Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Exogenous gibberellic acid shortening after-ripening process and promoting seed germination in a medicinal plant Panax notoginseng

Na Ge
Yunnan Agricultural University
Ling Yang
Yunnan Agricultural University
Rong-Mei Huang
Yunnan Agricultural University
Jin-Shang Jia
Yunnan Agricultural University
Qing-Yan Wang
Yunnan Agricultural University
Cui Chen
Yunnan Agricultural University
Zhen-Gui Meng
Yunnan Agricultural University
Long-Geng Li
Yunnan Agricultural University
Jun-Wen Chen (cjw31412@hotmail.com)
Yunnan Agricultural University

Research Article

Keywords: Dormancy, Recalcitrant seeds, Gibberellic acid, After-ripening process, Panax notoginseng

Posted Date: October 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2111983/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Plant Biology on February 1st, 2023. See the published version at https://doi.org/10.1186/s12870-023-04084-3.

Abstract

Background

Panax notoginseng (Burk) F.H. Chen is an important medicinal plant in the family of Araliaceae. Its seeds are classified as the type of morphophysiological dormancy (MPD), and are characterized by recalcitrance during the after-ripening process. However, it is not clear about the molecular mechanism on the dormancy in harvested recalcitrant seeds.

Results

In this study, exogenous supply of gibberellic acid (GA_3) with different concentrations shortened after-ripening process and promoted germination of *P. notoginseng* seeds. Among the identified plant hormone metabolites, exogenous GA_3 results in an increased levels of endogenous hormone GA_3 through permeation. A total of 2971 and 9827 differentially expressed genes (DEGs) were identified in response to 50 mg L^{-1} GA_3 (LG) and 500 mg L^{-1} GA_3 (HG) treatment, respectively, and the plant hormone signal and related metabolic pathways regulated by GA_3 was significantly enriched. GA_3 treatment upregulated the expression of *ent-copalyl diphosphate synthase* (*CPS*), *GA 20-oxidase* (*GA20ox*) and *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*), whereas it downregulated *DELLA*, *Pyrabactin resistance 1-like* (*PYL*) and *ABA-INSENSITIVE5* (*ABI5*). This effect was associated with higher expression of crucial seed embryo development and cell wall loosening genes, *Leafy Contyledon1* (*LEC1*), *Late Embryogenesis Abundant* (*LEA*), *expansins* (*EXP*) and *Pectinesterase* (*PME*).

Conclusions

Exogenous GA_3 application promotes the germination of *P. notoginseng* seeds by increasing GA_3 contents through permeation. Furthermore, the altered ratio of GA and ABA contributes to the development of the embryo, breaks the mechanical constraints of the seed coat and promotes the protrusion of the radicle in recalcitrant *P. notoginseng* seeds. These findings improve our knowledge of the contribution of GA to regulating dormancy of MPD seeds during the after-ripening process, and provide a new theoretical guidance for the application of recalcitrant seeds in agricultural production and storage.

Background

The seed is the most critical developmental period in the life cycle of a plant [1]. Seed dormancy is traditionally defined as an intrinsic obstacle to germination under a favorable conditions [2–4], it is crucial for the conservation of germplasm resources, and for the prevention of pre-harvest sprouting [5]. Seed dormancy is determined by the morphological and physiological characteristics [6], and it is

classified as physiological (PD), morphological (MD), physical (PY), combinational (PY + PD) and morpho-physiological (MPD) types [7, 8]. The MPD seeds have to undergo an after-ripening (AR) process characterized by a gradual reduction in dormancy level [9]. The seeds with the after-ripening process would undergo an intricate range of metabolic processes before germination, which is in preparation for the mobilization of food reserve and cell growth [10]. The after-ripening process depends on moisture and oil contents, seed covering structures and temperature [11]. With a low level of dormancy, temperatures and light could overcome the final limitations on germination, and the germination happens if water potential is adequate to allow the radicle to protrude [12]. The after-ripening is a process through which the dormancy is lost gradually with the increased duration of seed storage in a low hydrated state. However, it remains largely unknown about the mechanism of dormancy release of MPD seeds that show a high water content at the postharvest after-ripening process.

The elicitation, retention and reduction of seed dormancy is a highly intricate physiological process that relies on a multitude of endogenous and environmental factors [13]. Signals from hormones, essentially those of abscisic acid (ABA) and gibberellin (GA) are integrators between environmental cues and molecular signals, thus regulating gene expression [9, 14]. The ratio of GA and ABA is a critical factor to control seed dormancy and germination [4]. Fluctuating temperatures enhance the ratio of GA/ABA by decreasing ABA content in *Cynara cardunculus* seeds, and simultaneously the expression of *Nine-cis-epoxycarotenoid dioxygenase* (*NCED*) and *ABA-INSENSITIVE5* (*ABI5*) is inhibited [15]. In the *Arabidopsis* and tomato (*Lycopersicon esculentum* M.), the mutants with defects in the gene encoding GA biosynthetic enzymes are unable to germinate [16] Pimenta Lange and Lange, 2006). The dormancy loss in wheat seeds is accompanied by the increased expression of *TaGA20ox* and with the enhanced level of bioactive GA_1 during imbibition [17, 18]. *Arabidopsis* plants constitutively expressing GA catalytic enzyme GA 2-oxidase (GA2ox) reveal that the reduced accumulation of GA in seeds leads to the increased probability of seed abortion [19]. The seed germination is dependent on gibberellin (GA) and is inhibited by DELLA when GA concentration is relatively low in *Arabidopsis* (Cao et al., 2006; Sun and Gubler, 2004). Seeds of GA-deficient mutant *ga1* (*GA Requiring 1*) exhibit a failure to germinate phenotype in the lack of exogenous gibberellic acid (GA_3) [20]. In contrast, the mutant defective in GA2ox could deactivate bioactive GA, consequently accompanying with a decreased level of seed dormancy [21]. Overall, the signaling and content of ABA and GA play a critical role in regulating seed dormancy.

GA is required in seed development, and exogenous GA_3 has been applied to break seed dormancy. Exogenous GA_3 facilitates the germination of *Fraxinus hupehensis* seeds by enhancing the level of soluble sugars and weakening lipolysis [22]. Similarly, exogenous GA_3 might increase starch hydrolysis by stimulating the catalytic activity to mitigate oxidative damages in the early germination of *Zanthoxylum dissitum* seeds [23]. It has been found that exogenous GA_3 application prompts GA signal transduction and suppresses ABA synthesis to facilitate rice seed germination under low-temperature conditions [24]. Exogenous GA_3 might break seed dormancy and promote seed germination. However, little information is available about the response of dormancy release in recalcitrant seed to exogenous GA_3.

Panax notoginseng (Burkill) F. H. Chen (Sanqi in Chinese), a traditional Chinese medicinal plant, is a perennial herb from the family of Araliaceae[25]. Its seeds are classified into the group of morphophysiological dormancy (MPD), moreover it has been characterized by the typically recalcitrant trait that shows a high water content during the after-ripening process. The seeds need to undergo about 45 ~ 60 days of after-ripening process before germination (Duan et al., 2010). A preliminary study has demonstrated that incompletely developed embryos might result in the dormancy of *P. notoginseng* seed, and the embryo in the postharvest seed at a heart-shaped period has to be further differentiated and developed during the after-ripening process (An et al., 2006). Meanwhile, soluble sugar, starch and protein gradually decrease with the prolonged storage time in the seeds of *P. notoginseng* during the after-ripening process (An et al., 2010; Cui et al., 2010). *PE2*, *GAI*, *KS*, *PP2C*, *GA2OX* and other genes have been identified as the key genes involved in regulating the released dormancy of *P. notoginseng* seeds [26]. Our recent work has shown that exogenous GA_3 treatment could effectively shorten the after-ripening process and stimulate seed germination of *P. notoginseng* seeds (Ge et al., 2020). However, it is still unknown about the mechanisms through that GA_3 facilitates the germination of postharvest *P. notoginseng* seeds at the physiological and molecular levels.

In this study, we hypothesized that exogenous GA_3 application would enhance endogenous GA biosynthesis and accumulation, inhibit the expression of gene related to ABA signal transduction, and contribute to the embryo development, cell wall relaxation, and radicle protrusion, consequently shortening after-ripening process and promoting recalcitrant seed germination. Based on this hypothesis, we compared germination rate, hormonal content, and transcriptomic-related indicators of seeds treated with exogenous GA_3. We also identified differentially expressed genes (DEGs) associated with seed germination and the expression of genes related to seed germination verified by qRT-PCR. It would provide theoretical guidance for the application of recalcitrant seeds in agricultural production and seed storage.

Results

Effects of exogenous GA_3 treatment on embryo development and seed germination in P. notoginseng during the after-ripening process

Embryo development and seed germination in 50 mg L^{-1} GA_3-treated (LG), 250 mg L^{-1} GA_3-treated (MG), 500 mg L^{-1} GA_3-treated (HG) and control (CK) were as shown in Fig. 1. At 0 DAR, the embryos were enclosed by endosperms (Fig. 1A). The size of the embryos generally increased as the after-ripening process was prolonged (Fig. 1A-B). The embryo length was more than half of the seeds sections at 30 DAR, and the rates of Em/En of 50 mg L^{-1}, 250 mg $L^{-1}$and 500 mg L^{-1} GA_3-treated seeds were 61.98%, 60.50% and 63.01%, respectively. At 45 DAR, the 500 mg L^{-1} GA_3-treated the seeds had a significantly higher Em/En rate (92%) than 50 mg L^{-1} GA_3-treated ones and the control (Fig. 1B). Beginning 30 days after treatment, GA_3-treatment significantly enhanced the germination rate of *P. notoginseng* seeds (Fig. 1D). Compared with 30 DAR, the seed germination rate in control was raised by 10.0% and 27.0% at

45 DAR and 60 DAR, respectively, while the increase in the 50 mg L^{-1} GA_3-treated seeds were 30.0% and 53.0%, respectively, during the same period (Fig. 1D). Compared with CK, the external application of GA_3 significantly promoted seed germination of *P. notoginseng*, the rate of seed germination tended to raise as the GA_3 application increased (Fig. 1C).

RNA sequencing and gene annotation of the transcriptome

To acquire a general overview of the regulation of seed germination as impacted by exogenous GA_3 treatment with different concentrations, 27 cDNA libraries were selected to examine the transcriptome level of gene expression in *P. notoginseng* seeds. A total of 187.75 gigabytes (Gb) clean sequencing data was acquired (Table S1). For each sample, the average clear data was about 6.95 Gb, and Q30 (the percentage of bases with Phred > 30 to the total bases, Phrede = $-10\log_{10}(e)$) was about 94%. The data were used for comparisons with the reference genome of *P. notoginseng* [27]. 86.09% ~ 88.82% of the reads in the 27 libraries were uniquely mapped by alignment with the reference genome of *P. notoginseng* (Table 1). The transcript abundances of genes were assessed by fragments per kilo base of exon per million fragments mapped (FPKM). The distribution of the $\log_2$ (FPKM + 1) showed relatively high gene expression as shown in Fig. S2. In this experiment, all R^2 values between the three biological replicates were closer to 1 by using the Persons Correlation Coefficient (R) (Fig. S3), showing that the biological replicates of each sample had strong correlations.

