₄) during the process of breaking seed dormancy. Different bioactive GAs act in different biological processes in *P. polyphylla*; GA₄ may act in breaking seed dormancy, while GA₁ and GA₃ may act in breaking rhizome dormancy. Therefore, the choice of specific GA treatment in GA-dependent biological processes should be based on the habitats of *P. polyphylla* and the plant growth stage.

Declarations

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Authors' contributions

Guowei Zheng: methodology, validation, formal analysis, investigation, writing-original draft, visualization. Wenchun Li and Shunzhen Zhang: formal analysis, investigation. Qi Mi, Wenxiu Luo, Yanli Zhao and Xiangshi Qin: formal analysis, resources. Furong Xu: conceptualization, supervision, project administration, writing-review and editing. Weijiao Li: writing-review and editing. Shibiao Pu: writing-review and editing.

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Availability of data and materials

The raw sequencing data has been submitted to NCBI (PRJNA939355, <https://dataview.ncbi.nlm.nih.gov/object/PRJNA939355?reviewer=ege6n875uecv0e5jc643ocbmjl>). All remaining are included in this published article and its supplementary information files.

Ethics approval and consent to participate

The collection of plant material and all the methods complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures



Figure 1

Phenotypes of *Paris polyphylla* seeds at different germination stages.

