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Research Article

# Identification of a key signaling network regulating perennating bud dormancy in *Panax ginseng*

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ABSTRACT

*Background:* The cycle of seasonal dormancy of perennating buds is an essential adaptation of perennial plants to unfavorable winter conditions. Plant hormones are key regulators of this critical biological process, which is intricately connected with diverse internal and external factors. Recently, global warming has increased the frequency of aberrant temperature events that negatively affect the dormancy cycle of perennials. Although many studies have been conducted on the perennating organs of *Panax ginseng*, the molecular aspects of bud dormancy in this species remain largely unknown.

*Methods:* In this study, the molecular physiological responses of three *P. ginseng* cultivars with different dormancy break phenotypes in the spring were dissected using comparative genome-wide RNA-seq and network analyses. These analyses identified a key role for abscisic acid (ABA) activity in the regulation of bud dormancy. Gene set enrichment analysis revealed that a transcriptional network comprising stress-related hormone responses made a major contribution to the maintenance of dormancy.

*Results:* Increased expression levels of cold response and photosynthesis-related genes were associated with the transition from dormancy to active growth in perennating buds. Finally, the expression patterns of genes encoding ABA transporters, receptors (*PYRs/PYLs*), *PROTEIN PHOSPHATASE* 2Cs (*PP2Cs*), and *DELLAs* were highly correlated with different dormancy states in three *P. ginseng* cultivars.

*Conclusion:* This study provides evidence that ABA and stress signaling outputs are intricately connected with a key signaling network to regulate bud dormancy under seasonal conditions in the perennial plant *P. ginseng*.

## 1. Introduction

Korean ginseng (*Panax ginseng*) has long been used as a medicinal herb in the treatment of various diseases, including cancer, fatigue, and diabetes [1–3]. Its medicinal properties are primarily attributed to phenolic/alcohol-carbohydrate compounds known as saponins or ginsenosides, which accumulate to high levels in the root tissue [3]. Ginseng is a slow-growing perennial crop that requires at least 3–6 years of cultivation to develop its highly valued storage roots; in this time, the root increases in size and saponin content [4]. Therefore, ginseng exhibits a seasonal growth/dormancy cycle throughout the cultivation process, with perennating buds maintaining dormant in the cold winter and succeeding vigorous growth and development from spring to fall [3,4].

The optimal growth temperature range for *P. ginseng* cultivated under semi-shade conditions is generally between 20 and 25 °C. Recently, ginseng cultivation has become increasingly difficult due to global warming [5–7], which causes the temperature in ginseng

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cultivation areas to frequently deviate from the normal range. Abnormal high temperature in early spring can accelerate the breaking of bud dormancy, resulting in the early emergence of ginseng sprouts from the perennating buds [8]. However, the temperature fluctuations in early spring pose a serious threat to ginseng growth when abnormally warm periods are followed by sudden spring frosts [4,9,10]. Cold stress at this stage can cause severe damage, including chlorosis and death, resulting in negative effects on photosynthesis and overall ginseng growth and development [9,10]. Consequently, external temperature condition in early spring plays a key role in determining the life cycle and dormancy of the perennating buds, meaning this is one of the critical environmental factors for consistent ginseng yields and quality [5]. Although global warming and extreme weather occurrences present increasingly significant challenges to sustainable cultivation of ginseng, the physiological and genetic mechanisms involved in bud dormancy break of this important perennial crop remain largely unknown.

Plant hormones are central regulators of growth and development throughout a plant's life cycle. Abscisic acid (ABA) is a key regulator that is closely involved in several developmental processes, including seed germination, vascular development, and stress responses [11,12]. In perennial plants, ABA plays a central role in initiating the onset of bud dormancy by creating a symplastic blockage that prevents intercellular communication and ingress of growth-promoting signals [13]. Conversely, gibberellins (GA) are plant growth-promoting hormones that play essential roles in dormancy release and flowering in many plant species [14–16]. Recent research shows that exogenous GA enhances both root secondary growth and saponin content in *P. ginseng* [17,18]. GA also increases seed desiccation and germination rates in ginseng; these are both useful traits for shortening the breeding process [19]. Such studies indicate that GA function is broadly conserved across plant species and regulates important developmental traits associated with increased ginseng productivity. Understanding and characterizing the molecular network of ABA-GA interactions is necessary to identify target genes for genetic manipulation, thereby facilitating an increase in ginseng production and quality [20]. GA also positively regulates the breaking of bud dormancy in ginseng [10,21]. Since breaking dormancy is a temperature-sensitive process [4,22], this suggests that GA may play a role in the signaling crosstalk between cold responses and bud break. The complex interactions between ABA and GA, often characterized as an antagonistic relationship, have far-reaching implications for plant dormancy and development. These hormonal interactions are precisely balanced and contribute to plant development and fitness in response to diverse environmental cues [23]. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are also involved in regulating bud dormancy and dormancy release in perennial plants. JA and SA inhibit release from dormancy and suppress bud break, either by interaction with ABA or with GA [24]. ET plays a dual role in bud dormancy, working synergistically with ABA to induce dormancy but antagonistically with it to end dormancy [25,26]. These indicate that a complex hormonal interaction mechanism is likely involved in the control of bud dormancy in perennial plants. However, the functional signaling network responsible for regulating bud dormancy in a perennial *P. ginseng* has not been investigated thus far.

In this study, we conducted a comparative transcriptome analysis in perennating buds of three different ginseng cultivars, Geumsun (GS), Yeonpoong (YP), and Chunpoong (CP), which are characterized by different bud dormancy-release phenotypes under low temperature conditions in early spring. GS displays an early dormancy-release phenotype relative to YP (moderate bud dormancy) and CP (strong bud dormancy). Integrated bioinformatic analyses and physiological approaches for the investigation of perennating bud dormancy control in these cultivars revealed that a transcriptional network comprised of growth and dormancy-related hormones, including ABA, GA, JA, SA, and ET, was significantly connected to cold responses and photosynthesis to fine-tune the bud dormancy. In particular, transcriptional regulation of genes associated with key dormancy signaling pathways, encoding ABA receptors (*PYLs*), PROTEIN PHOSPHATASE 2C (*PP2C*), *DELLAs* and *DOG1*, is strongly linked with the perennating bud dormancy control of *P.* ginseng cultivars. In addition, the quantification of dormancy-related hormones, including ABA, GA, JA, and SA using an HPLC-QQ mass spectrometer revealed the significant roles of the antagonistic interaction between ABA and GA signaling outputs in fine-tuning the process of bud dormancy release. The novel insight provided by these results advances knowledge and understanding at the molecular level of the regulation of perennating bud dormancy in *P. ginseng*.

