

RNA-seq study reveals the signaling and carbohydrate metabolism regulators involved in dormancy release by warm stratification in *Paris polyphylla* var. *yunnanensis*

Bin Yang^{1,*}, Shan Sun², Shengyu Li¹, Jiali Zeng¹, Furong Xu^{3,**}

¹Guangzhou Key Laboratory for Research and Development of Crop Germplasm Resources, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China; ²The Laboratory of Seed Science and Technology, Guangdong Key Laboratory of Plant Molecular Breeding, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou 510642, China; ³College of Traditional Chinese Medicine, Yunnan University of Chinese Medicine, Kunming 650500, China

*E-mail: yangbin@zhku.edu.cn Tel & Fax: +86-020-89003026

**E-mail: xfrong99@163.com Tel & Fax: +86-0871-65933939

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Abstract Long-term seed dormancy of *Paris polyphylla* var. *yunnanensis* limits its large-scale artificial cultivation. It is crucial to understand the regulatory genes involving in dormancy release for artificial cultivation in this species. In this study, seed dormancy of *Paris polyphylla* var. *yunnanensis* was effectively released by warm stratification (20°C) for 90 days. The freshly harvested seeds (dormant) and stratified seeds (non-dormant) were used to sequence, and approximately 147 million clean reads and 28,083 annotated unigenes were detected. In which, a total of 10,937 differentially expressed genes (DEGs) were identified between dormant and non-dormant seeds. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) classification revealed that the majority unigenes involved in signaling transduction and carbohydrate metabolism. Of them, the signaling transduction-related DEGs were mainly hormones-, reactive oxygen species (ROS)-, and transcription factor (TF)-related genes. The largest number of signaling transduction-related DEGs were auxin-responsive genes (*SAUR*, *AUX/IAA*, and *ARF*) and AP2-like ethylene-responsive transcription factor (*ERF/AP2*). Moreover, at least 29 DEGs such as α -amylase (*AMY*), β -glucosidase (*Bglb/Bglu/Bglx*), and endoglucanase (*Glu*) were identified involving in carbohydrate metabolism. These identified genes provide a valuable resource to investigate the molecular basis of dormancy release in *Paris polyphylla* var. *yunnanensis*.

Key words: carbohydrate metabolism, *Paris polyphylla*, seed dormancy, signal transduction, warm stratification.

Introduction

Paris polyphylla var. *yunnanensis* (afterwards *P. polyphylla*) also named ‘dian chonglou’ in Chinese is an important medicinal plant. The dried rhizomes of this plant have become the indispensable ingredient of well-known traditional Chinese medicines such as “Yunnan Bai Yao” and “Gong Xue Ning”, which are utilized to improve blood circulation, stop bleeding, treat dispersing blood stasis and hemostasis and reduce inflammation and pain (Liao et al. 2019; Qi et al. 2013). *P. polyphylla* is primarily distributed in Yunnan, Sichuan, and Guizhou provinces of southwestern China (Ling et al. 2017). Unfortunately, the wild plants are confronted with the risk of extinction because of slow-growing and over-harvesting for recent decades (Liu et al. 2016). To ensure the sustainable utilization of wild resource of *P. polyphylla*, the successful artificial planting of this plant is indispensable. However, only 40% of *P. polyphylla*

seeds can germinate after experiencing a long dormancy period (18–24 months) under natural environment (Ling et al. 2017), which seems to place severe limitations on the large-scale artificial cultivation. Therefore, dormancy breaking is a main goal of seed treatments in *P. polyphylla*.

The freshly-harvested seeds of *P. polyphylla* contain an undeveloped globular embryo, and it is of the typical morphophysiological dormancy (Huang et al. 2008). These seeds can develop into the hypocotyl, radicle, and cotyledon after the morphological after-ripening process (Zhou et al. 2003). Stratification is a pre-treatment of seeds to release dormancy, which has been extensively reported in several species. For instance, the freshly harvested seeds of *Myrica rubra* require 8 weeks of warm stratification followed by 12 weeks of cold stratification for seed germination (Chen et al. 2008). Seed dormancy of *Punica granatum* is broken by warm and cold stratification (Shalimu et al. 2016). Cold stratification of

apple seeds for 90 days has a stronger dormancy breaking effect of isolated embryos (Ciacka et al. 2019). Similarly, the warm temperature stratification can effectively shorten the seed dormancy period of *P. polyphylla* and promote seed germination (Chen et al. 2015; Huang et al. 2008; Wang et al. 2012; Zhang et al. 2017). Studies have revealed that a constant exposure to warm conditions ($\sim 20^{\circ}\text{C}$) promotes the early seed germination and rapid radical growth of *P. polyphylla* when compared with cold or alternating cold–warm conditions (Chen et al. 2015; Huang et al. 2008; Wang et al. 2012; Zhang et al. 2017). However, the molecular mechanisms of warm stratification on the dormancy release remain unclear in *P. polyphylla*.