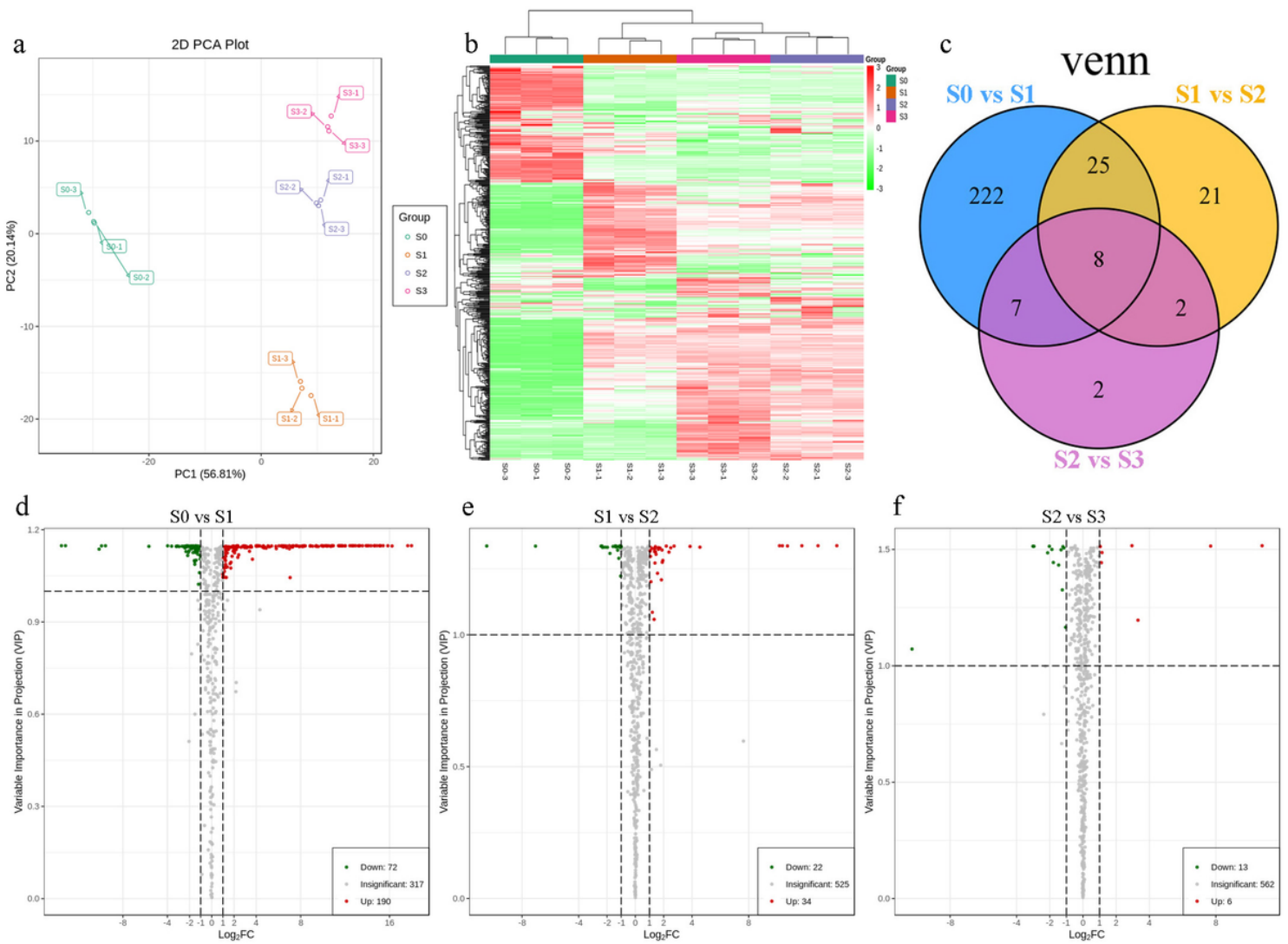


Figure 2

SCMs during the different germination stages of *P. polyphylla*. (a) PCA score plot of metabolite profiles from S0, S1, S2, and S3. (b) Venn diagram showing the number of total SCMs. (c) Accumulation patterns of significantly changed metabolites from S0, S1, S2, and S3. (d, e, f) Volcano plot of metabolites from S0, S1, S2, and S3.

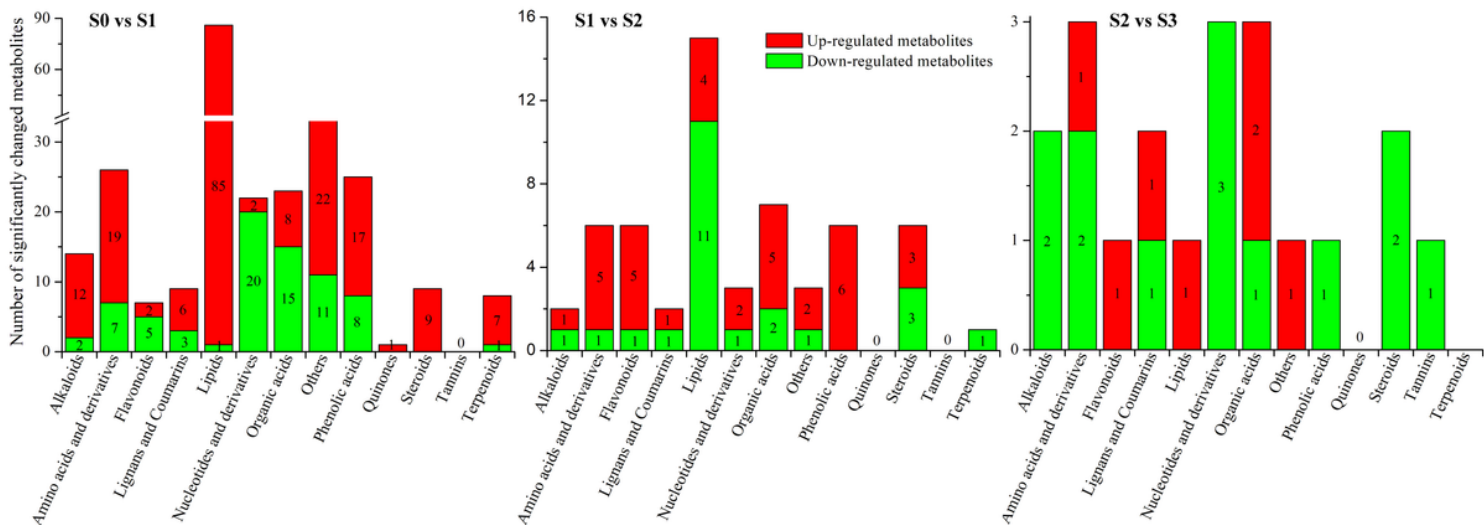


Figure 3

Number of SCMs during the different germination stages of *P. polyphylla*.