## 2. Materials and methods

### 2.1. Plant materials and bud dormancy test

*P. ginseng* cultivar samples, 'Guemsun (GS)', 'Yeonpoong (YP)', and 'Chunpoong (CP)', were provided by the National Institute of Horticultural and Herbal Science of Korea. To determine the bud dormancy breaking times of *P. ginseng* cultivars, the shoot emergency rate on the soil were observed at the field of the Department of Herbal Crop Research in Eumseong (for six-years-old ginsengs) and Ginseng & Medicinal Plant Research Institute in Keumsan (for three-years-old ginsengs), South Korea on April 4 and March 29, 2022, respectively (dormant release stage of GS). To validate the expression patterns of selected dormancy-related genes in different bud dormancy cultivars (six-years-old GS, CP, Kopoong (KP), Sunwon (SW), Cheonryang (CR), Cheongsun (CS), and Korea (KR), perennating buds were sampled from the field of the Ginseng & Medicinal Plant Research Institute in Keumsan on March 29, 2024 (dormant stage of all cultivars). To determine effects of GA and ABA on *P. ginseng* bud dormancy breaking, a Mock (DMSO), 10 μM $GA_3$ (Duchefa, Haarlem, Nederland), or 50 μM ABA (Sigma, St. Louis, USA) was treated once a day for a week. One-year old *P. ginseng* seedlings were grown at 22–23 °C with a 16 h/8 h (light/dark) cycle into the ginseng cultivation soil medium in green house.

### 2.2. RNA extraction and sequencing analysis

Total RNA was extracted from the perennating bud samples from three cultivars, GS, YP, and CP, using an Easy-Spin RNA Extraction Kit (iNtRON, Seongnam, Korea) according to the manufacturer's instructions. The TruSeq Stranded mRNA Library Prep Kit (Illumina, Inc., San Diego, CA) was used to produce total RNA for RNA-seq libraries using three biological replicates. End repair, single 'A' addition, and adapter ligation were performed on cDNA. PCR amplification was used to purify and enrich the libraries, which were subsequently sequenced on the Illumina HiSeq 4000 platform to obtain 100-bp paired-end reads. The raw data were submitted to the NCBI Short Read Archive database with the accession number PRJNA_1011468.

### 2.3. Differentially expressed genes analysis

Prinseq-lite version (0.20.4) was used to clean paired-end reads with the following parameters: min_len is 50, min_qual_score is 5, min_qual_mean is 20, derep is 14, trim_qual_left is 20, and trim_qual_right is 20 [27]. Bowtie 2 was used to match clean paired-end reads from each sample to the ginseng reference sequences [28]. For each transcript, read counts and TMM-normalized TPM (trimmed mean of M value-normalized transcripts per million) values were obtained using the RSEM 1.3.0 program [29]. The negative binomial dispersion across conditions was calculated using EdgeR version 3.16.5 for differential gene expression analysis [30]. Genes were considered significantly differentially expressed if they had a P 0.05 false discovery rate (FDR)-adjusted [31]. cDNA was produced using the TOP scriptTM RT Dry MIX (Cat. no. RT200, Enzynomics, Daejeon, Korea) to validate the RNA-seq data. To validate the transcripts level of Gene Set Enrichment Analysis (GSEA) transcriptome data that is down- or up-regulated in GS, YP, and CP, quantitative real-time reverse transcription-polymerase

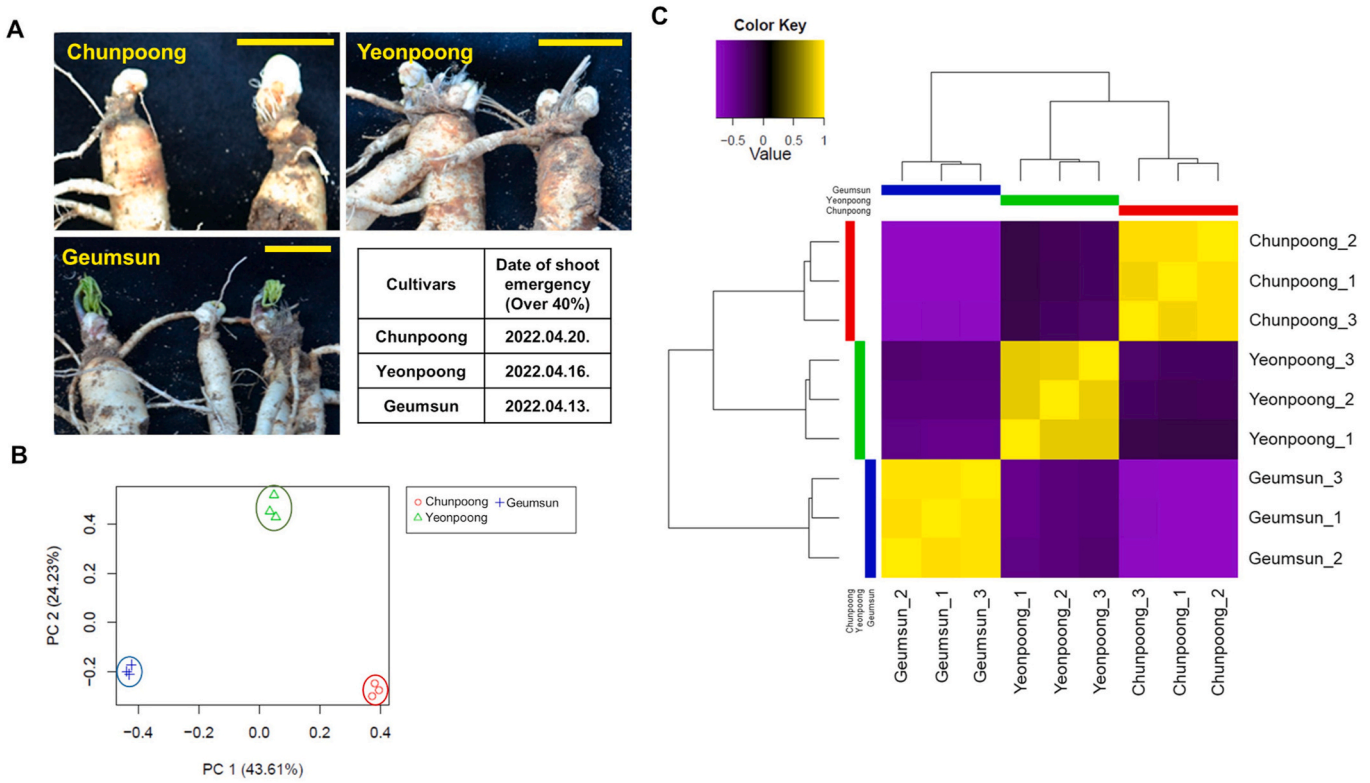


**Fig. 1.** Characterization of perennating bud dormancy phenotype in Guemsun (GS), Yeonpoong (YP), and Chunpoong (CP) cultivars. (A) Phenotype of perennating buds on April 4, 2022, and dates of 40 % or more shoot emergence for the 6-years old *P. ginseng* cultivars, Guemsun, Yeonpoong, and Chunpoong. Scale bar = 4 cm. (B, C) RNA-seq analysis for different perennating buds of Fig. 1A. Principal component analysis (PCA, B) and hierarchical clustering analysis of three ginseng samples (C).

chain reaction (qRTPCR) was done using the KOD SYBR qRT MIX (Cat. no. QKD-201, TOYOBO, Osaka, Japan). The internal control for qRT-PCR was *PgACT*. All used primers are listed inSupplementary Table 1. The Ginseng Genome Database (http://ginsengdb.snu.ac.kr/index.php) was used to get Ginseng reference sequences.

