Exogenous Gibberellin Promotes the Germination of *Phellodendron Chinense* Schneid Seeds by Regulating the Expression of Phytohormone Related Genes and Anabolic Proteins

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Research Article

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Abstract

Background: *Phellodendron chinense* Schneid is an important Chinese herb that contains berberine, phellodendrine, palmatine, and medicinal compounds. The germination rate of *Phellodendron chinense* Schneid seeds is lower after storage, and the exogenous gibberellin3 (GA_3) hormone promotes seed germination, but the mechanism is not cleared.

Results: Exogenous GA_3 hormone promoted germination of *Phellodendron chinense* Schneid seeds, elevated germination rates. It also increased the levels of activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Moreover, it enhanced the contents of berberine and endogenous GA_3, and increased the expression levels of *Pc(S)-GA2ox, Pc(S)-GA3ox*, and *Pc(S)-THBO*. However, it reduced the expression level of *Pc(S)-ABI5.* Furthermore, exogenous GA_3 up-regulated the protein levels of DNA guides RNA polymerase β'-subunits, Coffee coenzyme A oxymethyl transferase, and Histone H1.

Conclusion: These findings indicated that exogenous GA_3 promoted germination of *Phellodendron chinense* Schneid seeds by regulating the expression levels of phytohormone related genes and anabolic proteins.

Background

Phellodendron chinensis is a deciduous perennial tree species of Rutaceae, also known as Huangbai [1]. In China, there are two species and one variety, *Phellodendron amurense* Rupr, *Phellodendron Chinense* Schneid Var glabriusculum Schneid and *Phellodendron chinense* var meiguense Stian-lun Dai et Hong Wu [1]. The *Phellodendron amurense* Rupr is mainly distributed in the three eastern provinces, and the *Phellodendron chinense* Schneid is mainly distributed in Hubei, Hunan, Chongqing, Sichuan, and Yunnan provinces [2]. *Phellodendron chinensis* contains alkaloids and other medicinal compounds, which have been used for more than 2000 years in china [2]. The levels of the medicinal ingredients in the stem stark of *Phellodendron chinense* Schneid are higher than *Phellodendron amurense* Rupr. *Phellodendron chinense* Schneid mainly contains alkaloids, flavonoids, phenols, and terpenoids, with the alkaloids being the key medicinal compounds, which include berberine and phellodendrine [3, 4]. These two compounds have important physiological functions, such as immune modulation, anti-inflammatory, antimicrobial, antibacterial, anticancer, hypotensive, antiarrhythmic, antioxidant, antigastric ulcer, and antipyretic agents [5, 6].

Seed germination marks the beginning of plant growth and development, and is affected by the availability of water, oxygen, temperature, light and plant growth regulators [7]. Among them, the gibberellin3 (GA_3) plant growth regulator hormone belongs to diterpenoids that plays a key role during seed germination, vegetative growth, carbohydrate transformation, prevention of abscission, abloom period and accumulation of medicinal compounds [8, 9]. It has been documented that exogenous GA_3 promotes the seed germination of *F. hupehensis*, increases the levels of endogenous trans-Zetain (ZT) and GA_3 contents, but decreases the levels of endogenous abscisic acid (ABA) [10]. Another study

showed that exogenous GA_3 (1 μM) enhances the germination rate of *Stevia rebaudiana*, and improves the development of seedlings [11]. In *Phellodendron chinense* Schneid, low temperature storage gradually decreases the seed germination rate [12, 13]. However, the physiological mechanism through which exogenous GA_3 regulates *Phellodendron chinense* Schneid seed germination remains unclear. Herein, we analyzed the seed germination rates, endogenous phytohormone contents, gene expression levels, and protein expression levels, in order to clarify the physiological mechanism underlying the regulation of seed germination in *Phellodendron chinense* Schneid by exogenous GA3.

Methods

Materials

The seeds of *Phellodendron chinense* Schneid were picked from Xiangxi Autonomous Prefecture (Hunan province), and identified by professor Jiaxiang Li (College of Forestry, Central South University of Forestry and Technology).

Seeds germination

Seeds germination referred to published methods[14]. The full seeds were selected, and immersed in concentrated sulfuric acid (Sinopharm, CHN) for 30 min for shelling, than disinfected in 75% alcohol (Sinopharm, CHN) for 45 s, and washed three times using sterile distilled water. Subsequently, the seeds were sterilized in 0.1% HgCl (*w/v*, Sinopharm, CHN) for 20 min, and washed five times using sterile distilled water. Finally, one batch of the seeds was transferred to sterile distilled water (H_2O, control group) and another batch into 1 $mg{\cdot}L^{-1}$ Gibberellin3 (GA_3) solution (experimental group) for 24 h, respectively. The seeds were cleaned with sterilized filter paper, and transferred to Murashige and Skoog's (Murashige and Skoog, 1962, MS) medium (agar 0.8%, sugar 3%, pH5.8, Sinopharm, CHN) on a sterile ultra-clean table for culturing and germination. Each group had 20 bottles (20 seeds/bottle), photos were taken, and the germinating seeds were counted, and then collected materials of seeds at 0 d, 3 d, 6 d, and 9 d. At the same time, the seeds were collected and frozen in -80℃refrigerator for the analyses of enzyme activities, determination of endogenous phytohormone contents, detection of gene expression levels, and proteomic analysis. All the seeds were cultured in the tissue culture room and the culturing conditions were; in tissue culture room with temperature 25±1℃, light period 12 h light/12 h dark, light intensity 1000-1500 Lux. All the experiments were conducted in three replicates (n=400/20 bottles).