Table 1
Comparative genome statistics of samples.

sample	total reads	total map	unique map	multi map	mapping rate (%)
CK-0-1	46815702	42910019	40899591(87.36%)	2010428(4.29%)	91.66%
CK_0_2	46919124	42880938	40775136(86.91%)	2105802(4.49%)	91.39%
CK_0_3	45916162	42104871	40017119(87.15%)	2087752(4.55%)	91.70%
LG_0_1	46769434	42497124	40923834(87.5%)	1573290(3.36%)	90.87%
LG_0_2	44727404	40601250	39100103(87.42%)	1501147(3.36%)	90.77%
LG_0_3	43111468	39537923	37614568(87.25%)	1923355(4.46%)	91.71%
HG_0_1	46684610	42938303	40777893(87.35%)	2160410(4.63%)	91.98%
HG_0_2	45715822	41919574	39764745(86.98%)	2154829(4.71%)	91.70%
HG_0_3	51812850	47721232	45354018(87.53%)	2367214(4.57%)	92.10%
CK_30_1	46770276	43229185	41469671(88.67%)	1759514(3.76%)	92.43%
CK_30_2	46087118	42530822	40909769(88.77%)	1621053(3.52%)	92.28%
CK_30_3	50775950	46563721	44146740(86.94%)	2416981(4.76%)	91.70%
LG_30_1	44439024	41026549	39462059(88.8%)	1564490(3.52%)	92.32%
LG_30_2	46805200	43226074	41570186(88.82%)	1655888(3.54%)	92.35%
LG_30_3	44743018	40935834	39281645(87.79%)	1654189(3.7%)	91.49%
HG_30_1	48011460	44131637	42367279(88.24%)	1764358(3.67%)	91.92%
HG_30_2	47257916	43528792	41720947(88.28%)	1807845(3.83%)	92.11%
HG_30_3	46156508	42433078	40956718(88.73%)	1476360(3.2%)	91.93%
CK_50_1	46450880	41971359	39989166(86.09%)	1982193(4.27%)	90.36%
CK_50_2	44037090	40404986	38490509(87.4%)	1914477(4.35%)	91.75%
CK_50_3	49358510	45429117	43281381(87.69%)	2147736(4.35%)	92.04%
HG_50_1	44787148	40771025	39010023(87.1%)	1761002(3.93%)	91.03%
HG_50_2	48294500	44388897	42618190(88.25%)	1770707(3.67%)	91.91%
HG_50_3	42958006	39350379	37781609(87.95%)	1568770(3.65%)	91.60%
LG_50_1	45946054	42287132	40654703(88.48%)	1632429(3.55%)	92.04%
LG_50_2	45088374	41649863	40047536(88.82%)	1602327(3.55%)	92.37%

sample	total reads	total map	unique map	multi map	mapping rate (%)
LG_50_3	45204254	41657387	39812720(88.07%)	1844667(4.08%)	92.15%

Transcriptomic changes by treatment with exogenous GA_3

A total of 2971 and 9827 DEGs were common in *P. notoginseng* seeds with exogenous LG and HG treatment, respectively (Fig. 2B-C). Through pairwise comparisons, a total of 1064, 397, 1115, 2777, 792 and 6653 DEGs were identified at CK_0 d vs LG_0 d, CK_0 d vs HG_0 d, CK_30 d vs LG_30 d, CK_30 d vs HG_30 d, CK_50 d vs LG_50 d and CK_50 d vs HG_50 d, respectively (Fig. 2A-B). For DEGs in LG-treated seeds at 0 DAR, 497 were up-regulated and 567 down-regulated (Fig. 2B). In HG-treated seeds at 0 DAR, 265 were up-regulated and 132 down-regulated. At 30 DAR, 465 genes were up-regulated and 650 genes were down-regulated on the CK compared with LG-treated seeds; 1144 genes were up-regulated and 1633 genes were down-regulated on the CK compared with HG-treated seeds (Fig. 2A). At 50 DAR, 474 genes were up-regulated and 318 genes were down-regulated on the CK compared with LG-treated seeds (Fig. 2A); 3187 genes were up-regulated and 3466 genes were down-regulated on the CK compared with HG-treated seeds. To obtain the functional annotations of DEGs, GO annotation analysis was performed on DEGs (Fig. 3). The results showed that the most of DEGs were enriched in biological process and molecular function, while a number of DEGs were enriched in the cellular components in the comparisons at CK_30 d vs LG_30 d, CK_30 d vs HG_30 d, CK_50 d vs LG_50 d and CK_50 d vs HG_50 d (Fig. 3, Fig. S4). In biological process classification, these DEGs were specifically involved the metabolic processes, cells biological processes, and response the stress and abiotic stimulus. The molecular functions mainly included binding, catalysis, and transport activity. Besides, most of the gene products were located in cells and organelles.

Moreover, KEGG analysis was used to evaluate the biological functions of the DEGs (Fig. 4). In the comparisons at CK_0 d vs LG_0 d and CK_0 d vs HG_0 d, the DEGs were mainly identified in pentose and glucuronate interconversions, cyanoamino acid metabolism and protein processing in the endoplasmic reticulum (Fig. S5). In the comparisons at CK_30 d vs LG_30 d and CK_30 d vs HG_30 d, our results found that DEGs were mainly identified in plant hormone signal transduction, galactose metabolism, and amino sugar and nucleotide sugar metabolism. Importantly, in the comparisons at CK_50 d vs LG_50 d and CK_50 d vs HG_50 d, the results showed that the top KEGG pathways enriched were the plant hormone signal transduction, carbon metabolism and citrate cycle (Fig. 4C). Thus, DEGs functional enrichment suggested that plant hormone signal transduction and carbon metabolism were closely involved in GA_3-promoted seed development, and these pathways were further investigated.

Changes in profiles of plant hormone signal transduction and related metabolites in response to exogenous GA_3 treatment

In Fig. 5, the map showed the expression pattern of DEGs related to plant hormone ABA and GA signal transduction. Compared with the CK, GA_3-treatment up-regulated DEGs related to GA biosynthesis and signal transduction during the after-ripening process, including *ent-copalyl diphosphate synthase* (*CPS*), *GA20-oxidase* (*GA20ox*) and *GA INSENSITIVE DWARF1* (*GID1*), whereas down-regulated *DELLA* (Fig. 5A-B). Further, as shown in Fig. 5D, GA_3-treatment down-regulated most of the DEGs involved in ABA transport signal transduction pathway among them are *Pyrabactin resistance 1-like* (*PYL*) and *ABSCISIC ACIDINSENSITIVE 5* (*ABI5*), whereas upregulated *Protein Phosphatase 2C* (*PP2C*). To explore the functions of endogenous hormones in seed germination, ABA, GA_3 and IAA contents in the CK and GA_3-treated *P. notoginseng* seeds during the after-ripening process were detected using LC-MS (Table 2). Compared with the CK, endogenous hormones GA_3 content was significantly the highest at 0 DAR, but IAA content was not changed considerably in the GA_3-treated group. By contrast, ABA contents were the lowest in the GA_3-treated group. Thus, GA_3 treatment could stimulate seed germination by altering the accumulation of endogenous hormones. Moreover, exogenous GA_3 induced seed germination through increased GA_3 concentration and decreased ABA concentration. These results imply that exogenous GA_3 enhances GA biosynthesis and accumulation, while inhibits the gene expression related to ABA signal transduction.

Table 2
Changes of endogenous hormones in the control and GA_3-treated *P. notoginseng* seeds during after-ripening process.

Endogenous hormone (ng/g)	Time (d)	Sample		
		CK	GA_3 50mg/L	GA_3 500mg/L
GA_3	0	0.432 ± 0.078	4.006 ± 0.358	27.851 ± 2.118*
	30	0.197 ± 0.059	2.914 ± 2.582	15.118 ± 2.277*
	50	0.293 ± 0.244	0.788 ± 0.073	9.100 ± 0.948*
ABA	0	0.250 ± 0.045	0.228 ± 0.044	1.050 ± 0.474
	30	0.154 ± 0.013	0.154 ± 0.023	0.182 ± 0.016
	50	0.116 ± 0.026	0.105 ± 0.007	0.102 ± 0.007
IAA	0	20.465 ± 1.548	19.608 ± 1.091	19.756 ± 1.652
	30	12.695 ± 1.106	13.513 ± 2.230	11.191 ± 0.930
	50	7.108 ± 2.390	7.812 ± 1.471	8.167 ± 1.464

The values are means ± SD(n = 3). *$P < 0.05$, significant differences between the control and GA_3 treatments.

The expression of genes related to embryo development and cell wall relaxation in response to GA_3 treatment

The expression of gene involved in embryo development and cell wall loosening in *P. notogensing* seeds during the after-ripening process showed significant changes under exogenous GA_3 treatment. As shown in Fig. 6A, we focused on embryo development-related and cell wall-related DEGs, which were clustered into five gene groups: *Late Embryogenesis Abundant* (*LEA*), *Leafy Contyledon1*(*LEC1*), *Expansin* (*EXP*), *Xyloglucan Endotransglucosylase* (*XET*) and *Pectin Methylesterase* (*PME*). Compared with the CK, the expression level of *PME* and *LEA* did not change upon GA_3 treatment at 0 DAR, while GA_3 increased the expression level of *LEC1* and *EXP*. With the prolonged after-ripening process, *LEA* and *PME* were dramatically up-regulated in response to GA_3 applications. The expression of *LEC1*, *EXP* and other genes were found to be increased during after-ripening process after GA_3 treatment.

Verification of expression of DEGs using qRT-PCR

To test the reliability and the repeatability of RNA-seq, the DEGs related to GA biosynthesis and signal (*CPS*, *GA20ox*, *DELLA*), ABA signal and response (*PYL*, *ABI5*), embryo development (*LEA*) and cell wall metabolism (*PME*) were chosen for the confirmation of gene expression (Fig. 7). It indicated that the results of transcriptomic were reliable and accurate.

Discussion

Exogenous GA_3 effectively shortens the after-ripening process and promotes seed germination of P. notoginseng

Seed dormancy and germination are influenced by plant hormonals and the external environment [28–30]. Exogenous GA_3 treatment could significantly promote germination in wild species of pistachioo [8, 31]. It has been found that seed germination is substantially promoted by exogenous GA_3 treatment in *Fraxinus hupehensis* [22]. Consistently with the finding of previous studies, the present study revealed that exogenous GA_3 obviously promote seed germination of *P. notoginseng* (Fig. 1C-D). Compared with CK, the seed germination rate tended to be raised as GA_3 application increased, and the germination rate was highest in *P. notoginseng* seeds treated with 500 mg L^{-1} exogenous GA_3 (Fig. 1D). It has also been recorded that *Acer mono* Maxim. seeds are treated with 200 mg L^{-1} GA_3 and their germination rate effectively increased [32]. *Nitraria tangutorum* Bobr. seeds are treated with 150 mg L^{-1} GA_3 and germination rate, germination index and vigor index are the highest (Guo and Lin, 2009). These studies indicate that the appropriate concentration of exogenous GA_3 is one of the key factors in breaking dormancy and promoting seed germination. In our research, we verified the impacts of different concentrations of GA_3 (LG, MG and HG) on seed embryo development, germination rate and after-ripening process of *P. notoginseng* seeds (Fig. 1B-D), and found that the germination rate of *P. notoginseng* seed is gradually elevated with the increase of exogenous GA concentration (Fig. 1), indicating that 500mg L^{-1} of exogenous GA_3 is most appropriate to promote seed germination of recalcitrant *P. notoginseng* seeds.