### 2.4. Functional annotation and network analysis

For functional identification of differentially expressed genes, the BLAST tool was employed with an e-value threshold of 1 $E^{-5}$ against the Arabidopsis thaliana protein database. DAVID was used to perform gene ontology (GO) term enrichment analysis, and the enriched GO term was selected by the Fisher Exact test ($P < 0.05$) [32,33]. GSEA was used to further investigate enriched GO genes, as outlined in our earlier work [34]. The network analysis was carried out in Cytoscape (version 3.10.0) using the GeneMANIA program (version 3.5.2) [35,36]. The bar graph was created in Prism 6 (GraphPad, Boston, USA). The red line in the enrichment graphic depicts the gene subset that contributed the most to the enrichment score (ES). The ranking list metric in the graphic quantifies the relationship between a gene and a plant's phenotype. Positive values in the ranking list indicate genes increased in GS perennial bud samples with a red color gradient, whereas negative values show genes downregulated in GS perennial bud samples.

### 2.5. Phytohormones analysis

Phytohormones in ginsengs were quantified according to the methods of Schäfer et al. (2016), with modifications [37,38]. Samples were prepared as previously described [39]. Briefly, approximately 100 g of each frozen plant sample was homogenized twice with two steel beads in a 1600 MiniG (SPEX Sampleprep, NJ, USA) at 1300 rpm for 30 s each. The samples were evaporated to near dryness in a a HyperVAC (Hanil Scientific Inc., Daejeon, Korea) at 30 °C. The dried samples were dissolved in 500 ml 70 % (v/v) methanol: water for analysis with the HPLC-QQ mass spectrometer (LC-MS-8050, Shimadzu, Tokyo, Japan). Later, 1 μL (but samples for GA injected 5 μL) of the extracts was injected onto a C18 column (UPLC BEH, 1.7 μm particle size, 100 mm length x 2.1 mm inner diameter, Waters, Ireland) and separated using a HPLC system (Shimadzu). Solvent A consisted of deionized water containing 0.1 % (v/v) acetonitrile, 0.05 % formic acid. Solvent B consisted of 100 % MeOH. The following gradient conditions were used for the chromatography: 0.01–0.5 min 5 % B; 0.6–6.7 min linear gradient 50–95 % B; 6.7–8.7 min, 95 % B; and re-equilibration at 5 % B for 1 min. The flow rate was 400 μL/min. The phytohormones were detected in negative/-positive electrospray ionization mode. The amounts of JA, SA, gibberellic acid 3 ($GA_3$) and ABA were normalized by the exact fresh mass of plant materials and internal standards ($D_6$-JA, $D_4$-SA, 4-Methylumbelliferone (4-MU) and $D_6$-ABA, respectively) of each phytohormone. The levels of eluted analytes were determined by the mass spectrometer with the following molecular mass of ionized molecular fragments for the standard phytohormones: JA, precursor ion $m/z = (-)$ 209.20, product ion $m/z$ = 59.10 (CE 12.0) and 41.10 (CE 40), RT: 4.022 min; $D_6$-JA, precursor ion $m/z = (-)$ 215.20, product ion $m/z$ = 59.10 (CE 13.0) and 41.10 (CE 43.0), RT: 3.365 min; SA, precursor ion $m/z = (-)$ 137.00, product ion $m/z$ = 93.10 (CE 17.0) and 65.20 (CE 29.0), RT: 3.311 min; $D_4$-SA, precursor ion $m/z = (-)$ 141.10, product ion $m/z$ = 97.00 (CE 20.0) and 69.15 (CE 29.0), RT: 3.797 min; ABA, precursor ion $m/z = (-)$ 263.20, product ion $m/z$ = 153.30 (CE 11.0) and 219.25 (CE 13.0), RT: 3.365 min; $D_6$-ABA, precursor ion $m/z = (-)$ 269.20, product ion $m/z$ = 159.30 (CE 10.0) and 225.40 (CE 13.0), RT: 2.745 min: $GA_3$, precursor ion $m/z = (-)$ 345.20, product ion $m/z$ = 143.25 (CE 28.0) and 239.35 (CE 16.0), RT: 2.280 min; 4-MU, precursor ion $m/z = (+)$ 177.00, product ion $m/z$ = 77.00 (CE -35.0) and 105.20 (CE -21.0), RT: 2.573 min.