Seed germination is a critical developmental step regulated by multiple endogenous signals such as phytohormones, reactive oxygen species (ROS), and transcription factors (TFs). It is widely considered that abscisic acid (ABA) and gibberellin (GA) are the primary phytohormones that antagonistically controlled seed dormancy and germination (Finch-Savage and Leubner-Metzger 2006; Graeber et al. 2012). The enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) contributes to the ABA biosynthesis (Lefebvre et al. 2006) and ABA signal transduction such as *ABA-INSENSITIVE* (*ABI*) genes affecting seed germination (Finkelstein et al. 1998; Lopez-Molina et al. 2002). The GA signaling components such as DELLA proteins are reported to affect seed germination (Ravindran et al. 2017). Dormancy release is also associated with declined sensitivity to indole-3-acetic acid (IAA) in wheat (Liu A et al. 2013) and enhancing IAA signaling or biosynthesis in *Arabidopsis* (Liu X et al. 2013) and influencing IAA homeostasis in rice (Yang et al. 2019). ROS also emerged as key signaling molecules to promote dormancy release and stimulate seed germination (Bailly et al. 2008; Huang et al. 2018). Transcription factors, such as WRKY, MYB and AP2/EREBP, are another important signaling regulators in seed germination (He et al. 2020). However, the precise signaling responses in the dormancy release by warm stratification are not yet fully understood in *P. polyphylla*.

Seed germination involves in a complex series of metabolic processes such as seed respiration and storage reserves mobilization (Wanasundara et al. 1999). In which, the metabolic activities gradually increase with the absorption of water, and the enzymes related to storage reserves degradation are activated or resynthesized (Liu et al. 2018). Starch is generated from carbohydrates, which is mainly stored in the endosperm of seeds. The α -amylase and β -amylase are the key enzymes involved in the process of starch decomposition, affecting seed germination and seedling growth (Farooq et al. 2017). The starch granules are firstly degraded by α -amylase during seed germination, and then the linear glucans can be attacked by β -amylase

and the maltose product can be further degraded into glucose by α -glucosidase (Guo et al. 2020). The degraded starch is absorbed by the scutellum, and sucrose is then synthesized and transported to the embryo or seedling growth (Aoki et al. 2006). The carbohydrates are necessary for the provision of energy and essential nutrients and their carbon skeletons are used for different biosynthetic processes (Cao et al. 2019). The enzymes of starch degradation are mostly formed during the post-ripening stage (Bewley and Black 1994). However, the precise roles of starch degradation in the dormancy release by warm stratification are not yet fully understood in *P. polyphylla*.

To date, several transcriptome researches have been conducted to reveal the molecular mechanisms of *P. polyphylla* on dormancy release (Liao et al. 2019; Ling et al. 2017; Qi et al. 2013). However, the precise roles of signaling responses and starch degradation in dormancy release by warm stratification are not yet fully understood in *P. polyphylla*. Therefore, we performed de novo transcriptome sequencing using Illumina Nova platform and compared the transcriptome of freshly harvested seeds (FHS, dormant) and stratified seeds (SS, non-dormant) to identify key candidate genes involved in seed dormancy. Especially, the potential genes related to signaling responses and starch degradation involving in dormancy release were highlighted in this study. Our results provide new insights into the molecular regulation of dormancy release in *P. polyphylla*.

Materials and methods

Plant materials and seed treatment

Freshly matured fruits of *P. polyphylla* were collected in Yunnan Province of China in October 2019. Seeds were separated from the fruits and washed to remove the pulpy outer layer prior to use in experiments. For warm stratification, the freshly harvested seeds were placed into moistened quartz sand at 20°C for 3 months to break seed dormancy. The freshly harvested seeds and warm stratified seeds were snap-frozen in liquid nitrogen and stored in a refrigerator at -80°C until use.

RNA extraction and quality determination

Total RNA was isolated separately from freshly harvested seeds and warm stratified seeds using a Total RNA Rapid Extraction Kit RP3301 (BioTeke Corporation, Wuxi, China) according to the manufacturer's instruction. The concentration, purity and integrity of total RNA were determined using agarose electrophoresis and the Bioanalyzer 2100 (Agilent Technologies, CA, USA). Ribosomal RNA (rRNA) in the samples was depleted using the Ribo-Zero™ Magnetic Kit (Epicentre, Madison, USA) according to the manufacturer's protocol.

cDNA library construction and sequencing

Sequencing libraries were generated using NEB Next[®] Ultra[™] Directional RNA Library Prep Kit (NEB, Ipswich, USA) following manufacturer's protocol. The mRNA was fragmented by heat treatment (94°C) for 15 min in First Strand Synthesis Reaction Buffer. First strand cDNA was generated using ProtoScript II Reverse Transcriptase with random primers. Second strand cDNA synthesis was subsequently performed by adding Second Strand Synthesis Reaction Buffer with dUTP Mix, and Second Strand Synthesis Enzyme Mix. In order to select ligated cDNA fragments (300–500 bp in length), the library fragments were purified with AMPure XP Beads (Agencourt, Beverly, USA). Adaptor-ligated products were amplified with PCR using USER Enzyme, Universal PCR Primer and Index (X) Primer. PCR products were then purified with AMPure XP Beads. DNA library quality and quantity were assessed with the Qubit Fluorometer and High Sensitivity DNA chip. The TruSeq PE Cluster Kit (Illumina, San Diego, USA) was used for cluster generation on the cBOT according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina sequencing NovaSeq platform (Illumina, San Diego, USA).

Transcriptome assembly and functional annotation

Clean reads were obtained by removing adaptor sequences, unknown sequences (indicated as “N” in a sequence) and low-quality reads from raw data using Trimmomatic v0.32 (Bolger et al. 2014). The percentage of bases with a Phred value >20 (Q20) or >30 (Q30) and GC-content of the clean data were simultaneously calculated. Sequence quality was evaluated with Fastqc v0.10.0 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Transcriptome de novo assembly was generated using Trinity v2.06 with min_kmer_cov set to 2 and all other parameters set to default (Grabherr et al. 2011). To annotate the assembled transcripts, the unigene sequences were aligned against the databases of NCBI non-redundant protein sequences (NR) database, Swiss-Prot database, euKaryotic Ortholog Groups (KOG), Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), respectively, using Blastp v2.2.22 with a significance threshold of E -value $<10^{-5}$.