GA essentially stimulates endosperm weakening and embryo expansion [33], and promotes the protrusion of radicle by breaking through the confines of the seed coat [34, 35]. Herein, we found that the endosperm tissue around seed embryos treated with HG is softened at 30 DAR compared with CK (Fig. 1A), implying that GA_3 treatment might stimulate the softening of tissues around seed embryo, thus providing sufficient space for embryo development. Our results agreed with the observation that GA accelerates the growth potential of the embryo and weakens the structures surrounding the embryo in tomato [36]. Above all, our results reveal that 500 mg L^{-1} GA_3 treatment might effectively shorten the after-ripening process and promote seed germination by stimulating seed embryo development and softening the tissues around the embryos of *P. notoginseng.*

Exogenous GA_3 application accelerates P. notoginseng **seed germination by changing endogenous hormone accumulation**

ABA and GA antagonistically regulate seed dormancy and germination [28, 37]. The induction and maintenance of dormancy are positively regulated by ABA, while germination is enhanced by GA [38]. Consistently, our results showed that GA_3 content is highest, and ABA content is the lowest in *P. notoginseng* seeds treated with GA_3 (Table 2). This is agreement with other study showing an increase in energy requirements and endogenous GA_3 content but a decrease in ABA content during germination and growth of seeds[22]. Compared with the CK, our study found that the GA_3 content in *P. notoginseng* seeds had a 60-fold increase after treatment with 500 mg L^{-1} GA_3, followed by a 9-fold increase in seeds after treatment with 250 mg L^{-1} GA_3 at 0 DAR (Table 2). The levels were much too high to be endogenous GA_3 and they were reduced with time after treatment. It could be the result that the penetration of exogenous GA_3 into the seed tissues was caused by the concentration difference between the soak solution and the cytolymph during the soak treatment. Those results indicated that a part of measured endogenous GA_3 is likely to be remaining from the GA_3 treatment, but both of them contribute to alter the ratio of GA and ABA. Besides, our results found that exogenous GA_3 application could not cause auxin (IAA) content to be different in *P. notogensing* seeds, and this is contrary to the finding that exogenous GA_3 increases IAA content in the tiller node of rice (*Oryza sativa* L.) [39], implying that IAA responds diversely in the regulation network of plant development upon GA_3 treatment. Thus, we consider that exogenous GA_3 release dormancy to promote seed germination mainly through changing the ratio of GA and ABA.

Cellular ABA and GA levels are controlled by the balance between their biosynthesis and catabolism [40]. Our transcriptomic analysis revealed that the total of 2971 and 9827 DEGs are dramatically affected by exogenous LG and HG treatment, respectively (Fig. 2A). Meanwhile, it was significantly enriched for plant hormone signal transduction and related metabolic pathways regulated by GA (Fig. 4C-D), suggesting that GA induces dramatic responses at the transcriptional level. Some candidate genes in GA_3 and ABA signaling pathways also determine seed germination [37, 41]. In our study, the expression level of *CPS*, *GID1* and most of *GA20ox* were downregulated by GA_3 treatment at 0 DAR (Fig. 7C-D), and this effect gradually weakens and was lost with decreasing levels of GA_3 in seeds (Table 2), suggesting that high

concentrations of GA_3 in treated seeds might be a negative regulator to suppress GA biosynthesis and signaling by reducing expression of some GA-biosynthesis genes in a homeostasis mechanism (Binenbaum et al., 2018). A study on barley, wheat and rice has shown that *HvGA20ox* is a pivotal gene for regulate seed germination in barley [42]. *OsGA20ox2* and *OsGA2ox3* were essential genes to control seed germination in rice [43], and the mutation *OsGA20ox2* shows the reduced GA level and enhanced seed dormancy [44]. Likewise, our study found that *CPS*, *GA20ox*, *GID1* and *DELLA* genes involved in GA hormone biosynthesis and catabolism pathways are affected by exogenous GA_3 treatment (Fig. 5A-B). GA_3 upregulated the expression of *CPS*, *GA20ox* and *GID1*, and downregulated *DELLA* at 30 DAR and 50 DAR (Fig. 7C-D). DELLA is a plant growth suppressor, while GID1 is a receptor for GA_3, it acts by binding to GID1 receptor to degrade DELLA protein in plants [45, 46]. Overall, the expression levels of *GA20ox* and *GID1* were upregulated, and the expression level of *DELLA* was downregulated by GA_3 treatment during the after-ripening process, thereby perturbing GA_3 signal transduction in recalcitrant *P. notogensing* seeds.

A comparative analysis of *PP2C* mutants suggests that AtPP2CA is a significant player in seeds [47, 48]. Of these, the ABA receptors PYR1/PYL proteins might confer a prominent function in seed ABA responsiveness through regulating PP2C activity [49, 50], and the *pyr1 prl1 prl2 prl4* quadruple mutant shows ABA insensitive the germination [49]. Genetic analysis reveals that ABA-INSENSITIVE 3(ABI3), ABA-INSENSITIVE 4 (ABI4) and ABA-INSENSITIVE 5 (ABI5) are the key transcription factors that confer seed ABA responsiveness [51]. The seeds of *abi5* mutants reduce transcript levels of *Early Methionine-labelled 1* and *6* (*EM1* and *EM6*), which are associated with germination process [52, 53]. The transcriptomic analysis showed that the expression of *PYL*, *PP2C* and *ABI5* has a significant change in *P. notoginseng* seeds treated with exogenous GA_3 (Fig. 5C). *PYL* and *ABI5* showed a higher expression level at the 0 DAR, and they gradually decreased with the prolonged after-ripening process in *P. notoginseng* seeds. Surprisingly, compared with CK, the expression of *PYL* and *ABI5* tended to decline as the GA_3 application increased, and it was lowest in *P. notoginseng* seeds treated with 500 mg L^{-1} exogenous GA_3 at 50 DAR (Fig. 7E-F), and the expression trend of *PP2C* was reversed during the after-ripening process (Fig. 5C). Our results suggest that exogenous GA_3 regulates the essential genes to perturb endogenous GA and ABA biosynthesis and catabolism in *P. notoginseng* seeds. This might partly contribute to the antagonistic action of GA and ABA on seed germination and growth.

The elevated endogenous hormone GA effectively promote the expression of genes related to embryo development and cell wall loosening

The synthesis and catabolism of GA_3 vigorously promotes cell division during seed development and germination [54, 55]. A previous study has shown that the incomplete development of embryos could result in seed dormancy of *P. notoginseng* [56]. Recent studies revealed that *LEAFY COTYLEDON 1* (*LEC1*) is a critical regulator of seed development, its loss of function results in a short embryo axis and intolerance to desiccation [57, 58]. Consistently, Late *Embryogenesis Abundant* (*LEA*) and *LEC1* are required for seed maturation and acquisition of desiccation tolerance [59, 60]. In our study, we found that the expression of *LEC1* and *LEA* is lower in CK at 0 DAR, and they are dramatically up-regulated in

response of *P. notoginseng* seeds to GA_3 applications during the after-ripening process (Fig. 6A), demonstrating that the embryo development is relatively vigorous under GA_3 treatment (Fig. 1A). These results support the view that GA_3 treatment could promote the embryo development to boost seed germination of postharvest *P. notogensing*.

The architecture of cell wall is a key determinant for plant growth [61]. The dormancy or germination is determined by the balance between the resistance strength of the surrounding tissues and the growth potential of the elongating radicle [62]. There are a series of evidence that GA_3 could facilitate radicle protrusion by breaking through the mechanical constraints of the seed coat during seed germination [54, 63]. The cell wall-degrading enzymes, such as cellulases, xyloglucan endotransglucosylase-hydrolase (XTH), pectinesterase (PME), expansins (EXP) and hemicellulases, have been proved to contribute to cell wall loosening [61, 64–66]. In our study, DEGs (*PME*, *EXP* and *XTH*) involved in cell wall development were up-regulated by exogenous GA_3 treatment (Fig. 6A-B). Our result is consistent with observations that *xyloglucan endotransglucosylase* (*XET*), *xyloglucan endohydrolase* (*XEH*) and *EXP* are upregulated during *Arabidopsis* seed germination [67, 68]. Our observations confirmed that a series of cell wall-degrading genes is up-regulated significantly in *P. notoginseng* seeds treated with exogenous GA_3 (Fig. 7G), and it suggests that exogenous GA_3 might promote cell wall metabolism and endosperm degradation. Compared with CK, the expression of *EXP* and *PME* were both upregulated in *P. notoginseng* seeds treated with LG and HG. Differently, the up-regulated expression level in the seeds treated with HG was significantly higher than those treated with LG (Fig. 6B). Thus, although LG, MG and HG treatments all promote seed germination, to a higher degree HG accelerate the degradation of cell wall to create more spaces for seed germination by up-regulating *EXP* and *PME*. This could be regarded as the reason for the highest germination rate of *P. notoginseng* seeds treated with HG. In general, we believe that the genes (*LEA*, *LEC1*, *EXP*, *PME* and *XEH*) involved in embryo development and the cell wall degradation might create more spaces for radicle elongation to accelerate the germination in postharvest *P. notoginseng* seeds treated with GA_3.

Conclusion

Exogenous GA_3 increases the content of endogenous hormones GA_3 through permeation and alters the ratio of GA and ABA to promote seed germination of *P. notogensing*. GA-treated *P. notogensing* seeds maintain higher development and germination than CK treatment. We also find that GA_3 upregulate DEGs involved in GA biosynthesis and catabolism, embryo development and the cell wall loosening, while downregulate ABA biosynthesis and catabolism. Based on the findings of the present work, a model is proposed to explain the dormancy mechanism in recalcitrant *P. notogensing* seeds regulated by GA_3 (Fig. 8). Exogenous GA_3 application increases the content of endogenous hormones GA_3 through permeation to alter the ratio of GA/ABA, and this would contribute to the development of the embryo, break the mechanical constraints of the seed coat and promote the protrusion of the radicle in postharvest recalcitrant seeds. These findings would comprehensively improve our understanding of the potential roles of GA in regulating the dormancy of recalcitrant seeds during the after-ripening process.

Materials And Methods

Materials and treatments

Seeds were routinely harvested from the plants of 3-year-old *P. notoginseng* (Fig. S1A) that were cultivated at the experimental farm of Wenshan Miao Xiang *P. notoginseng* Industrial Co., Ltd., China (Longitude 104°32', latitude 23°53'). In November, 3-year-old mature and plump seeds of *P. notoginseng* were selected (Fig. S1B). After artificial peeling (Fig. S1C), the seeds were washed in ddH_2O, soaked and disinfected with 5% $CuSO_4$ bactericidal solution and washed twice, then the seeds were obtained by indoor shade drying out surface moisture. The seeds were submitted to one of three different treatments by soaking for 24 h, the concentrations 50 mg L^{-1} (LG), 250 mg L^{-1} (MG) and 500 mg L^{-1} (HG) of exogenous hormones GA_3 were selected for soaking treatment, and ddH_2O-soaked treatment was defined as the control (CK). The soaked seeds with three replicates were placed in a ventilated net basket for 50 days in a sandy stratification chamber at 15 ± 5℃ to accomplish after-ripening process. The start point before the seeds was stored in sandy stratification is defined as the time point of 0 days after-ripening (DAR). At each sampling point (0, 30, 50 DAR), the samples from the concentrations of 50 mg L^{-1} (LG) and 500 mg L^{-1} (HG) of GA_3 were selected based on the results from experiments (Fig. 1) for further assays.