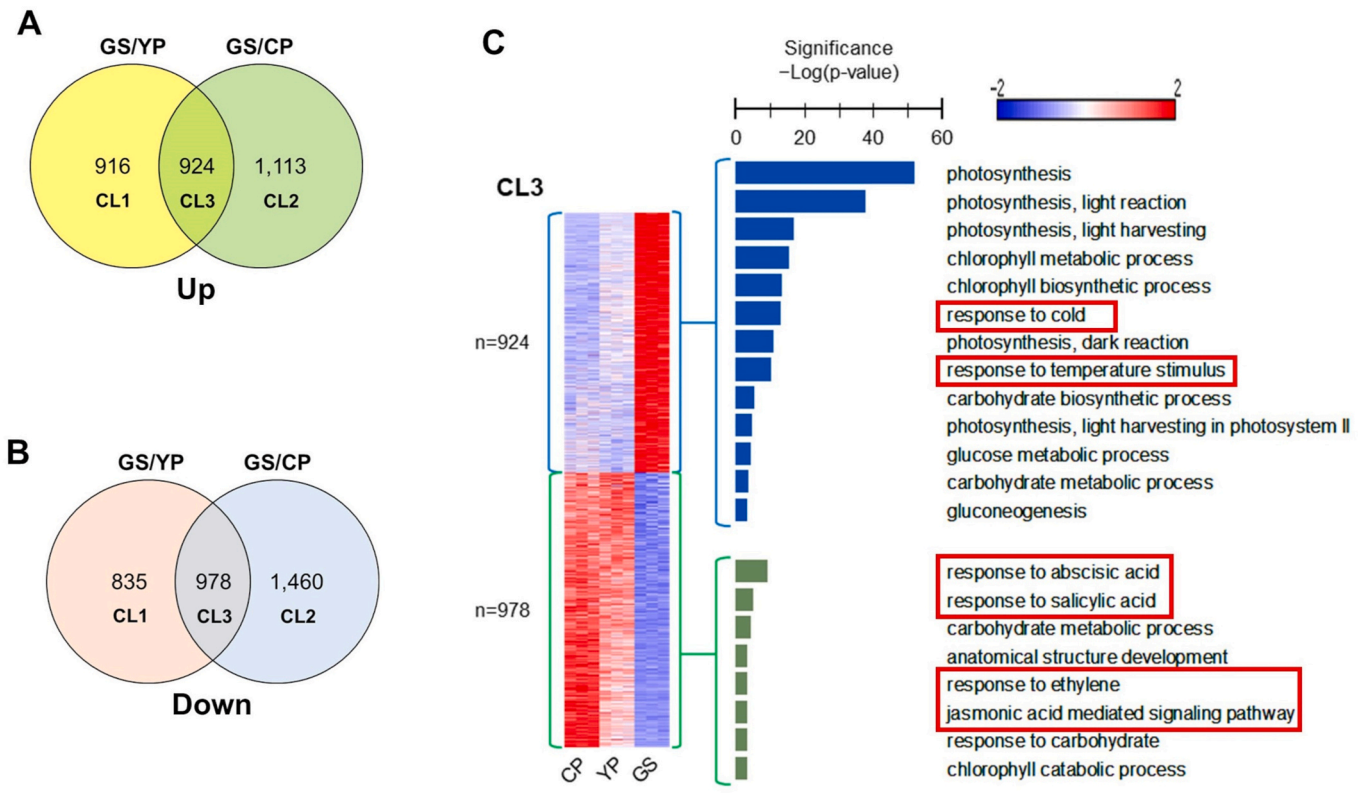


**Fig. 2.** Transcriptome profiling of perennating buds in GS, YP, and CP. (A, B) Venn diagram analysis of visualize GS, YP, and CP dependent (A) up-regulated DEGs and (B) down-regulated DEGs with false discovery rate [FDR] $< 0.05$ and $\geq |1.5|$-fold change. CL indicates clade. (C) GO analysis was performed using the clade 3. GO terms in biological process of level 3 and level 5, with EASE score <0.01, were selected. GS, YP, and CP indicated Geumsun, Yunpoong, and Chunpoong cultivars, respectively.

## 3. Results

### 3.1. Transcriptomic analysis of *P. ginseng* cultivars with differing perennating bud dormancy traits

The selective breeding program for *P. ginseng* in Korea has developed cultivars with diverse phenotypic characteristics [40]. The timing of release from dormancy of perennating buds in *P. ginseng* is one of the key phenotypic traits and this phenotypic difference in the spring have led to the identification of early, mid, and late season ginseng varieties. Guemsun (GS) is a representative early season variety [40]. We reconfirmed its early bud break phenotype by comparing GS with the middle (YP) and late season (CP) varieties in agricultural fields trials in 2022. The point of over 40 % shoot emergence from 6-year-old perennating buds occurred 3 and 7 days earlier in GS than in YP and CP, respectively (Fig. 1A). We also observed an earlier bud break phenotype in GS than in YP in 3-year-old perennating buds in another agricultural field (Fig. S1). These results indicated that control of perennating bud dormancy was regulated by unknown genetic factors in *P. ginseng* cultivars. To investigate the molecular mechanism controlling early breaking of bud dormancy in GS, we performed a transcriptomic analysis on the perennating buds of the three *P. ginseng* cultivars. Principal component analysis and a sample correlation matrix identified sample correlations within and between genotypes for further transcriptomic analysis (Fig. 1B and C). In particular, we identified specific patterns of differential gene expression in the three ginseng cultivars (Fig. 1B and C).

### 3.2. Functional annotation of the transcriptome in three different *P. ginseng* cultivars with differing bud dormancy phenotypes

To investigate the transcriptomic changes associated with the different bud dormancy phenotypes, we conducted in silico bioinformatic comparative analysis on the RNA-seq data from the three cultivars CP, YP and GS. We selected 916 upregulated and 835 downregulated differentially expressed genes (DEGs) in clade 1 (YP-specific DEGs), 1113 upregulated and 1460 downregulated DEGs in clade 2 (CP-specific DEGs), and 924 upregulated and 978 downregulated DEGs in clade 3 (GS-specific DEGs); in all cases, |fold change| $\geq 1.5$ and false discovery rate [FDR] $< 0.05$ (Fig. 2A and B; Table S2). We then performed a gene ontology (GO) enrichment analysis by clustering the comparisons between GS and CP/YP pairing sets. These analyses indicated that dynamic changes in gene expression occurred during the breaking of bud dormancy in *P. ginseng* cultivars (Fig. S2 and Table S2). GO enrichment analysis of the selected DEGs revealed that bud dormancy was significantly associated with the following functional categories: 'transport' (*P*-values of sub-GO terms in the representative category: $P = 0.044$ for auxin transport), 'response to temperature' ($P = 4.8 \times 10^{-8}$ for response to temperature stimulus; $P = 2.2 \times 10^{-9}$ for response to cold), 'photosynthesis' ($P = 1.9 \times 10^{-31}$ for photosynthesis), 'response to hormone' ($P = 0.048$ for JA-mediated signaling pathway; $P = 0.014$ for response to SA; $P = 0.000016$ for response to ABA), 'biosynthetic process' ($P = 0.00063$ for carbohydrate biosynthetic process; $P = 6.7 \times 10^{-7}$ for chlorophyll biosynthetic process), and 'metabolic process' ($P = 0.034$ for JA metabolic process; $P = 0.000008$ for carbohydrate metabolic process) (Fig. S2). In particular, we observed a notable increase in the expression of gene sets associated with pathways related to the cold response and photosynthesis in the GS cultivar (CL3, Fig. 2C). Stress-related hormones, including ABA, SA, JA, and ethylene, were more abundant in the dormant perennating buds of CP and YP (Fig. 2C). These results suggested that signaling pathways associated with cold responses and photosynthesis played important roles in triggering the breaking of bud dormancy and initiating the autotrophic life cycle of the shoot system in *P. ginseng*.