Analysis of different expression genes

Differentially-expressed genes (DEGs) between freshly harvested seeds and warm stratified seeds were determined using the DEGseq v1.20.0 with the method MARS (MA-plot-based method with random sampling model) (Wang et al. 2010). Adjusted p values (q) were generated using the Benjamini and Hochberg correction to control the false discovery rate (FDR). In this analysis, the genes with a threshold q -value <0.001 , fold change ≥ 2 and RPKM ≥ 5 in at least one sample were identified as differentially expressed.

Gene validation and expression analysis

Approximately 4 μ g of total RNA was reverse transcribed to

cDNA using the HiScript[®] II Reverse Transcriptase system (Vazyme Biotech Co., Ltd., Nanjing, China). The quantitative real-time PCR (qRT-PCR) was performed by 20 μ l reaction mixture in 96-well plate with the CFX96 Real-Time System (Bio-Rad, CA, USA). Primers were designed using Primer Premier 5 and listed in Supplementary Table S1. The *Actin* gene (contig12067; Liao et al. 2019) and *GAPDH* gene (contig14070; Qi et al. 2013) were used as internal references to normalize gene expression levels. Levels of gene expression were calculated using the comparative CT value method (Livak and Schmittgen 2001).

Results

Illumina sequencing and de novo assembly

To investigate the transcriptomic responses to dormancy release of *P. polyphylla* seeds, two cDNA libraries were constructed from the mRNA of the freshly harvested seeds (FHS, dormant) and stratified seeds (SS, non-dormant; germinated seeds with approximately 2 mm length protruding radicles), which were sequenced using Illumina Nova. After removing the adapters, low-quality data and ambiguous reads, approximately 75 million and 72 million clean reads were obtained in FHS (FHS-1, FHS-2, and FHS-3) and SS (SS-1, SS-2 and SS-3) samples, respectively (Supplementary Table S2). In addition, Q20 and Q30 are greater than 95% in both samples, and GC percentage are about 50% on average (Supplementary Table S2). A total of 147,222,491 clean reads (including FHS and SS samples) were used to assemble the transcriptome data using the Trinity software. Based on overlapping information in high-quality reads, 317,811 transcripts with a mean length of 957 bp and 90,246 unigenes with an average length of 1,022 bp were generated (Supplementary Table S3). The N50 and N90 are 1,552 bp and 393 bp for the transcripts, respectively, and the corresponding unigenes is 1,692 bp and 418 bp. The smallest and largest length of unigenes is 201 bp and 60,742 bp, respectively (Supplementary Table S3). The length distribution of the transcripts and unigenes is shown in Figure 1.

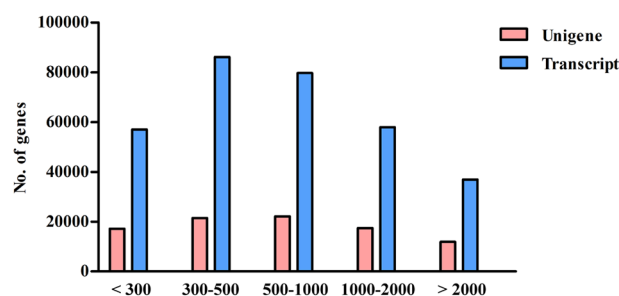


Figure 1. Length distributions of the transcripts and unigenes from de novo assembly.

Functional classification of unigenes

For functional annotation, the public databases including NR, Swiss-Prot, KOG, GO and KEGG were used to annotate the assembled unigenes. A total of 28,083 unigenes were successfully matched with the searched databases, suggesting that a substantial fraction of the expressed genes were identified in this study (Figure 2). However, we found that 68.88% of the unigenes were un-matched, and these unaligned genes may be novel transcripts in *P. polyphylla*. The mapping rates of unigenes against the databases of NR, Swiss-Prot, KOG, GO and KEGG are 29.41%, 19.54%, 15.81%, 10.5%, and 3.2%, respectively (Figure 2).

To further reveal the potential functions of transcripts, functional classification of the predicted unigenes were performed using KEGG assignments. The unigenes annotated by KEGG were clustered into 33 functional KEGG pathways, in which four in the cellular processes, three in the environmental information processing,

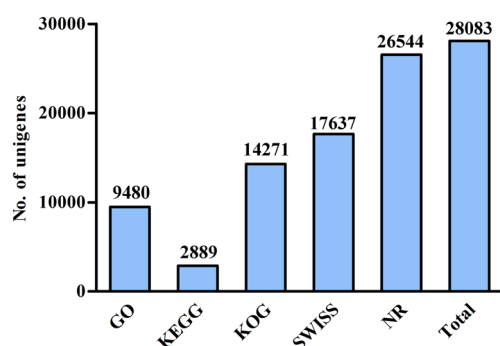


Figure 2. The numbers of annotated unigene in five public databases.

four in the genetic information processing, twelve in the metabolism and ten in the organismal systems (Figure 3). By comparison, the unigenes were mainly associated with “signal transduction” (26.93%) and “carbohydrate metabolism” (21.15%).