Microscopic inspection and morphological measurements

A number of seeds were fixed using FAA (70% alcohol: acetic acid: formalin = 18:1:1) for microscopic inspection. The determination of embryo (Em), endosperm (En) and embryo rate (Em/En) was carried out at four time points of 0, 15, 30 and 45 DAR. Seeds were divided in half lengthwise by using a razor blade. Seed sections were examined using a stereoscopic microscope (ZEISS, SteREO Discovery.V20, Germany) equipped with a digital camera. The pictures were processed (brightness and contrast adjusted) and combined using Photoshop CS6 (Adobe, USA).

HPLC-MS analysis of endogenous hormone GA_3, ABA and IAA content

Samples used for the determination of endogenous hormone content were frozen in liquid nitrogen at three time points of 0, 30 and 50 DAR. Endogenous hormone ABA, GA_3 and IAA content in seed of *P. notoginseng* was examined as reported by Pan et al [69] with some modification.

Total RNA extraction and transcriptome analysis

For LG, HG and CK treatment, the sample at 0, 30 and 50 DAR were separated and applied for RNA extraction. Using the Plant Plus Kit (Tiangen, Beijing, China) to extract the total RNA with three replications, and RNA quality was monitored on 1% agarose electrophoresis. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Using NEBNext® UltraTM RNA Library Prep Kit, sequencing libraries were generated for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences. The libraries were sequenced on an Illumina platform by the Novegene Technology Company (Beijing, China). Raw reads were analyzed and low quality reads and reads containing adapters were removed, resulting in clean reads. The clean reads were mapped to the *P. notogensing* reference genome (Fan et al., 2020) using HISAT2 v2.0.5, and novel genes prediction were made with String Tie (1.3.3b) [70].

Differential expression analysis of paired conditions with three biological replicates per condition was performed using the DESeq2 R package (1.16.1). According to the method of Benjamini and Hochberg [71], the *P*-values were adapted to control the false discovery rate. Genes with an adjusted *P*-value < 0.05 found by DESeq2 were designated as differential expression. Gene Ontology (GO) analysis and KEGG analysis of differentially expressed genes (DEGs) were performed by the clusterProfiler R package. The terms with corrected *P*-value < 0.05 were identified as significant enrichment of differently expressed genes.

Gene expression assessment

Gene expression was assayed by Quantitative Real-time PCR (qRT-PCR). The total RNA was isolated from seeds of *P. notogensing* samples using the same scheme described for RNA-Seq. The cDNA was synthesized by using Prime Script RT reagent kit (Takara Bio, Kyoto, Japan). As shown in Table S2, primers were accessed using Premier 3.0 [72] for quantitative PCR (qRT-PCR) and synthesized by Tsingke Biotech Co., Ltd. (Kunming, China). The qRT-PCR reaction was performed with three technical replicates using the Quant studio12 K Flex System (Thermo Fisher Scientific). The *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE(GAPDH)* was chosen as the internal reference for *P. notoginseng* seeds. Candidate genes were analyzed for relative expression levels using the $2^{-\Delta\Delta Ct}$ algorithm[73] by standardizing their transcript levels of related genes in control. Each sample was analyzed in three replicates.

Statistical analysis

The experiment was performed in three biological replicates. Statistical analyses were carried out using the SPSS software package (Chicago, IL, USA) and SigmaPlot 10.0, where the variables were present as the mean ± SD (n = 3). The least significant difference (LSD) was calculated and $P < 0.05$ was deemed statistically significant.

Abbreviations

ABA: Abscisic acid; *ABI3*: ABA-INSENSITIVE 3; *ABI4*: ABA-INSENSITIVE 4; *ABI5*: ABA-INSENSITIVE5; *CPS*: Ent-copalyl diphosphate synthase; CK: Control; DAR: Days after-ripening; DEGs: Differentially expressed genes; *EXP*: Expansin; Em: Embryo; En: Endosperm; Em/En: Embryo rate; GA: Gibberellin; GA_3: Gibberellic acid; *GA20ox*: GA 20-oxidase; IAA: Auxin; GO: Gene Ontology; *LEA*: Late Embyogenesis Abundant; *LEC1*: Leafy Cntyledon1; MD: Morphologcal; *NCED*: Nine-cis-epoxycarotenoid dioxygenase; *PYL*: Pyrabactin

resistance 1-like; *PME*: Pectinesterase; PD: Physiological; PY: Physical; PY + PD: Combinational; *PME*: Pectin Methylesterase; *PP2C*: Protein Phosphatase 2C; *XET*: Xyloglucan Endotransglucosylase; *XEH*: Xyloglucan endohydrolase.

Declarations

Ethics approval and consent to participate

Not applicable. The authors declared that a permission to collect *Panax notoginseng* material has been obtained, and experimental research works on the plants described in this paper comply with institutional, national and international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequencing data from this study have been deposited in the Genome Sequence Archive in BIG Data Center (https://bigd.big.ac.cn/), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under the accession number: CRA008378. Other data generated or analyzed during this study are included in this published article and its supplementary information files. Hoo & Tseng firstly undertook the formal identification of the plant material *Panax notoginseng* (Burkill) (Journal of Systematics and Evolution 11: 435, 1973) in Flora of China.

Competing Interests

The authors report no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work is funded by the Major Special Science and Technology Project of Yunnan Province (202102AA310048); and the National Key Research and Development Plan of China (2021YFD1601003).

Authors’ contributions

Chen Jun-Wen directed the experiment and made suggestions for the writing of the manuscript. Ge Na wrote the manuscript, Yang Ling and Ge Na participated most of the experiments. Huang Rong-Mei, Jia

Jin-Shang, Wang Qing-Yan, Chen Cui, Meng Zhen-Gui and Li Long-Geng analyzed the relevant experimental data. All authors contributed to the article and approved the submitted version.

Acknowledgements

We thank Dr JYZ for technical assistance. We are also grateful to KLL and MJM for their assistance with data analysis.

Authors' information

[1] College of Agronomy & Biotechnology, Yunnan Agricultural University, Kunming, Yunnan 650201, China. [2] The Key Laboratory of Medicinal Plant Biology of Yunnan Province, Yunnan Agricultural University, Kunming, Yunnan 650201, China. [3] National & Local Joint Engineering Research Center on Germplasm Innovation & Utilization of Chinese Medicinal Materials in Southwestern China, Yunnan Agricultural University, Kunming, Yunnan 650201, China.

References

1. Soppe WJJ, Bentsink L. Seed dormancy back on track; its definition and regulation by DOG1. New Phytol. 2020, 228(3):816-819.
2. 2. Finkelstein R, Reeves W, Ariizumi T, Steber C. Molecular aspects of seed dormancy. Annu Rev Plant Biol. 2008, 59:387-415.
3. 3. Brandizzi F: To grow or not to grow…. Plant J. 2020, 103(2):479-480.
4. 4. Tuan PA, Nguyen TN, Jordan MC, Ayele BT. A shift in Abscisic acid/gibberellin balance underlies retention of dormancy induced by seed development temperature. Plant, Cell Environ. 2021, 44(7):2230-2244.
5. 5. MacGregor DR, Zhang N, Iwasaki M, Chen M, Dave A, Lopez Molina L, Penfield S. *ICE1* and *ZOU* determine the depth of primary seed dormancy in *Arabidopsis* independently of their role in endosperm development. Plant J. 2019, 98(2):277-290.
6. 6. Nikolaeva MG: On criteria to use in studies of seed evolution. Seed Sci Res. 2004, 14(4).
7. 7. Baskin JM, Baskin CC. A classification system for seed dormancy. Seed Sci Res. 2007, 14(1):1-16.
8. 8. Finch Savage WE, Leubner Metzger G. Seed dormancy and the control of germination. New Phytol. 2006, 171(3):501-523.
9. 9. Carrera-Castaño G, Calleja-Cabrera J, Pernas M, Gómez L, Oñate-Sánchez L. An Updated Overview on the Regulation of Seed Germination. Plants. 2020, 9(6):703.
10. Liu Y, Han C, Deng X, Liu D, Liu N, Yan Y. Integrated physiology and proteome analysis of embryo and endosperm highlights complex metabolic networks involved in seed germination in wheat (*Triticum aestivum* L.). J Plant Physiol. 2018, 229:63-76.

11. Manz B, Muller K, Kucera B, Volke F, Leubner-Metzger G. Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. Plant Physiol. 2005, 138(3):1538-1551.
12. Batlla D, Benech-Arnold RL. A framework for the interpretation of temperature effects on dormancy and germination in seed populations showing dormancy. Seed Sci Res. 2015, 25(2):147-158.
13. Yang L, Liu S, Lin R. The role of light in regulating seed dormancy and germination. J Integr Plant Biol. 2020, 62(9):1310-1326.
14. Shu K, Zhou W, Chen F, Luo X, Yang W. Abscisic acid and gibberellins antagonistically mediate plant development and abiotic stress responses. Front Plant Sci. 2018, 9:416.
15. Roberto Huarte H, Luna V, Pagano EA, Zavala JA, Benech-Arnold RL. Fluctuating temperatures terminate dormancy in *Cynara cardunculus* seeds by turning off ABA synthesis and reducing ABA signalling, but not stimulating GA synthesis or signalling. Seed Sci Res. 2014, 24(2):79-89.
16. Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, Kato T, Tabata S, Kamiya Y, Sun TP. Distinct and overlapping roles of two gibberellin 3-oxidases in *Arabidopsis* development. Plant J. 2006, 45(5):804-818.
17. Zuo J, Lin C, Cao H, Chen F, Liu Y, Liu J. Genome-wide association study and quantitative trait loci mapping of seed dormancy in common wheat (*Triticum aestivum* L.). Planta. 2019, 250(1):187-198.
18. Nakamura S. Grain dormancy genes responsible for preventing pre-harvest sprouting in barley and wheat. Breeding Sci. 2018, 68(3):295-304.
19. Singh DP, Jermakow AM, Swain SM. Gibberellins are required for seed development and pollen tube growth in *Arabidopsis*. Plant Cell. 2002, 14(12):3133-3147.
20. Cao D, Cheng H, Wu W, Soo HM, Peng J. Gibberellin mobilizes distinct DELLA-dependent transcriptomes to regulate seed germination and floral development in *Arabidopsis*. Plant Physiol. 2006, 142(2):509-525.
21. Li C, Zheng L, Wang X, Hu Z, Zheng Y, Chen Q, Hao X, Xiao X, Wang X, Wang G, Zhang, Y. Comprehensive expression analysis of Arabidopsis *GA_2-oxidase* genes and their functional insights. Plant Sci. 2019, 285:1-13.
22. Song Q, Cheng S, Chen Z, Nie G, Xu F, Zhang J, Zhou M, Zhang W, Liao Y, Ye J. Comparative transcriptome analysis revealing the potential mechanism of seed germination stimulated by exogenous gibberellin in *Fraxinus hupehensis*. BMC Plant Biol. 2019, 19(1):199.
23. Sun J, Jia H, Wang P, Zhou T, Wu Y, Liu Z. Exogenous gibberellin weakens lipid breakdown by increasing soluble sugars levels in early germination of *zanthoxylum* seeds. Plant Sci. 2019, 280:155-163.
24. Wang Y, Cui Y, Hu G, Wang X, Chen H, Shi Q, Xiang J, Zhang Y, Zhu D, Zhang Y. Reduced bioactive gibberellin content in rice seeds under low temperature leads to decreased sugar consumption and low seed germination rates. Plant Physiol Biochem. 2018, 133:1-10.