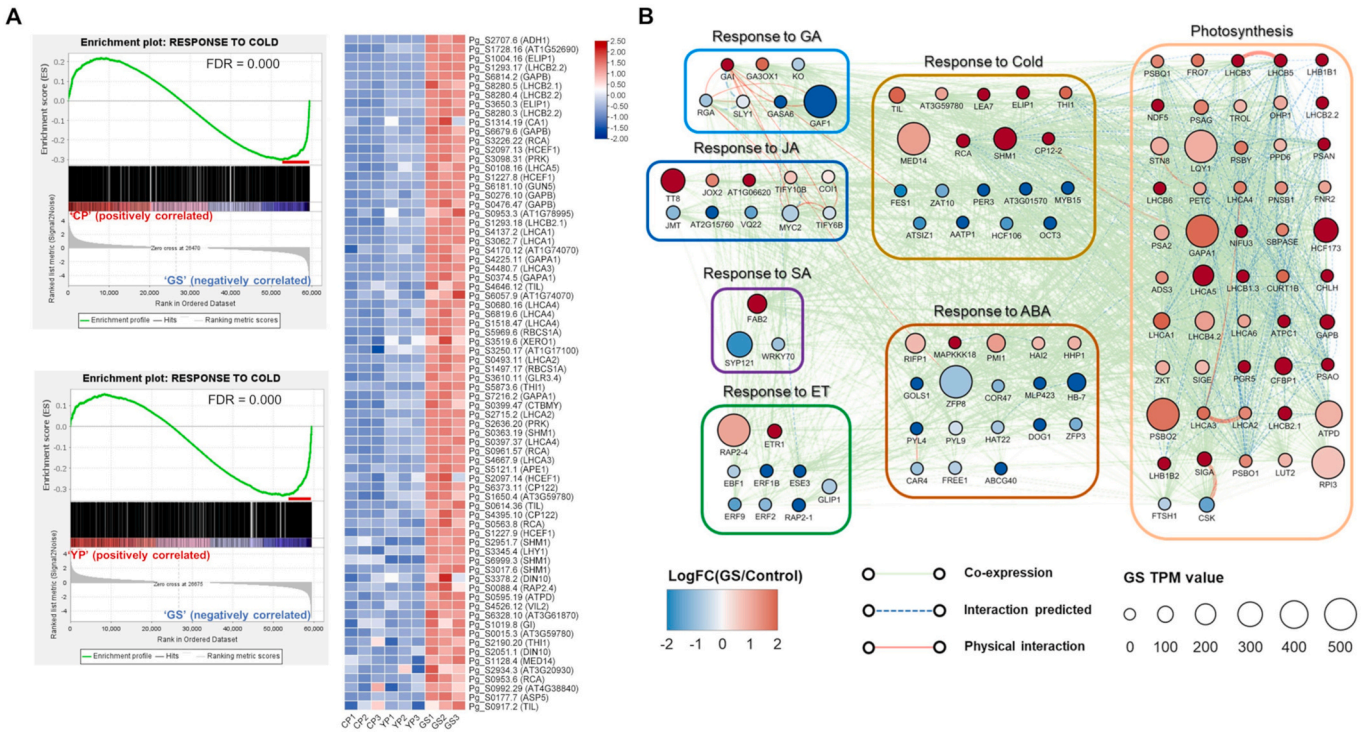


**Fig. 3.** Functional enrichment of cold response related genes in germinating buds of *P. ginseng* and protein-protein interaction (PPI) network analysis of DEGs. (A) Enrichment plot for the response to cold (GO:0009409) in the transcriptome data of GS and CP (upper panel, FDR = 0) or YP (lower panel, FDR = 0.025) perennating bud samples. In the enrichment plot, the red lines (leading-edge subset) represent the gene subset that made the largest contribution to the enrichment score (ES) (false discovery rate [FDR] < 0.05). The ranking list metric in the plot measures the correlation between a gene and genotypes. In the ranking list, positive values indicate genes up-regulated in GS samples, and negative values indicate genes down-regulated in CP or YP samples. The expression heat maps of leading-edge subset genes were presented. (B) Protein-protein interaction (PPI) network analysis of the selected leading-edge subset genes.

### 3.3. *The cold-related pathway plays a central role in breaking bud dormancy in P. ginseng*

We focused next on the functional enrichment analysis of a GO term associated with 'response to cold' in *P. ginseng*. Gene set enrichment analysis (GSEA) confirmed that the GO term 'response to cold' was significantly enriched in the perennial buds of GS relative to both YP and CP (both FDR = 0.0; Fig. 3A). In the GSEA, 107 genes were identified as a crucial leading-edge subset, which was an enriched gene set group leading to enrichment scores (ES) with respect to expression changes (Fig. 3A). These leading-edge subset genes were notably upregulated in germinated buds of GS; they included *EARLY LIGHT-INDUCABLE PROTEIN 1* (*ELIP1*), *TEMPERATURE-INDUCED LIPOCALIN* (*TIL*), *LATE ELONGATED HYPOCOTYL* (*LHY1*), *VERNALIZATION INSENSITIVE 3-LIKE 2* (*VIL2*), and *GIGANTEA* (*GI*), which play critical roles in cold response and tolerance (Fig. 3A). Furthermore, the selected leading-edge subset encompassed numerous genes associated with photosynthesis (Fig. 3A: right panel heatmap) and GSEA showed a significant enrichment of photosynthesis in the GS buds ([FDR] = 0.0, Fig. S3), indicating a link between cold signaling and photosynthesis. To investigate this, we focused on identifying the key signaling network associated with the selected cold response-related leading-edge subset genes during bud dormancy. Using the STRING database (https://string-db.org/) and homology to *Arabidopsis thaliana* genes, we constructed a protein–protein interaction network of the selected cold-response genes. Our network analysis revealed that the cold-response pathway was tightly connected with photosynthesis-related pathways, as well as with seed dormancy and stress-related hormones such as GA, ABA, JA, SA and ET (Fig. 3B). These interaction networks were further validated using GSEA (Fig. 4A and Figs. S3 and Fig. 5). Stress-related hormones. including JA, SA, and ET, were significantly downregulated in GS (Fig. 4A; Figs. S4–6). GSEA also identified the leading-edge subsets of each pathway, which were likely to be involved in regulating perennating bud dormancy (Tables S3 and 4). However, as levels of SA and JA did not differ in the perennating buds of the ginseng cultivars tested (Figs. S5C and D), it appeared unlikely that JA and SA played major roles in regulating bud dormancy. We used qRT-PCR to validate the expression patterns of DEGs associated with selected GO terms (R = 0.79; Fig. S6). These results, together with the co-expression and protein–-protein interaction patterns observed for cold response-related genes (Fig. 3B), highlighted the importance of photosynthesis and hormone signaling pathways during the post-dormancy phase of perennating buds in *P. ginseng*.