Identification and functional classification of DEGs

Differentially expressed genes (DEGs) were identified from the 90,246 unigenes if RPKM ≥ 5 in at least one sample, fold change was ≥ 2 and q -value < 0.001 . According to the standard, a total of 10,937 DEGs were identified between FHS and SS, in which 8,393 were up-regulated and 2,544 were down-regulated (Figure 4A). The functional classification of DEGs was performed by GO and KEGG enrichment analysis. GO analysis showed that 724 DEGs were significantly enriched in 25 GO terms (Figure 4B). For example, the DEGs were enriched in the “O-methyltransferase activity”, “transferase activity, transferring hexosyl groups”, “translation”, “structural constituent of ribosome”, “hydrolase activity, hydrolyzing O-glycosyl compounds”, “response to oxidative stress”, “response to hormone”, and “sequence-specific DNA binding transcription factor activity”. Further KEGG enrichment analysis revealed that 249 DEGs were significantly enriched in 9 KEGG pathways (Figure 4C). The most significant enriched pathway was “plant hormone signal transduction”, followed by “DNA replication”, “flavonoid biosynthesis”, “MAPK signaling pathway”, and “starch and sucrose metabolism” and so on. These results indicated that the signaling and metabolism pathways might play important roles on dormancy release by warm stratification in *P. polyphylla*.

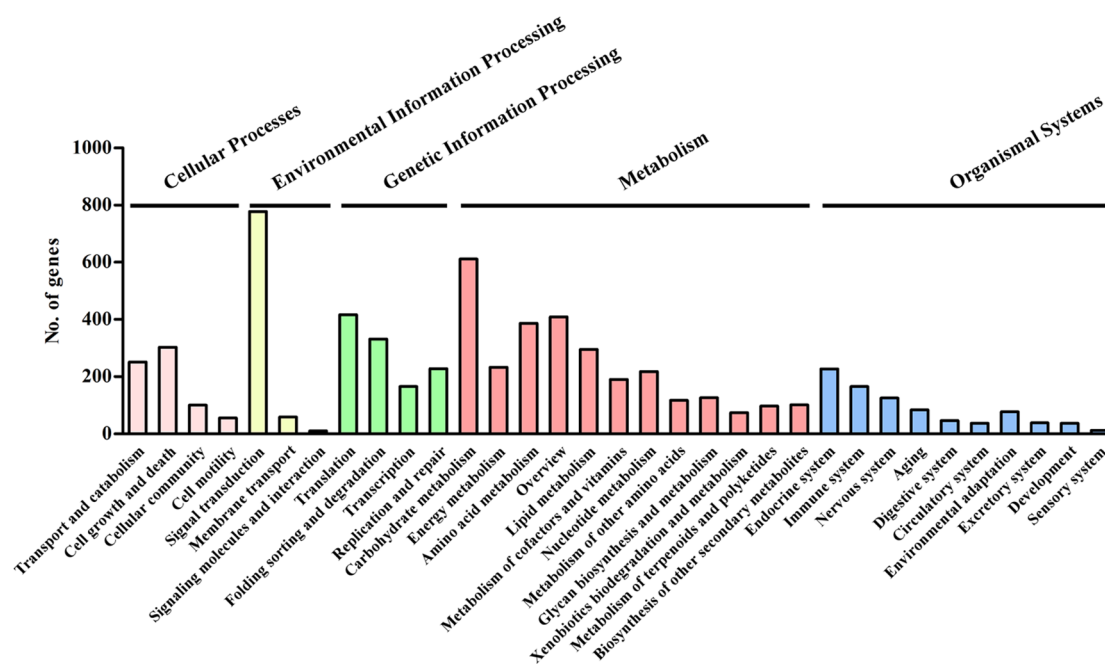


Figure 3. KEGG classifications of unigenes.