25. Chen J, Kuang S, Long G, Yang S, Meng Z, Li L, Chen Z, Zhang G. Photosynthesis, light energy partitioning, and photoprotection in the shade-demanding species *Panax notoginseng* under high and low level of growth irradiance. Funct Plant Biol. 2016, 43(6):479.
26. Yang K, Yang L, Fan W, Long GQ, Xie SQ, Meng ZG, Zhang GH, Yang SC, Chen JW. Illumina-based transcriptomic analysis on recalcitrant seeds of *Panax notoginseng* for the dormancy release during the after-ripening process. Physiol Plant. 2019, 167(4):597-612.
27. Fan G, Liu X, Sun S, Shi C, Du X, Han K, Yang B, Fu Y, Liu M, Seim I *et al*. The Chromosome Level Genome and Genome-wide Association Study for the Agronomic Traits of P*anax notoginseng*. iScience. 2020, 23(9):101538.
28. Miransari M, Smith DL. Plant hormones and seed germination. Environ Exp Bot. 2014:110-121.
29. Penfield S. Seed dormancy and germination. Cur Biol. 2017, 27(17): 874-878.
30. Willis CG, Baskin CC, Baskin JM, Auld JR, Venable DL, Cavender-Bares J, Donohue K, Rubio DCR. The evolution of seed dormancy: environmental cues, evolutionary hubs, and diversification of the seed plants. New Phytol. 2014, 203(1):300-309.
31. Rahemi M, Baninasab A. Effect of gibberellic acid on seedling growth in two wild species of pistachio. J Hortic Sci Biotechnol. 2000, 75(3):336-339.
32. Tang SY, Cui NH, Feng YC, Zhang J, Liu Y. Effect of gibberellin concentration on seed germination of *Acer mono* Maxim. Chin J Appl Environ Biol. 2021, 3 (27): 555-559.
33. Cao X, Li M, Li J, Song Y, Zhang X, Yang D, Li M, Wei J. Co-expression of hydrolase genes improves seed germination of *Sinopodophyllum hexandrum*. Ind Crop Prod. 2021, 164:113414.
34. Tuan PA, Kumar R, Rehal PK, Toora PK, Ayele BT. Molecular mechanisms underlying abscisic acid/gibberellin balance in the control of seed dormancy and germination in cereals. Front Plant Sci. 2018, 9:668.
35. Sun T, Gubler F. Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol. 2004, 55(1):197-223.
36. Yamaguchi S, Kamiya Y. Gibberellins and Light-Stimulated Seed Germination. J Plant Growth Regul. 2001, 20(4):369-376.
37. Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y. Abscisic acid and the control of seed dormancy and germination. Seed Sci Res. 2010, 20(2):55-67.
38. Nee G, Xiang Y, Soppe WJ. The release of dormancy, a wake-up call for seeds to germinate. *Curr Opin Plant Biol.* 2017, 35:8-14.
39. Liu Y, Ding Y, Wang Q, Li G, Xu J, Liu Z, Wang S. Effect of plant growth regulators on growth of rice tiller bud and changes of endogenous hormones. Acta Agronomica Sinica. 2011, 37(4):670-676.
40. Yan A, Chen Z. The pivotal role of Abscisic acid signaling during transition from seed maturation to germination. Plant Cell Rep. 2017, 36(5):689-703.
41. Hauvermale AL, Steber CM. GA signaling is essential for the embryo-to-seedling transition during *Arabidopsis* seed germination, a ghost story. Plant Signal Behav. 2020, 15(1):1705028.

42. Li C, Ni P, Yu J, Bellgard M, Lance R, Appels R, Francki M, Hunter A, Zhang Y, Schibeci D, Li H, Tarr A, Wang J, Cakir M. Genes controlling seed dormancy and pre-harvest sprouting in a rice-wheat-barley comparison. Funct Integr Genomic. 2004, 4(2):84-93.
43. Ye H, Feng J, Zhang L, Zhang J, Mispan MS, Cao Z, Beighley DH, Yang J, Gu X. Map-based cloning of seed dormancy1-2 identified a gibberellin synthesis gene regulating the development of endosperm-imposed dormancy in rice. *Plant Physiol.* 2015, 169(3): 2152-2165.
44. Magwa RA, Zhao H, Xing Y. Genome-wide association mapping revealed a diverse genetic basis of seed dormancy across subpopulations in rice (*Oryza sativa* L.). BMC Genet. 2016, 17:28.
45. Ito T, Okada K, Fukazawa J, Takahashi Y. DELLA-dependent and -independent gibberellin signaling. Plant Signal Behav. 2018, 13(3):e1445933.
46. Binenbaum J, Weinstain R, Shani E. Gibberellin localization and transport in plants. Trends Plant Sci. 2018, 23(5):410-421.
47. Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami T, Shinozaki K, Hirayama T. ABA-hypersensitive germination3 encodes a Protein Phosphatase 2C (AtPP2CA) that strongly regulates Abscisic acid signaling during germination among *Arabidopsis* Protein Phosphatase 2Cs. Plant Physiol. 2006. 140(1), 115-126.
48. Dupeux FF, Antoni RR, Betz KK, Santiago JJ, Gonzalez-Guzman MM, Rodriguez LL, Rubio SS, Park SS, Cutler SRSR, Rodriguez PLPL *et al*. Modulation of Abscisic acid signaling in vivo by an engineered receptor-insensitive Protein Phosphatase Type 2C Allele1. Plant Physiol. 2011, 156(1):106-116.
49. Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. Regulators of PP2C phosphatase activity function as Abscisic acid sensors. Science. 2009, 324(5930):1064-1068.
50. Fuchs S, Tischer SV, Wunschel C, Christmann A, Grill E. Abscisic acid sensor RCAR7/PYL13, specific regulator of protein phosphatase coreceptors. *PNAS.* 2014, 111(15):5741-5746.
51. Maia De Oliveira J, Dekkers SJW, Dolle M, Ligterink W, Hilhorst HWM. Abscisic acid (ABA) sensitivity regulates desiccation tolerance in germinated *Arabidopsis* seeds. New phytol. 2014, 203(1):81-93.
52. Lopez-Molina L, Mongrand S, Chua NH. A post germination developmental arrest checkpoint is mediated by Abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. PNAS. 2001, 98(8):4782-4787.
53. Kanai M, Nishimura M, Hayashi M. A peroxisomal ABC transporter promotes seed germination by inducing pectin degradation under the control of ABI5. Plant J. 2010, 62 (6), 936-947.
54. Hedden P, Thomas SG. Gibberellin biosynthesis and its regulation. Biochem J. 2012, 444(1):11-25.
55. Pimenta Lange MJ, Lange T. Gibberellin biosynthesis and the regulation of plant development. Plan Biol. 2006, 8(3):281-290.
56. An N, Cui XM, Xiao FH, Chen ZJ, Duan CL. Dynamic studies on physiological and biochemical changes during fruit development of *Panax notoginseng*. Chin Trad Herb Drugs. 2006, 37(7): 1086-1088.

57. Jo L, Pelletier JM, Harada JJ. Central role of the LEAFY COTYLEDON1 transcription factor in seed development. J Integr Plan Biol. 2019, 61(5):564-580.
58. Song J, Xie X, Chen C, Shu J, Thapa RK, Nguyen V, Bian S, Kohalmi SE, Marsolais F, Zou J, Cui Y. LEAFY COTYLEDON1 expression in the endosperm enables embryo maturation in *Arabidopsis.* Nat Commun. 2021, 12(1):3963.
59. Plancot B, Gugi B, Mollet JC, Loutelier-Bourhis C, Ramasandra GS, Lerouge P, Follet-Gueye ML, Vicre M, Alfonso C, Nguema-Ona E, Bardor M, Driouich A. Desiccation tolerance in plants: Structural characterization of the cell wall hemicellulosic polysaccharides in three Selaginella species. *Carbohydr Polym*. 2019, 208:180-190.
60. Battaglia M, Covarrubias AA. Late Embryogenesis Abundant (LEA) proteins in legumes. Front Plant Sci. 2013, 4:192.
61. Hayashi T, Kaida R. Functions of xyloglucan in plant cells. Mol Plant. 2011, 4(1):17-24.
62. Graeber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJJ. Molecular mechanisms of seed dormancy. Plant Cell Environ. 2012, 35(10):1769-1786.
63. Davière J, Achard P. Gibberellin signaling in plants. Development. 2013, 140(6):1147-1151.
64. Liepman AH, Nairn CJ, Willats WG, Sorensen I, Roberts AW, Keegstra K. Functional Genomic Analysis Supports Conservation of Function among Cellulose Synthase-like a Gene Family Members and Suggests Diverse Roles of Mannans in Plants. Plant physiol. 2007, 143(4):1881-1893.
65. Zhu XF, Shi YZ, Lei GJ, Fry SC, Zhang BC, Zhou YH, Braam J, Jiang T, Xu XY, Mao CZ, Pan YJ, Yang JL, Wu P, Zheng SJ. XTH31, encoding an in vitro XEH/XET-active enzyme, regulates aluminum sensitivity by modulating in vivo XET action, cell wall xyloglucan content, and aluminum binding capacity in *Arabidopsis*. Plant cell. 2012, 24(11): 4731-4747.
66. Cosgrove DJ. Loosening of plant cell walls by expansins. Nature. 2000, 407(6802):321-326.
67. Sechet J, Frey A, Effroy-Cuzzi D, Berger A, Perreau F, Cueff G, Charif D, Rajjou L, Mouille G, North HM, Marion-Poll A. Xyloglucan metabolism differentially impacts the cell wall characteristics of the endosperm and embryo during *Arabidopsis* seed germination. Plant physiol. 2016, 170(3):1367-1380.
68. Sampedro J, Guttman M, Li LC, Cosgrove DJ. Evolutionary divergence of β-expansin structure and function in grasses parallels emergence of distinctive primary cell wall traits. Plant J. 2015, 81(1):108-120.
69. Pan X, Welti R, Wang X. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat Protoc. 2010, 5(6):986-992.
70. Pertea M, Pertea GM, Antonescu CM, Chang T, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol. 2015, 33(3):290-295.
71. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Statist. Soc. B. 1995, 57(1):289-300.

72. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012, 40(15):e115.
73. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods. 2001, 25(4):402-408.