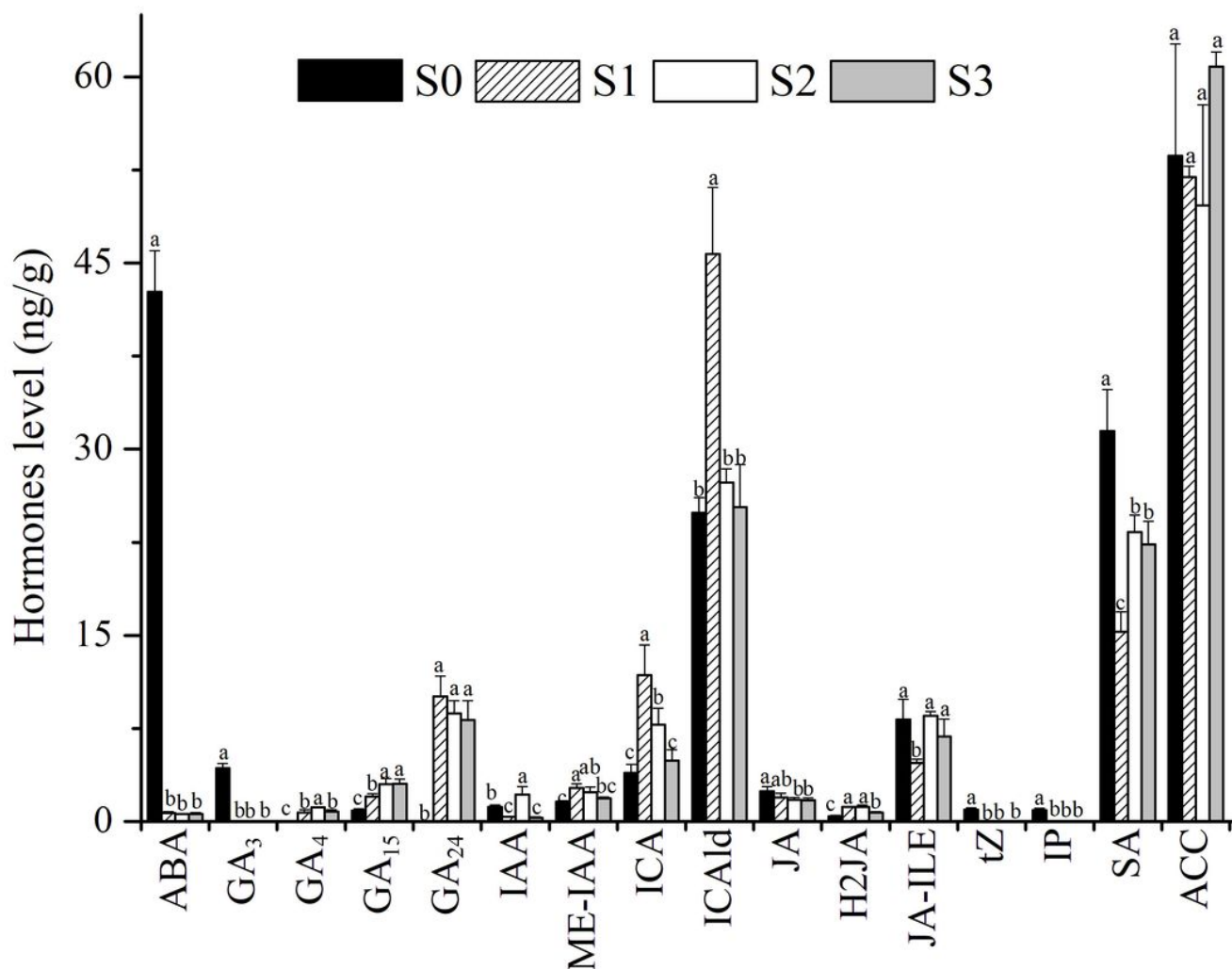


Figure 4

Changes in hormone levels during the germination of *P. polyphylla* seeds. The bars marked with different letters are significantly different from the others ($P < 0.05$). The values are means $\pm$ standard deviations ($n=3$).