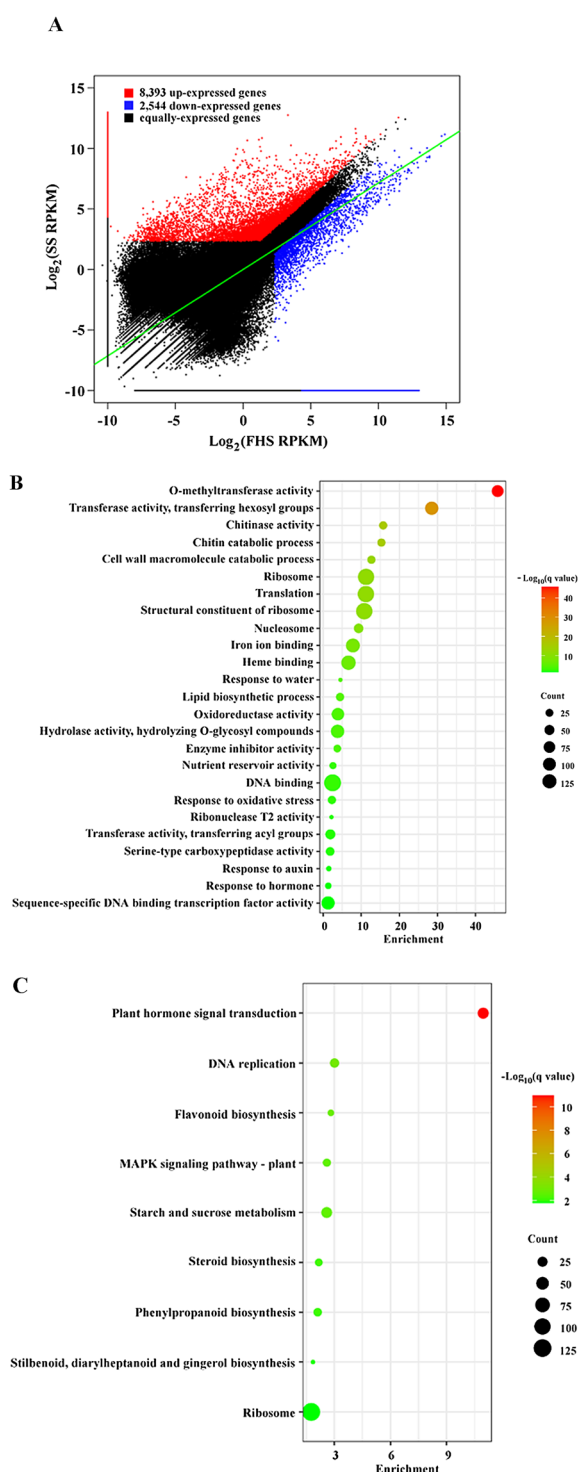


Figure 4. Differentially expressed genes between the freshly harvested seeds and stratified seeds. (A) The distribution of differentially expressed genes. The red points represent the up-regulated unigenes, and the blue points represent the down-regulated. The black points indicate that the unigenes have no expression difference between FHS and SS. (B) GO classification of differentially expressed genes. (C) KEGG pathways enrichment analysis of differentially expressed genes.

Thus, the DEGs involved in signaling and metabolism pathways were detailly analyzed.

DEGs involved in signaling pathways

The above GO and KEGG enrichment analysis highlighted that phytohormones, ROS and transcription factors are involved in the signal transduction during dormancy release in *P. polyphylla*. In which, a total of 45 DEGs related to phytohormone signaling were identified. For example, 30 DEGs (28 up-regulated and 2 down-regulated) were involved in IAA signaling pathway, including three transport inhibitor response 1 (*TIR1*), one indole-3-acetic acid-amido synthetase (*GH3*), and 26 auxin-responsive genes (7 for *SAUR*, 8 for *AUX/IAA*, and 11 for *ARF*) (Figure 5A). Ten DEGs (7 up-regulated and 3 down-regulated) were involved in ABA signaling pathway, including 3 for ABA receptors (*PYR/PYL*), 1 for protein phosphatase 2C (*PP2C*), 4 for ABA-insensitive 5 (*ABI5*), and 2 for ABA-responsive element binding factors (*ABF*) (Figure 5B). Five up-regulated DEGs were involved in GA signaling pathway, including 2 for DELLA proteins (*SLR1*), 1 for GA receptor (*GID1*), and 2 for transcription factors (*GAMYB*) (Figure 5C). Moreover, a total of 53 other transcription factor DEGs (28 up-regulated and 25 down-regulated) were detected, including 24 for *AP2/ERF*, 9 for *WRKY*, 8 for *bZIP*, 5 for *HOX*, 3 for *DREB*, 2 for *MADS* and 2 for *HSF* (Figure 5D). Additionally, a total of 23 DEGs related to ROS signaling were identified. Of them, four *POD* genes were down-regulated while three *CAT*, one *SOD* and the other 15 *POD* genes were up-regulated (Figure 5E). To validate the reliability of RNA-seq results, seven randomly selected DEGs related to signaling transduction were conducted for expression qualification using qRT-PCR approach. The expression patterns of these genes were generally consistent with the results of transcriptome data (Figure 5F, G).

DEGs involved in carbohydrate metabolism

The above KEGG enrichment analysis highlighted that carbohydrate metabolism pathway involved in dormancy release in *P. polyphylla*. According to the KEGG annotation result, a total of 29 DEGs (23 up-regulated and six down-regulated) were detected involving in starch and sucrose metabolism pathway; these DEGs were mapped and visualized onto the KEGG pathway graphs (map00500, starch and sucrose metabolism; Figure 6A, B). For example, two α -amylase (*AMY*) genes were up-regulated while two β -amylase (*BAM*) genes were down-regulated during dormancy release. Most of β -glucosidase (*Bglb/Bglu/Bglx*) and endoglucanase (*Glu*) were up-regulated during dormancy release in *P. polyphylla*. Moreover, each two genes for starch synthase (*glgA*) and trehalose 6-phosphate phosphatase (*otsB*) were up-regulated and down-regulated, respectively, in