### 3.4. *ABA signaling outputs are critical for bud dormancy in P. ginseng*

The essentially antagonistic roles of GA and ABA-mediated signaling pathways for seed or bud germination have been extensively investigated. In particular, *RCAR3 INTERACTING F-BOX PROTEIN 1* (*RIFP1*), *HIGHLY ABA-INDUCED PP2C 2* (*HAI2*) and *HEPTAHELICAL PROTEIN 1* (*HHP1*), positive regulators of the seed germination process that act by direct inhibition of a canonical ABA signaling pathway, were greatly enhanced in the GS cultivar (Fig. 3B) [41–43]. To determine the correlation between ABA signaling activity and bud dormancy, we carried out GSEA on selected DEGs. This revealed that the ABA-related pathway (response to ABA [FDR = 0]) and the ABA-activated signaling pathway ([FDR = 0.025]) were significantly enriched in CP (Fig. 4A), but a GA-mediated signaling pathway was not (Fig. S8A). However, although the response to ABA was enriched in the YP cultivar (moderate bud dormancy), genes related to the ABA-activated signaling pathway showed similar patterns of expression to GS (FDR = 0.212; Fig. S7). These results suggested that ABA signaling output could be a factor in determining the dormant state of perennating buds in *P. ginseng*.

To confirm the functional roles of ABA and GA, we tested the effects

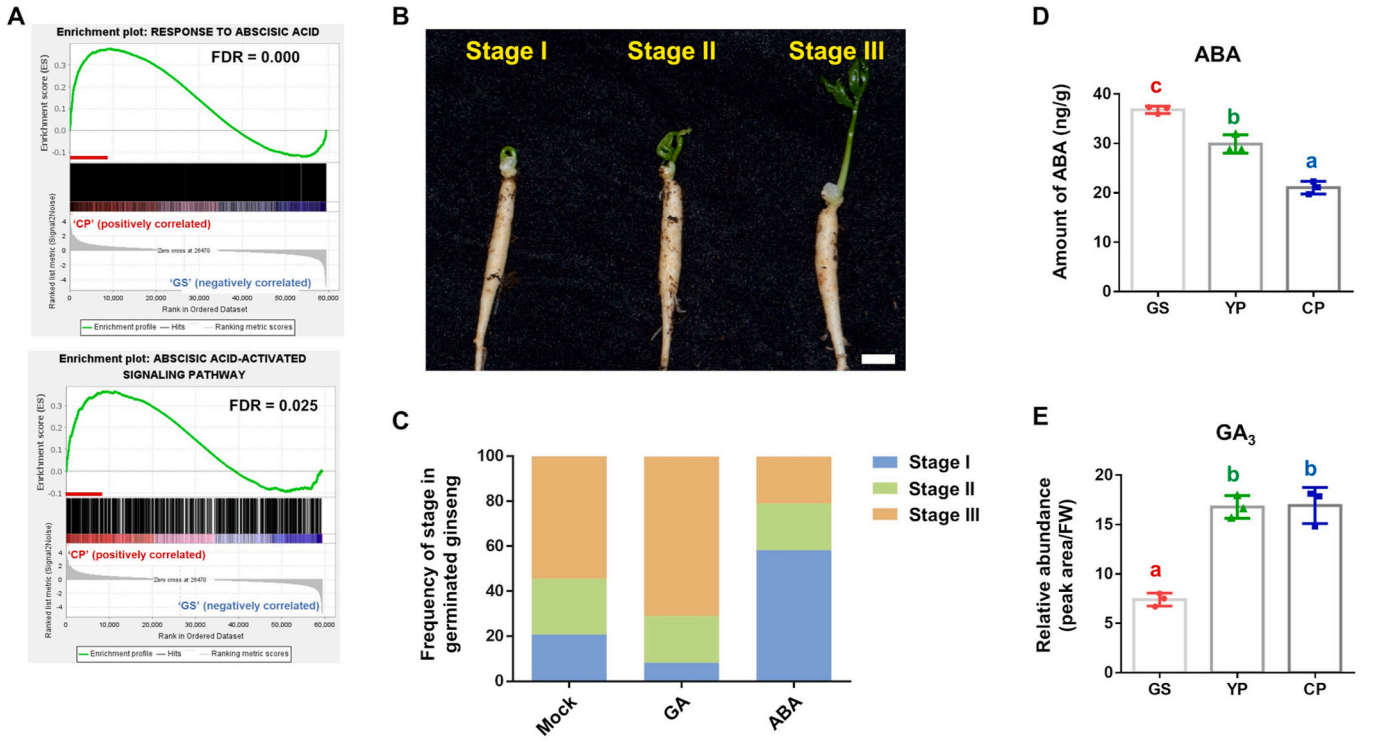


**Fig. 4.** ABA and GA play critical roles in bud dormancy regulation in *P. ginseng*. (A) Enrichment plot for a response to ABA (upper panel, GO:0009737) and ABA-activated signaling pathway (lower pannel, GO:0009738). (B) Developmental stages of bud germination in *P. ginseng*. Scale bar = 1 cm. (C) Measurement of the frequency of developmental stages in perennating buds (one-year-old YP roots) treated with a mock control (Mock), 10 μM $GA_3$ or 50 μM ABA once a day for a week. (D) Measurement of ABA and (E) $GA_3$ contents in the perennating buds of GS, YP, and CP presented in Fig. 1A. Error bars indicate SEM (n = 3, $P < 0.05$, one-way ANOVA with Tukey's multiple range test).

of exogenous application on bud germination. We categorized the germination phenotypes of ginseng buds into three developmental stages (Fig. 4B) and measured the changes in germination rate in response to exogenous GA and ABA (Fig. 4C). As shown in Fig. 4C, GA treatment enhanced the proportion of buds in stage 3 but exogenous ABA greatly reduced the germination rate, compared to a mock-treated control. These results indicated that increased specific ABA-mediated signaling outputs in CP and YP contributed to maintaining bud dormancy. This conclusion was further supported by differences in GO enrichment and GSEA between the cultivars YP (moderate bud dormancy) and CP (strong bud dormancy). GO terms related to cold and stress hormones (ABA, JA, and SA) involved in bud dormancy were highly enriched in CP compared with YP (Fig. S9 and Table S6).

Next, we used HPLC-QQ mass spectrometry to measure endogenous ABA and active $GA_3$ levels in perennating buds of the CP, YP and GS cultivars. Interestingly, ABA levels were lower in YP and CP than in GS, in which bud germination was promoted, but $GA_3$ levels were higher (Fig. 4E), suggesting the hormone metabolism pathway contained a negative feedback loop. This possibility was supported by observing significant enrichment of GA biosynthetic processes in the GS cultivar relative to YP and CP (Fig. S10 and Table S7).