Figures

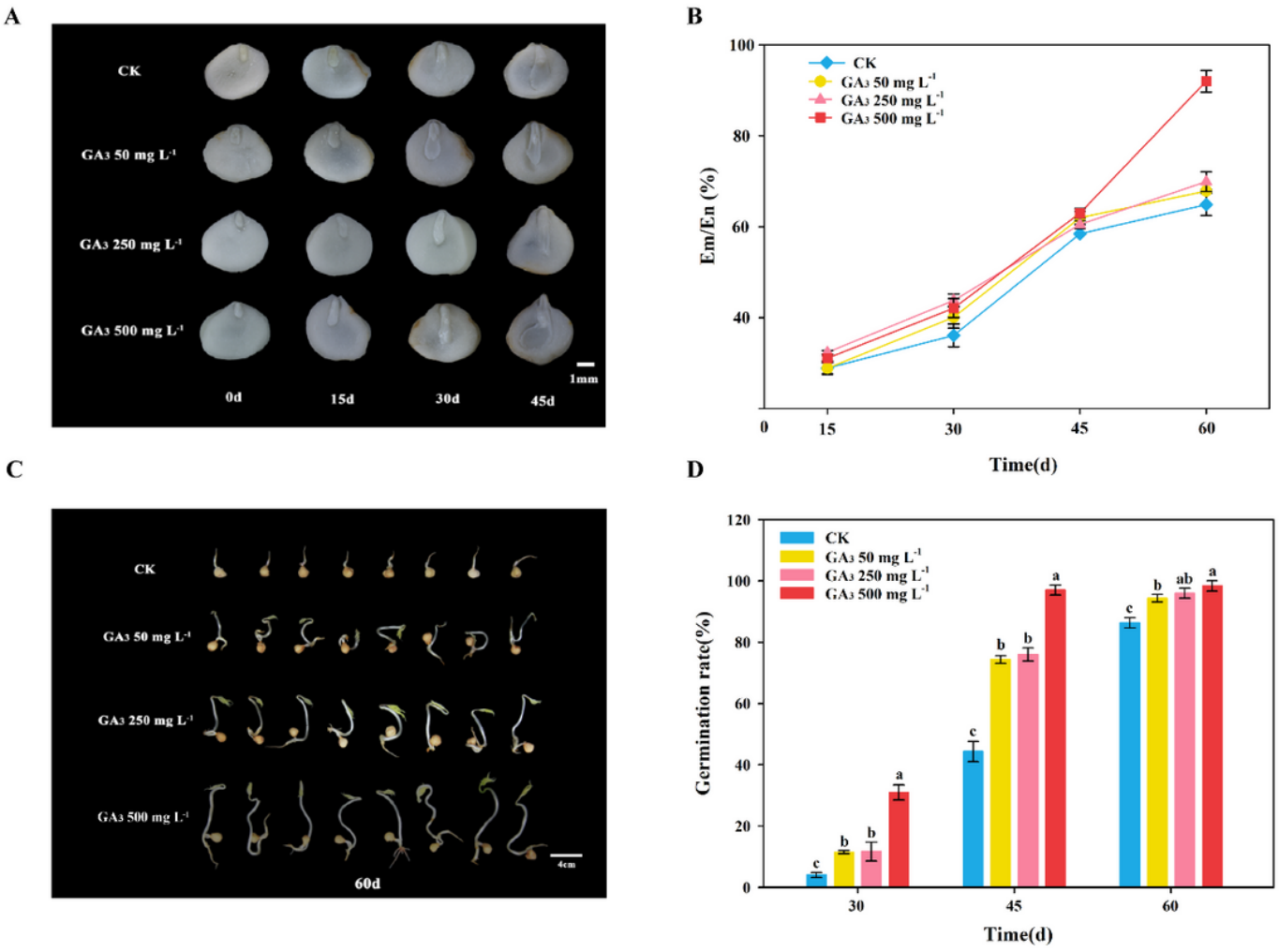

Figure 1

Application of exogenous GA_3 to promote seed germination of *P. notoginseng*. (A) Appearance and stereoscopic micrographs of control and GA_3-treated *P. notoginseng* seeds during the after-ripening process. (B) Changes in Em/En ratio of control and GA_3-treated *P. notoginseng* seeds during the after-ripening process. (C) Appearance and morphology of control and GA_3-treated *P. notoginseng* seeds after

germination (t=60d). (D) Changes in the rate of germination in control and GA_3-treated *P. notoginseng* seeds during the after-ripening process. The values presented are the means ± SE (n = 3). Different letters indicate significant differences among treatments in the same period using Duncan's test ($P < 0.05$).

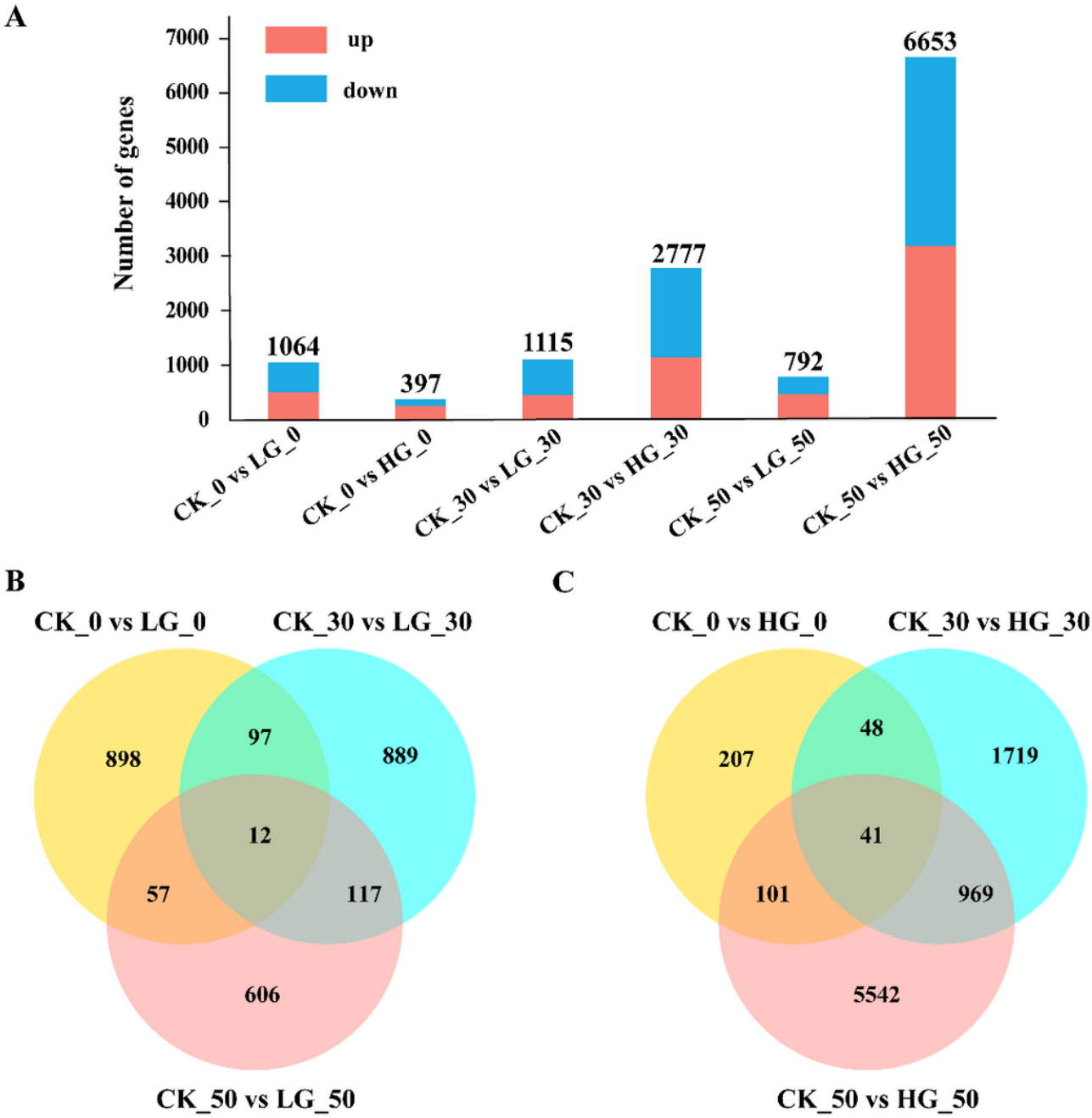

Figure 2

Statistical analysis of differentially expressed genes (DEGs) in control and GA_3-treated *P. notoginseng* seeds during the after-ripening process. (A) Histogram of DEGs. (B) Venn diagram showing the DEGs between the control and Low concentration GA_3-treated (LG) *P. notoginseng* seeds during the after-ripening process. (C) Venn diagram showing the DEGs between the control and the High concentration GA_3-treated (HG) *P. notoginseng* seeds during after-ripening process.

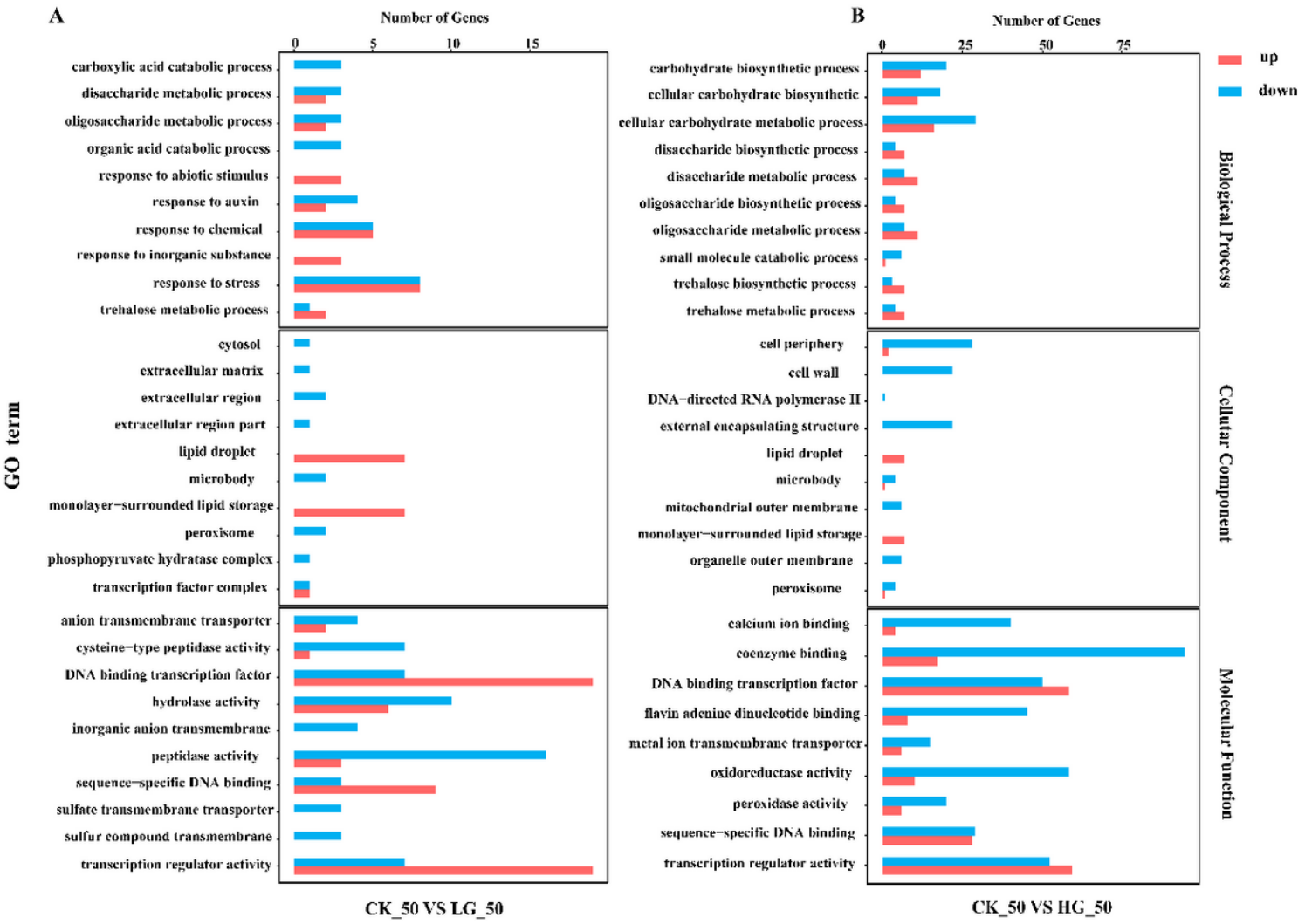

Figure 3

GO analysis of differentially expressed genes (DEGs) in control and GA_3-treated *P. notoginseng* seeds during after-ripening process. (A) Top 30 most enriched GO terms of DEGs between CK_50 VS LG_50. (B) Top 30 most enriched GO terms of DEGs between CK_50 VS HG_50. The Y-axis on the left represents GO terms, including biological process, cellular component, and molecular function, the X-axis indicates genes number of each term. Up-regulated genes are shown in the red bar, and down-regulated genes are shown in the blue bar.