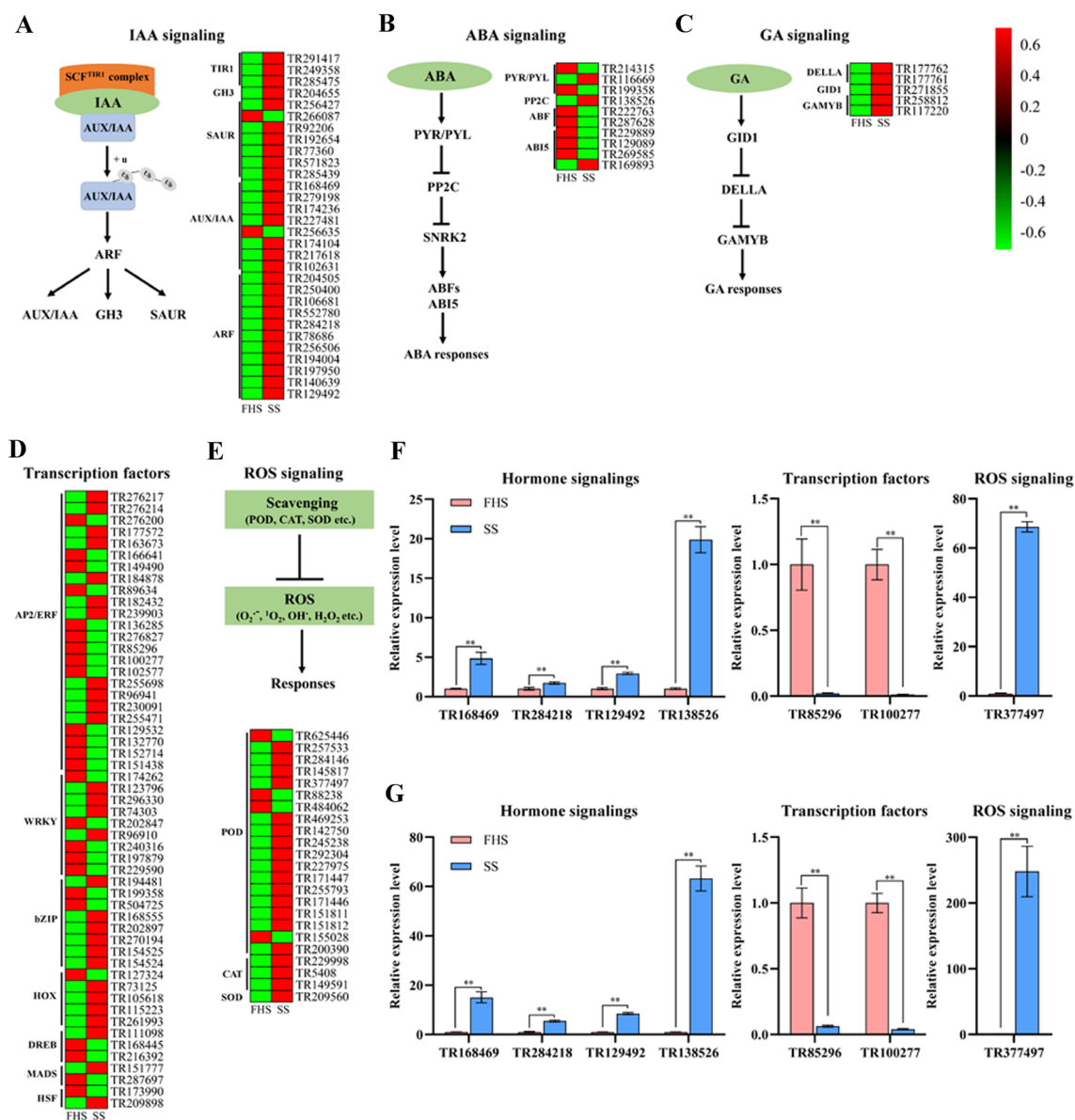


Figure 5. Differentially expressed genes related to signal transduction between the freshly harvested seeds and stratified seeds. Differentially expressed genes related to the IAA signaling (A), ABA signaling (B), GA signaling (C), transcription factors (D) and ROS signaling (E). Heatmap represent the gene expression levels (RPKM values) in the freshly harvested seeds and stratified seeds. The relative expression levels of seven differentially expressed genes related to hormone signaling, transcription factors and ROS signaling were calibrated against those of the *Actin* gene (F) and *GAPDH* gene (G) quantified by qRT-PCR, respectively. ** indicates significant differences between the freshly harvested seeds and stratified seeds at the 1% level.

dormancy release by warm stratification in *P. polyphylla*. Additionally, several other DEGs related to starch and sucrose metabolism, including hexokinase (*HK*), α -glucosidase (*malZ*), 1,4- α -glucan branching enzyme (*glgB*), glucan endo-1,3- β -glucosidase (*GN1_2_3*), fructokinase (*scrK*), and trehalose 6-phosphate synthase (*TPS*), were detected for dormancy release. To validate the reliability of RNA-seq results, seven randomly selected DEGs related to starch and sucrose metabolism were conducted for expression qualification using qRT-PCR approach. The expression patterns of these genes were generally consistent with the results of

transcriptome data (Figure 6C, D).

Discussion

Seeds with morphophysiological dormancy require a period of cold and/or warm stratification to break dormancy in plants. Seed dormancy of *P. polyphylla* is a typical morphophysiological dormancy type (Qi et al. 2013). Previously, a constant exposure to warm conditions (around 20°C) promotes the early seed germination and rapid radical growth of *P. polyphylla* (Liao et al. 2019). Similarly, seed dormancy