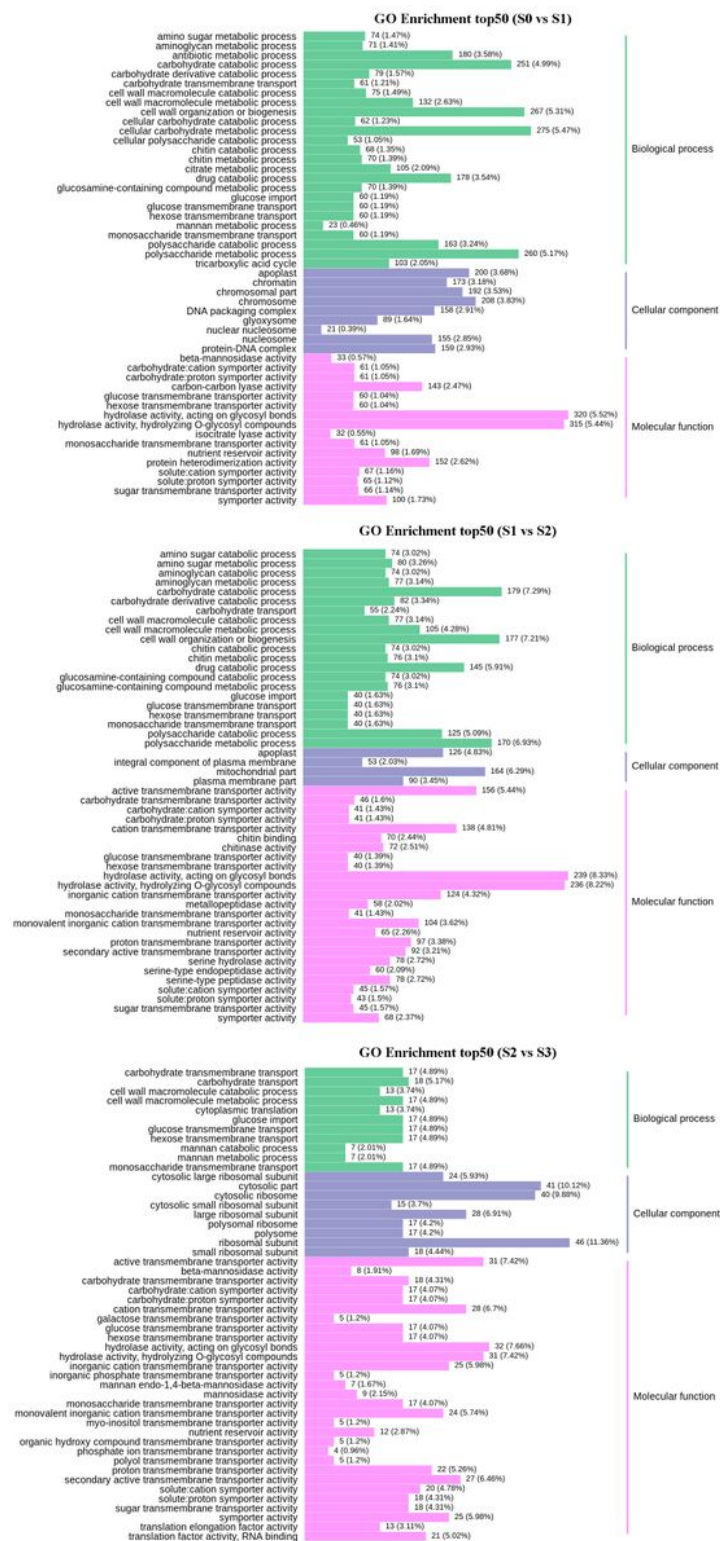


Figure 5

GO classification of DEGs between different comparisons (S0 vs. S1, S1 vs. S2, and S2 vs. S3).