### 3.5. *Differential expression patterns of dormancy-related hormone components in P. ginseng in different states of bud dormancy*

Our comparative transcriptomic analysis shed light on the importance of ABA signaling output for modulating bud dormancy in *P. ginseng*. Furthermore, we found that 22 leading-edge subset genes in an ABA-activated signaling pathway, including both canonical and non-canonical signal transduction components, ABA transporters, and positive regulators for seed dormancy, were significantly reduced in the GS cultivar (Fig. 4A and Fig. S5B). To dissect the molecular mechanisms in ABA-mediated regulation of ginseng bud dormancy in more detail, we examined the expression patterns of ABA-related genes in the three *P. ginseng* cultivars with differing dormancy phenotypes early in the spring season (Fig. 5A). Expression of many vascular tissue-specific ABA exporters (*ATP-BINDING CASSETTE (ABC) SUBFAMILY G 25* (*ABCG25*) and *ABCG31*), influx carriers (*ABCG40* and *ABA-IMPORTING TRANSPORTER 1* (*AIT1*)), and ABA receptors (*PYRABACTIN RESISTANCE 1-LIKE* (*PYL*)) was decreased in the weakly dormant GS cultivar [44–46]. Conversly, expression levels of *HAI2* and *HAI3* were enhanced in the GS cultivar, which functioned as negative regulators of ABA signaling by inhibiting a downstream SUCROSE NON-FERMENTING 1-RELATED PROTEIN KINASE (SnRK). Furthermore, we found that expression of most *DELLA* genes, which are central inhibitors of GA signaling pathways, was higher in CP and YP, the cultivars with stronger bud dormancy (Fig. 5A). These differences in regulation of ABA signaling activity correlated with lower levels of expression of key dormancy regulators, such as *DELAY OF GERMINATION 1* (*DOG1*) and *DREB AND EAR MOTIF PROTEIN 2* (*DEAR2*) in the GS cultivar [47–49]. Finally, we used qRT-PCR to confirm the expression patterns of the key dormancy-related genes *PYL*, *PP2C* and *DOG1* in the tested cultivars (Fig. 5B). In addition, we found that the identified dormancy-related genes involved in ABA (*PYLs* and *DOG1*), SA and JA responses were highly upregulated in moderate and strong dormancy cultivars (CP, KP and SW). In contrast, weak dormancy cultivars (GS, CS, and KR) had mostly low expression levels of the dormancy-related genes. However, CR, a moderate dormancy cultivar, had relatively low expression levels of ABA-responsive genes compared to other strong dormancy cultivars, but significantly upregulated patterns of SA- and JA-responsive genes (Fig. S11). These indicate that ABA and stress signaling outputs are highly correlated with the maintenance of perennating bud dormancy, although cultivar-specific differences could also be specified in this process. Taken together, these findings indicate that bud dormancy in

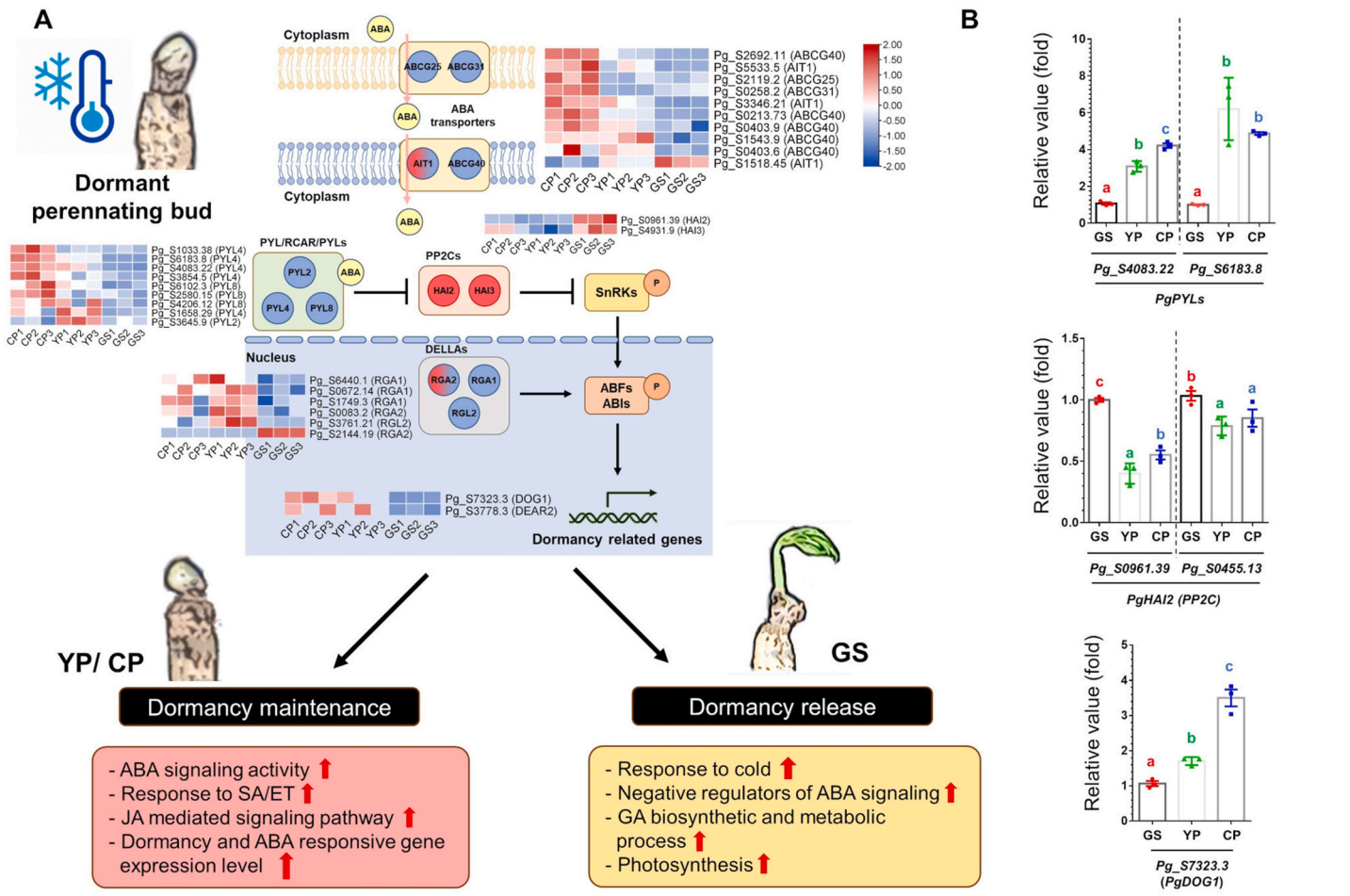


**Fig. 5.** Fine-tuning ABA signals is critical for bud dormancy in the low temperature. (A) A schematic model for regulation of perennating bud dormancy by fine-tuning of ABA signaling activity in *P. ginseng*. Transcriptome analysis of canonical ABA signaling pathway and ABA transporters in GS, CP and YP perennating buds. The expression heatmaps of genes involved in ABA transporters (ABCGs), signaling pathways (PYLs, PP2Cs, DOG1 and DEAR2) and DELLAs were obtained from the RNA-seq data. (B) The expression level of response to ABA related genes were analyzed by qRT-PCR. *PgACT* was used as an internal control. Error bars indicate SEM (n = 3, P < 0.05, one-way ANOVA with Tukey's multiple range test).

ginseng is controlled by the complex regulation of stress and dormancy-related hormonal activity and cold signaling. Our results also suggest that artificial selection of *P. ginseng* from different genetic resources for traits such as the differential breaking of bud dormancy has acted upon the still unidentified fine-tuning mechanism regulating the balance between the ABA and GA signaling pathways.