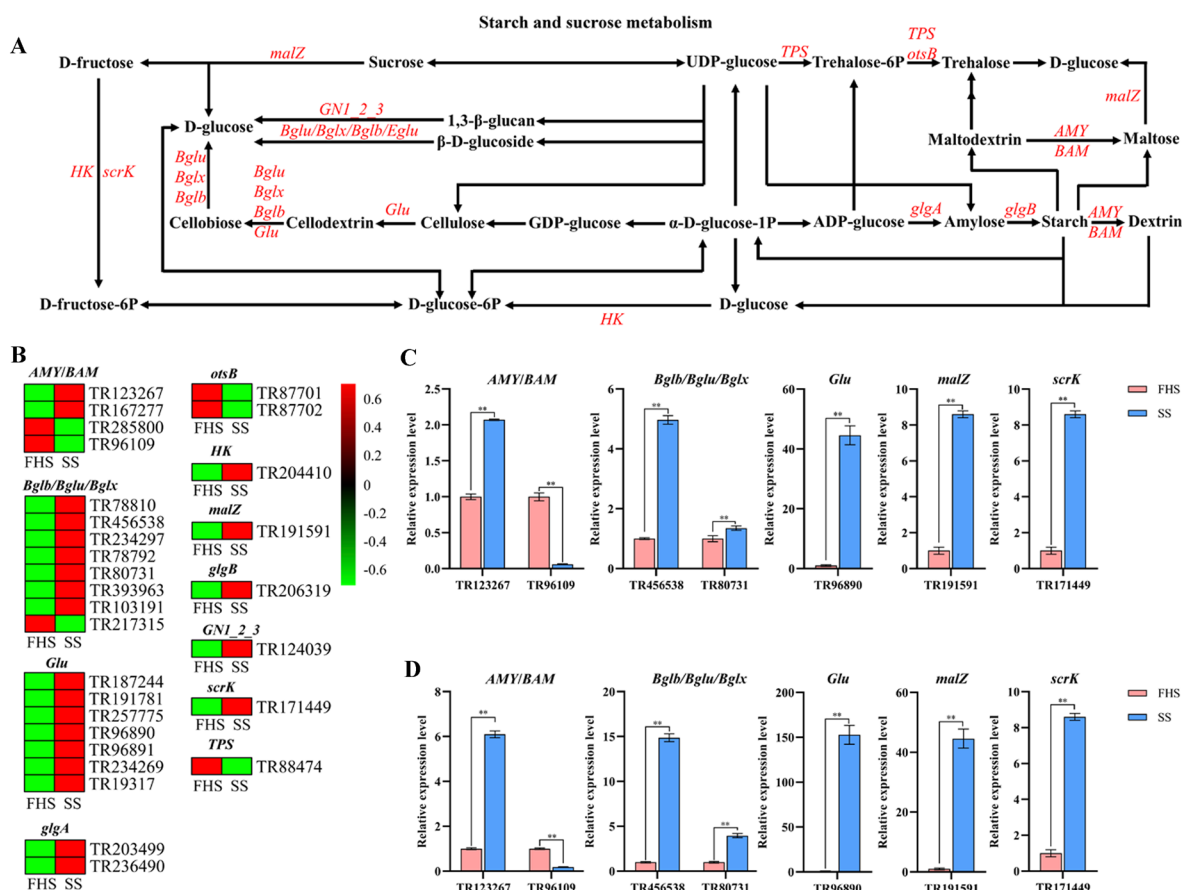


Figure 6. Differentially expressed genes related to carbohydrate metabolism between the freshly harvested seeds and stratified seeds. (A) Differentially expressed genes related to starch and sucrose pathway. The diagram was manually drawn according to the KEGG pathway map (map00500, starch and sucrose metabolism, <https://www.genome.jp/pathway/map00500>). (B) Heatmap represent the gene expression levels (RPKM values) in the freshly harvested seeds and stratified seeds. The relative expression levels of seven differentially expressed genes related to starch and sucrose metabolism were calibrated against those of the *Actin* gene (C) and *GAPDH* gene (D) quantified by qRT-PCR, respectively. ** indicates significant differences between the freshly harvested seeds and stratified seeds at the 1% level.

of *P. polyphylla* can be effectively released by warm stratification at 20°C for 90 days in this study. In order to reveal the mechanisms of dormancy release of *P. polyphylla* during a warm stratification, we performed a de novo transcriptomes of dormant and germinated seeds. Finally, a total of 90,246 unigenes were identified, while only 31.12% of unigenes were annotated in the public databases. These results are similar with previous study in *P. polyphylla* (Liao et al. 2019). It is possible that the genome and EST information are insufficient for *P. polyphylla*. Our further GO and KEGG analysis showed that the most abundant of genes associated with signal transduction and carbohydrate metabolism. Thus, the details of DEGs involved in signal transduction and carbohydrate metabolism during dormancy release were focused in this study.

It has been well known that phytohormones especially ABA and GA play a crucial role in controlling seed dormancy and germination (Nonogaki et al. 2014; Yano et al. 2013). Previously, a total of 136 DEGs were identified involving in phytohormone (ABA, GA, IAA,

CK, JA, SA and ET) signaling pathways during warm stratification in *P. polyphylla* (Liao et al. 2019). Similarly, we observed that the mainly IAA, ABA, and GA related DEGs involved in dormancy release in *P. polyphylla* during warm stratification. The higher expression of *ABI5* has been detected in sorghum cultivars with deeper seed dormancy (Rodríguez et al. 2009). The mutation of rice *PYL1* and *PYL12* exhibited significant defects in seed dormancy (Miao et al. 2018). GA promotes seed germination through degradation of DELLA proteins, which repress GA activated responses and regulate seed dormancy (Tyler et al. 2004). Moreover, *AtARF10/16* act as upstream of the ABA signaling by controlling *AtABI3* expression in Arabidopsis, and *Atarf10arf16* mutant showed significantly decreased seed dormancy (Liu X et al. 2013). IAA signaling repressor *AtIAA8* enhances seed germination through the inhibition of *AtABI3* expression in Arabidopsis (Hussain et al. 2020). Rice *OsSAUR33* regulates seed germination through sugar signaling pathway (Zhao et al. 2021). In this study, we speculated that dormancy release is regulated by ABA signaling

pathway via down-regulating *ABI5* and *PYR/PYL* expression, and by GA signaling pathway via up-regulating *DELLA* expression, as well as by IAA signaling pathway via up-regulating *ARF*, *AUX/IAA* and *SAUR* expression in *P. polyphylla* during warm stratification. However, how these genes regulate seed dormancy needs to be investigated in the future.