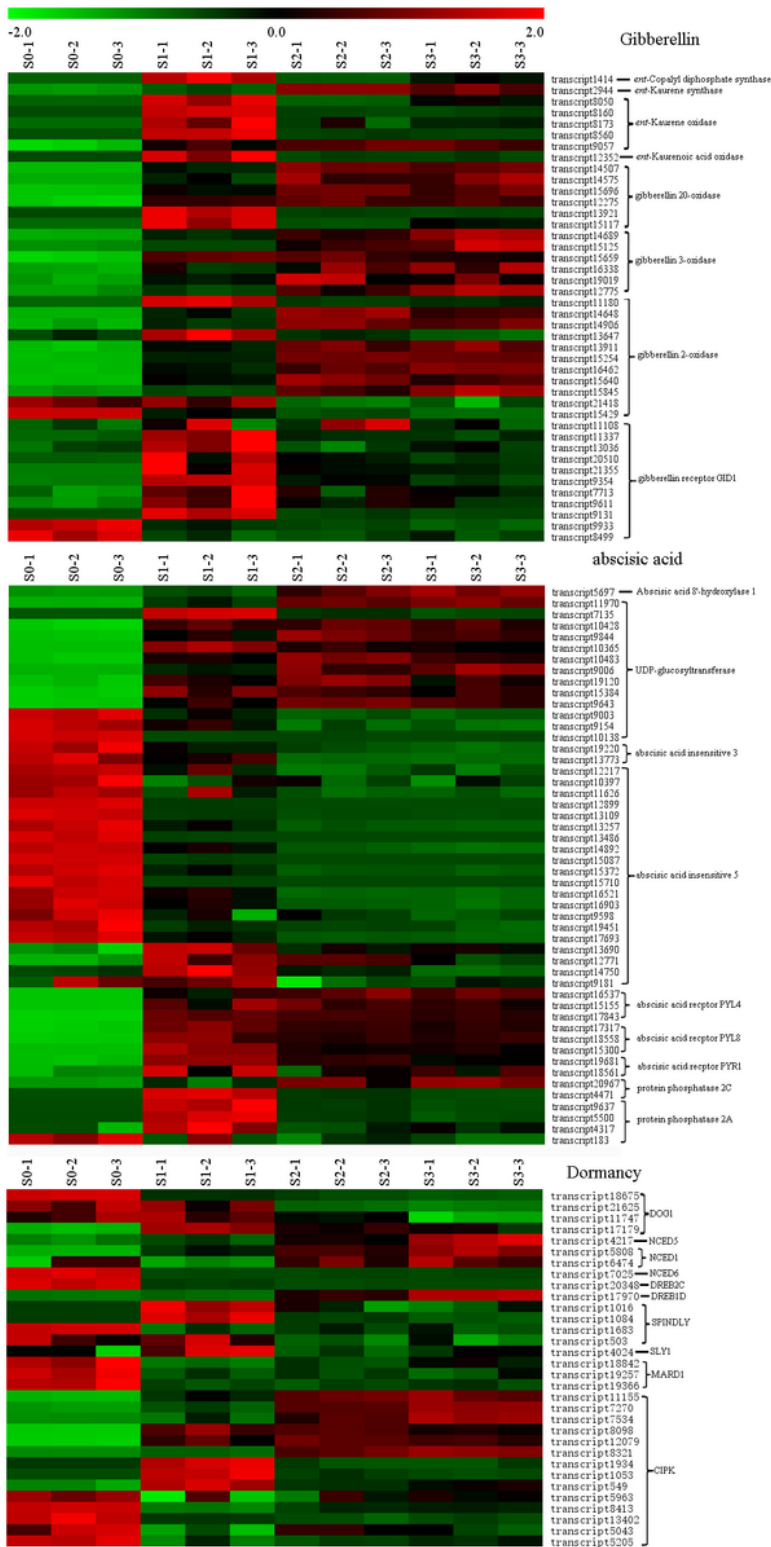


Figure 6

Hierarchical cluster of GA, ABA and dormancy -related DEGs in the different germination stages of *P. polyphylla*.

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