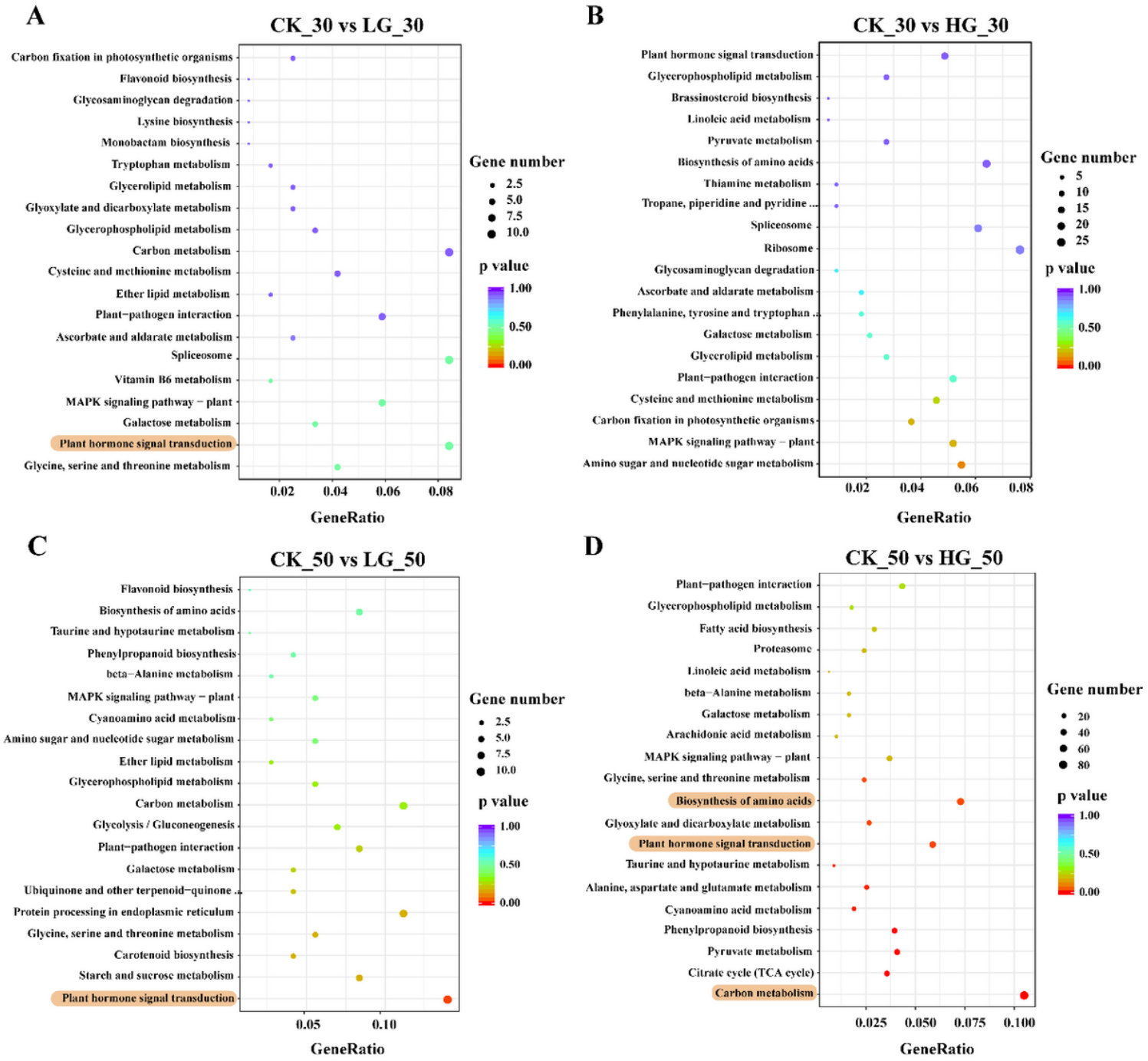

Figure 4

KEGG analysis of differentially expressed genes (DEGs) in control and GA_3-treated *P. notoginseng* seeds during the after-ripening process. (A) Top 20 most enriched KEGG pathways of DEGs between CK_30 vs LG_30. (B) Top 20 most enriched KEGG pathways of DEGs between CK_30 vs HG_30. (C) Top 20 most enriched KEGG pathways of DEGs between CK_50 vs LG_50. (D) Top 20 most enriched KEGG pathways of DEGs between CK_50 vs HG_50. The Y-axis on the left represents GO KEGG pathways, the X-axis indicates the “Gene Ratio” represented by the ratio of DEGs numbers to the total annotated gene numbers of each pathway. Low *P* values are shown in the red circle, and high *P* values are shown in the purple circle. The area of a circle represents DEGs number.

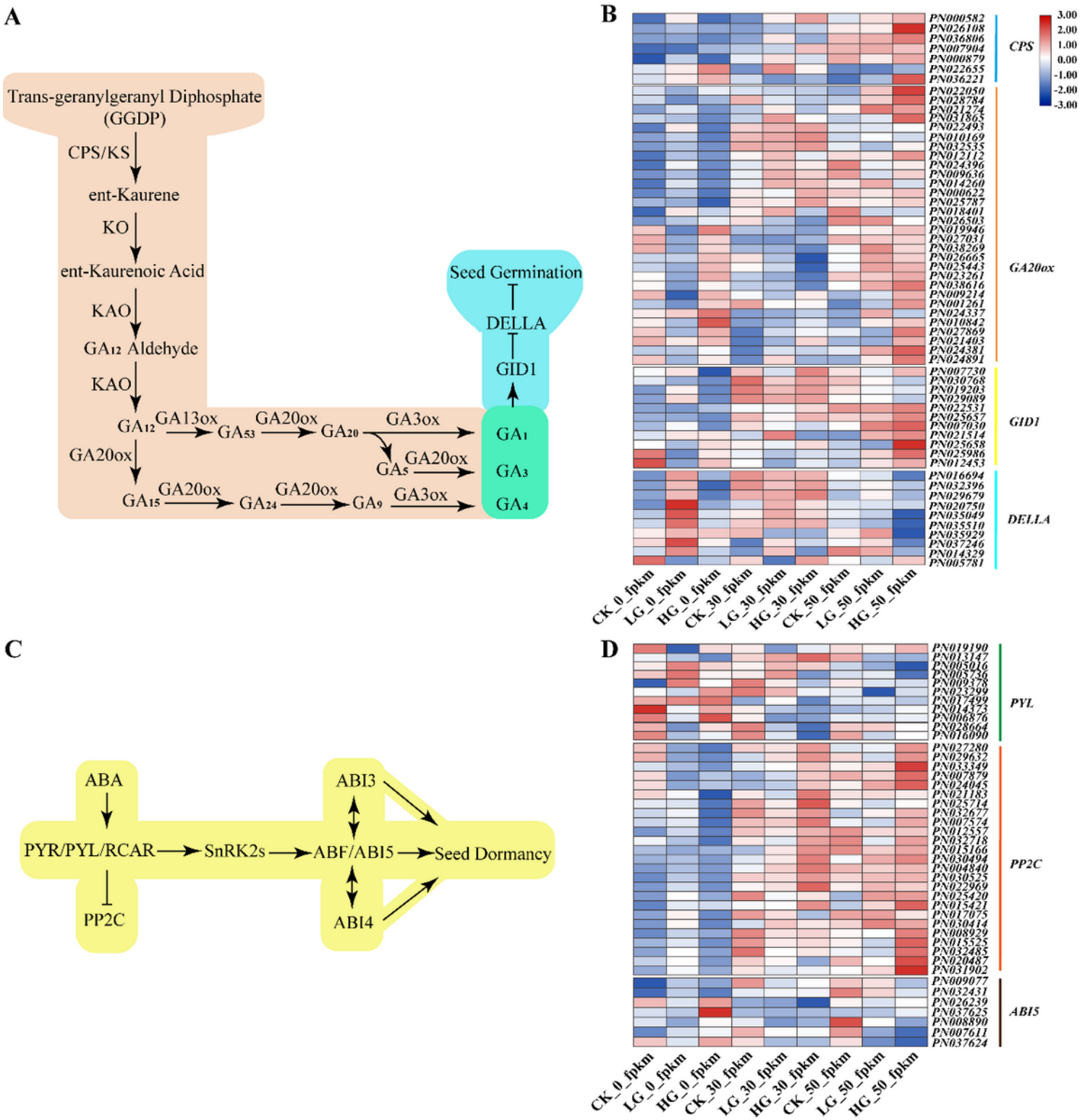

Figure 5

Expression pattern analysis base on RNA-seq of GA and ABA biosynthesis and signal transduction pathway-related genes. (A) The pathway of GA biosynthesis and signal transduction. (B) Heat maps of genes associated with GA biosynthesis and signal transduction of *P. notoginseng* seeds treated with GA_3 during the after-ripening process. (C) The pathway of ABA signal transduction. (D) Heat maps of genes associated with ABA signal transduction of *P. notoginseng* seeds treated with GA_3 during the after-

ripening process. In the heat map, different color indicates the expression level changes in GA_3-treated seeds compared with the control during the after-ripening process.