The accumulation of ROS not only leads to cell injury and disturbances but also functions as a signaling molecule during seed germination (Bailly 2004). In plant, the antioxidative mechanisms are important for the regulating ROS accumulation. Levels of antioxidant compounds, such as ascorbate, glutathione, and peroxidases, increase during seed germination (De Gara et al. 1997; Tommasi et al. 2001). In which, peroxidases are evolutionarily conserved antioxidant enzymes encoded by a large multi-gene family (Mei et al. 2009). Seed germination requires the specific peroxidase activity to facilitate cell wall loosening during imbibition (Jemmat et al. 2020). Peroxidase activity has been shown to be associated with endosperm weakening and radicle emergence in tomato (Morohashi 2002) and lettuce (Zhang et al. 2014). The activity of peroxidase was significantly increased in imbibed seeds by cold stratification compared to that in freshly harvested seeds in rice (Yang et al. 2019). Similarly, we observed that the higher peroxidase expressions occurred in dormancy release in *P. polyphylla*, suggesting dormancy release might be via peroxidase-mediated cell wall loosening (Goggin et al. 2011). Moreover, the involvement of ROS in seed dormancy and germination might be through the regulation of ABA and GA metabolisms in seeds (Anand et al. 2019). The roles of ROS on the early signaling responses of dormancy release need to be further investigated in *P. polyphylla*.

The TFs of *AP2/ERF* are key genes serving in ethylene signaling transduction pathway (Lai et al. 2021). Overexpression of soybean *AP2/ERF* gene *GmSGR* exhibited reduced ABA-inhibition of seed germination in *Arabidopsis* (Wang et al. 2008), while overexpression of *AtERF15* was hypersensitive to ABA at the germination stage (Lee et al. 2015). The *WRKY* TFs regulate seed germination involving in ABA pathway (Rushton et al. 2010). In rice, *OsWRKY29* negatively regulates seed dormancy by directly repressing the *OsABF1* and *OsVP1* expression (Zhou et al. 2020). Overexpression of maize *ZmWRKY65* enhances the sensitivity to ABA during seed germination (Huo et al. 2021). In many plants, the *bZIP* TFs have been shown to function in the regulation of seed germination (Nijhawan et al. 2008). Four *bZIP* TFs, *OsbZIP23*, *OsbZIP66*, *OsbZIP72*, and *OsbZIP09*, regulate seed germination via affecting ABA signaling in rice (Song et al. 2020; Zhu et al. 2021). Previously, the TFs families of *ERF*, *BHLH*, and *HD-ZIP* were identified by transcriptome in the early germination stage in rice,

while *WRKY*, *TCP*, and *NAC* families were involved in the late germination stage (Wei et al. 2015). Similarly, we found that 53 TFs, such as *AP2/ERF*, *WRKY*, and *bZIP*, were differentially expressed between germinating seeds and dormant seeds in *P. polyphylla*. These observations imply that these TFs play an important role in dormancy release during warm stratification. TFs can regulate the expression of their target genes either individually or cooperatively, and the regulation can be positive or negative. In this study, different members of the same TF family showed different expression patterns between germinating seeds and dormant seeds in *P. polyphylla*. This result implied that different members of the same TF family have distinct biological functions or regulatory mechanisms in dormancy release. However, the roles of here identified TFs on dormancy release need to be further investigated in *P. polyphylla*.

With the absorption of water, seed metabolic activities gradually increase and the storage materials such as starch, protein, and lipids begin to decompose into small and soluble molecules to support the early embryo growth (Dal Degan et al. 1994). The degraded starch is absorbed by the scutellum, and sucrose is then synthesized and transported to the embryo or seedling growth (Aoki et al. 2006). Previous observations showed that the expression of *OsGAMYB* and α -amylase genes was significantly higher in imbibed seeds by cold stratification relative to that in freshly harvested seeds in rice (Yang et al. 2019). Overexpression of wheat α -amylase type 2 *TaAMY2* led to an absence of seed dormancy due to ABA insensitivity (Zhang Q et al. 2021). Similarly, we observed that *GAMYB* and α -amylase genes were significantly upregulated during dormancy release by warm stratification in *P. polyphylla*. We speculated that *GAMYB* might bind to the α -amylase promoter and activate its transcription during warm stratification. Moreover, endoglucanases (*Glu*) and β -glucosidases (*Bglb/Bglu/Bglx*) are the key enzymes that catalyze cellobiose synthesis from cellulose producing D-glucose (Zhang M et al. 2021). Previously, rice *Os4BGlu10*, *Os6BGlu24*, and *Os9BGlu33* has been reported involving in seed germination (Ren et al. 2019), and the expression of β -glucosidase and endoglucanase involved in dormancy release in wheat (Zhang M et al. 2021). Similarly, several β -glucosidase (*Bglb/Bglu/Bglx*) and endoglucanase (*Glu*) genes were differentially expressed in dormancy release of *P. polyphylla* in this study. These results demonstrated that starch and sucrose metabolism might play important roles on dormancy release by warm stratification in *P. polyphylla*.

In summary, a comprehensive transcriptome was performed to reveal the signaling and starch metabolism regulators involving in dormancy release of *P. polyphylla* by warm stratification in this study. We observed that the signaling associated DEGs including hormone-, ROS-

and TF-related genes and the carbohydrate metabolism associated DEGs including α -amylase, β -glucosidase, and endoglucanase genes might play important roles in dormancy release in *P. polyphylla*. The identified genes provide a valuable resource to investigate the molecular mechanism of dormancy release in *P. polyphylla* in the future.

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Author contributions

BY and FX planned the research. BY, SS, SL and JZ performed all important experiments. BY and FX analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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