## 4. Discussion

Bud dormancy is an important adaptive mechanism in temperate perennials, protecting plants that are vulnerable to harsh winter conditions. Due to climate change, the frequency of periods of abnormally low and high temperatures is increasing [50]. Bud dormancy release in ginseng is generally influenced by internal and abiotic factors, with low external temperature being the most detrimental environmental factor [6,9,10]. Recent investigations have revealed the molecular genetic mechanisms underlying the physiological processes associated with the onset and breaking of bud dormancy in perennial plants such as poplar [51–53]. Few studies, however, have attempted to identify the regulatory networks involved in the dormancy cycle of perennial buds in *P. ginseng* [54]. In this study, we identified the molecular mechanisms responsible for breaking dormancy of perennating ginseng buds, as well as the formation of the key transcriptional networks involved in this process (Fig. 4). ABA activity was the main regulator of bud dormancy in ginseng (Figs. 2 and 3). In addition, the early breaking of shoot dormancy in the GS cultivar in early spring appears to be caused by specific alleles of ABA or GA signaling genes (Fig. 3).

The complex regulation of plant hormonal pathways underlying bud dormancy has been extensively investigated to date [24,51,55,56]. Our findings suggest a complex interplay of hormone signaling regulates perennial bud dormancy in *P. ginseng*. Notably, ABA and GA emerged as key regulators of bud dormancy. ABA plays an essential role in bud dormancy in perennial plants through the initiation and progression of dormancy and arrest of the cell cycle [24,26]. GA, however, controls the dormancy cycle, and the transition between dormancy and growth, by promoting growth of primordia and thereby the release from bud dormancy. GA and ABA levels are inversely correlated during dormancy, with the ABA/GA ratio varying with the degree of dormancy [57]. Consistent with previous findings, ABA had an inhibitory effect on dormancy breaking in *P. ginseng*, while GA had positive effects on bud germination (Fig. 4) [22]. Integration of transcriptional network analysis and GSEA also revealed that bud dormancy in the YP and CP cultivars was closely related to the activation of ABA signaling (Figs. 3 and 4). ABA signaling activity was repressed during the release from bud dormancy [24,55], suggesting that ABA directly modulated perennating bud dormancy-release cycles. Our transcriptomic analysis revealed the downregulation of ABA response and ABA signaling-related genes in the GS cultivar, suggesting that the early breaking of bud dormancy in early spring resulted from an ABA-insensitive phenotype. The upregulation of GA biosynthesis-related genes in GS, coupled with the reduction in

expression of most *DELLA* genes, further supported the suggestion of active GA responses in this cultivar (Fig. 5). These results indicate there is a balance between ABA and GA signaling in the regulation of bud dormancy and that the early release from dormancy in GS probably results from a deviation from this balance. More detailed studies are needed to elucidate the specific genetic mutations responsible for the ABA-insensitive and GA-sensitive phenotypes seen in GS.

GO enrichment and network analysis revealed that other stress-related hormones, such as JA, SA, and ET, were involved in the regulation of bud dormancy and release (Fig. 4). The positive enrichment of GO terms related to JA and SA in YP and CP was consistent with previous studies that showed the JA and SA pathways are involved in inhibiting dormancy release, either by inducing ABA or by inhibiting GA to suppress bud break [24,58]. ET plays a dual role during bud dormancy, acting synergistically with ABA during dormancy initiation but antagonistically during dormancy release [25,26]. Furthermore, ET homeostasis and signaling pathways are required for dormancy formation, and both ABA and GA responses are downstream of ET-mediated dormancy regulation [24]. This is consisted with the reduced pattern of expression of ET-related genes in GS (Fig. 4 and Fig. S7). We uncovered a complex transcriptional network that included genes involved in JA, SA, ET, ABA, and GA signal pathways, as well as in low temperature signaling. The upregulation of low temperature response genes in GS (Fig. 4A) indicated an enhanced low temperature response, which contributed to the early release from bud dormancy in this cultivar. Genes such as *ELIP1, TIL, LHY1, VIL2*, and *GI*, which are involved in the response to low temperature, play a pivotal role in promoting low temperature adaptation [59–63]. This network appeared to orchestrate the breaking of bud dormancy in ginseng, highlighting the complex crosstalk and homeostasis between these hormones and the response to cold. In addition, *DOG1*, which acts together with ABA to delay dormancy breaking, showed distinctive expression patterns in the cultivars tested, suggesting this gene played a role in regulating perennating bud dormancy in *P. ginseng* (Fig. 5). Our results also showed a significant increase in the expression of photosynthesis-related genes immediately after dormancy release in ginseng (Fig. S8). This finding was supported by GSEA, which revealed the enrichment of genes associated with photosynthesis in perennating buds after dormancy release in GS. This unexpected role for photosynthesis in dormancy release highlights the complex metabolic regulation that accompanies the transition from dormancy to active growth.

The early breaking of bud dormancy observed in GS has significant implications for ginseng cultivation. Improving our understanding of the genetic and hormonal basis of bud dormancy will elucidate the mechanisms underlying perennating bud dormancy and release in *P. ginseng*. This study provides evidence that ABA is involved in the regulation of perennating bud dormancy in ginseng, but the exact downstream signaling pathways that interact with the hormone signaling pathways to regulate dormancy release remain to be elucidated. In conclusion, this study provides valuable novel insight into the complex hormonal regulatory and genetic pathways involved in bud dormancy release in *P. ginseng*.

## Author contributions

H.R designed the experiments and supervised this study. J.H, W.B and D.S. were contributed to RNA extraction and sequencing analysis. J. H, K.R.G, S.H, W.B., M.G.J, J.W.L and J.U.K carried out P. ginseng growth experiments and histological sectioning analysis. J.H., K.R.G, D. S. and H.R. analyzed bioinformatic analysis and qRT-PCR validation. J. K., G.L. and Y.J. analyzed hormone contents in perennating buds. J.H, S. H, Y.J. and H.R wrote the manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.04.004.

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