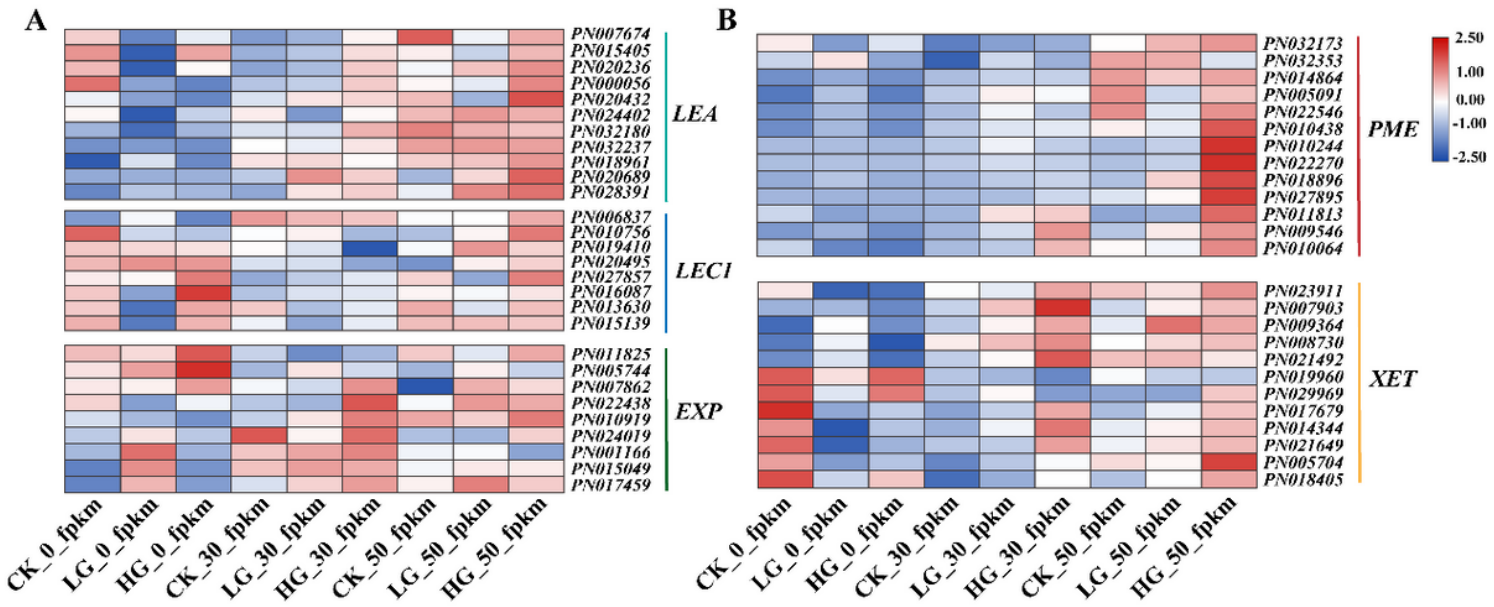

Figure 6

Effects of GA_3 on profiles of transcriptome of *P. notoginseng* seeds during the after-ripening process. (A) Heat map showing the expression patterns of the candidate genes involved in embryo development in *P. notoginseng* seeds. (B) Heat map showing the expression patterns of the candidate genes involved in cell wall metabolism in *P. notoginseng* seeds. In the heat map, different color indicates the expression level changes in GA_3-treated seeds compared with the control during the after-ripening process.

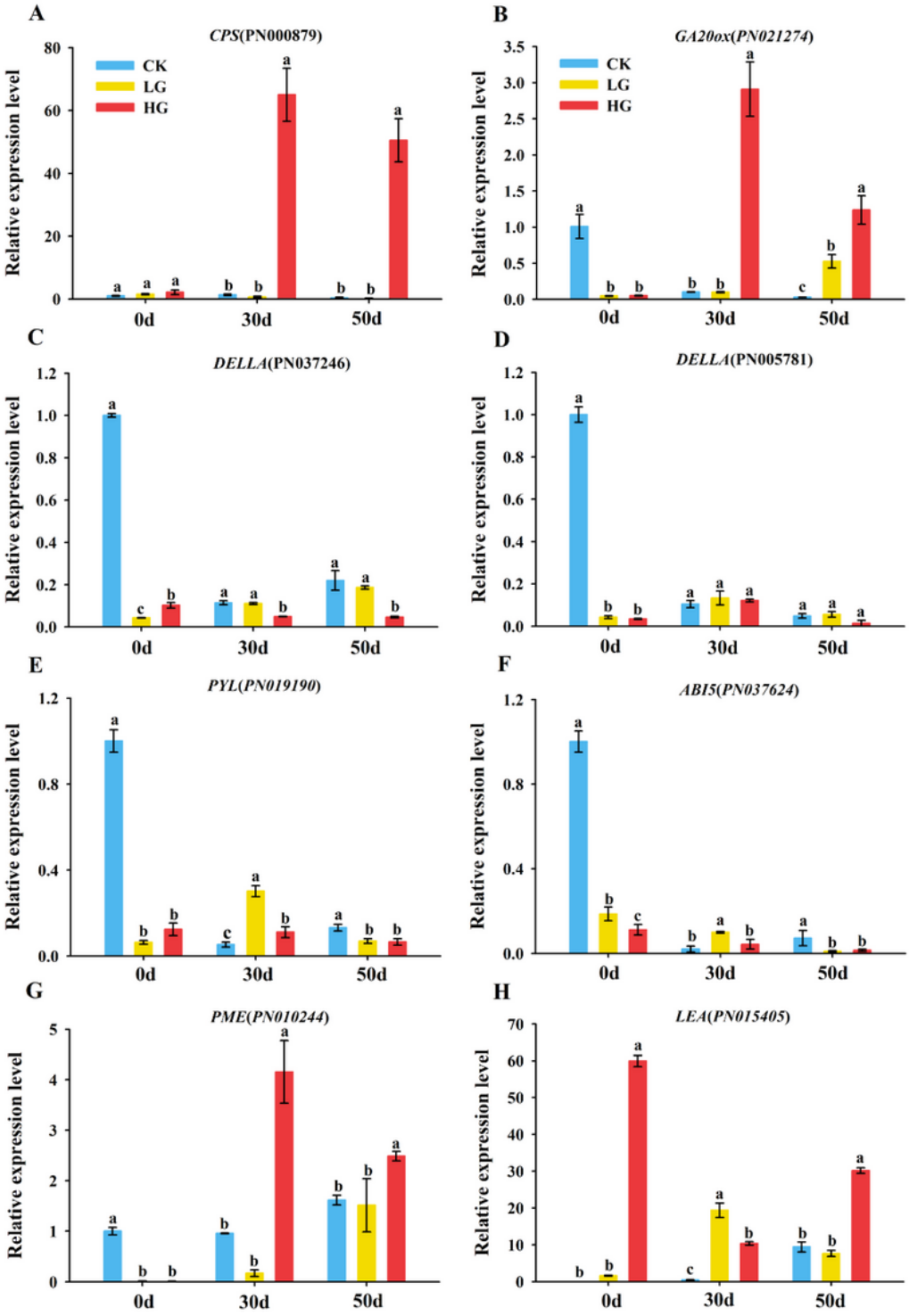

Figure 7

The DEGs expression in response to GA_3 treatment of *P. notoginseng* seeds during the after-ripening process. Genes expression was analyzed by RT-qPCR. (A) *CPS*, *ent-copalyl diphosphate synthase*. (B) *GA20ox*, *GA20-oxidase*. (C, D) *DELLA*. (E) *PYL*, *Pyrabactin resistance 1-like*. (F) *ABI5*, *ABA-INSENSITIVE5*. (G) *PME*, *Pectinesterase*. (H) *LEA*, *Late Embryogenesis Abundant*. The values presented are the means ±

SE (n = 3). Different letters indicate significant differences among treatments in the same period using Duncan's test ($P < 0.05$).

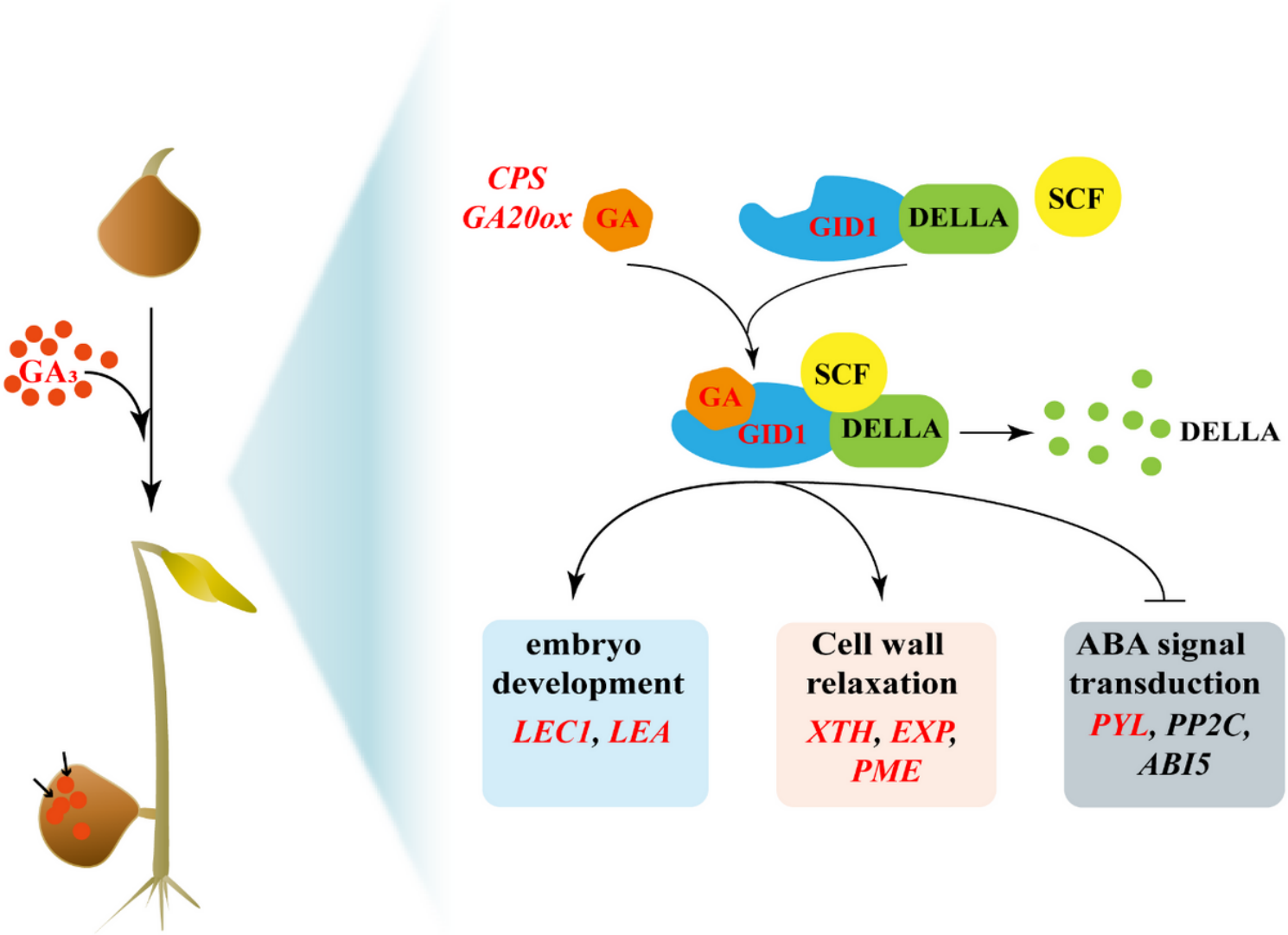

Figure 8

A model for the possible mechanism of exogenous GA_3 regulation of germination in *P. notoginseng* seeds during the after-ripening process at the transcriptional levels. Genes marked in red indicate that these genes were GA-induced, and likewise, black ones suggest that the genes were GA-repressed. Arrows and blunted lines designate positive and inhibitory interactions, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 04Supplementaryfigures.docx
- 05Supplementarytables.docx