Enzyme activity assay

The collected seeds (0.2 g) were placed into a pre-cooled mortar containing 1 mL pre-cooled phosphate buffer (0.05 $mol{\cdot}L^{-1}$), and then ground to pulp. Finally, the volume was adjusted to a fixed volume of 8 mL using phosphate buffer. The extracts were transferred to the centrifugal tube, and centrifuged at 12000 g at 4℃ for 20 min; the supernatants were the enzyme extracts. The analysis of the activities of superoxide

dismutase (SOD), peroxidase (POD), and catalase (CAT) were performed as previously described [15-17]. All the experiments were conducted in three replicates (n=3).

Berberine and phellodendrine analyses

The extraction and determination of berberine and phellodendrine in *Phellodendron chinense* Schneid seeds was conducted as previously described [18, 19]. The standards of berberine and phellodendrine were purchased from Beijing Solarbio Science & Technology Company of China. All the experiments were performed in three replicates (n=3).

Endogenous phytohormones assay

Endogenous phytohormones assay referred to published methods [20]. Firstly, the collected seeds were ground into powder in liquid nitrogen, and then 100 mg samples were placed into pre-cooled centrifuge tubes containing 1.5 mL extraction solution (50% acetonitrile containing 0.3 ng ABA isotope as the internal standard, Sigma, USA). Afterwards, the samples were ultrasonicated for 3 mins under ice bath conditions, then placed into the rotating instrument overnight at 4℃. Thereafter, the samples were centrifuged at 13000 rpm at 4℃ for 10 min, and the supernatants were transferred into new centrifuge tubes (2 mL). Subsequently, the precipitation were re-dissolved by 1.0 mL extraction solution, and extracted again at 4℃ for 60 min, and then two supernatants were combined after centrifugation for determination. Then, the Oasis HLB RP (1 cc/30 mg, Merck, GER) column was activated by washing with 1mL methanol and 1mL water, followed by equilibration with 50% acetonitrile (ACN). The extracts were then added to Oasis HLB RP column, and the filtrates collected, followed by washing of the column with 30% (v/v) can. The filtrates were combined. The filtrates were dried with nitrogen at low temperature, then resuspended in 300 μL 30% acetonitrile (Sigma, USA) and filtered with 0.22 μm nylon membrane. The filtrates were analyzed by mass spectrometry. The ExionLC AD System of SCIEX company (USA) chromatographic system was used in the study. According to the properties of phytohormones, the Waters BEH C18 RP Column (1.7 μm, 2.1×150 mm) was used. The mobile phase was 0.01% formic acid water (A) - acetonitrile (B) (Sigma, USA). The elution gradient was 0-1 min, 5% B; 1-7 min, 5% -100% B; 7-9 min, 100% B; 9-9.3 min, 100% -5% B; 9.3-12 min, 5% B. The flow rate was 0.3 $mL \cdot min^{-1}$, the injection volume 10 μL, and the column temperature was 45℃.

UHPLC-ESI-MS/MS was used for qualitative and quantitative detection of plant hormones in the samples [21]. Mass spectrometry system (AB SCIEX QTRAP 5500, USA) detection system with electrospray ion source (ESI) in multi response monitoring (MRM) mode analysis. Mass spectrometric analysis conditions were as follows: air curtain gas (PSI): 40; temperature (centigrade): 600; spray gas 1 (PSI): 60. Gas 2 (PSI): 60; ionization voltage (V): -4500 or +5500.

Gene Expression analysis

The conserved regions of the sequences of the *GA2-OXIDASE* (*GA2ox*), *GA3-OXIDASE* (*GA3ox*), ABSCISIC *ACID-INSENSITIVE 5* (*ABI5*), and *TETRAHYDROPROTOBERBERINE OXIDASE* (*THBO*) genes were cloned by

polymerase chain reaction (PCR) from leaves of *Phellodendron chinense* Schneid, and then primers designed and their stability for quantitative reverse transcription PCR (qRT-PCR) determined on an ABI 7500 (ABI, USA) detection system. The total RNA was extracted from the collected seeds using the RNA extraction Kit (OMEGA, USA). Afterwards, cDNA synthesis was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). Then, q-PCR reactions were performed with the SYBR Green Premix Pro Taq HS qPCR Kit (without ROX) (Accurate Biotechology(Hunan)Co.,Ltd, CHN). The expression levels of *Pc(S)-GA2ox*, *Pc(S)-GA3ox*, *Pc(S)-ABI5* and *Pc(S)-THBO* were determined on the ABI 7500 detection system according to operation procedure. Each qRT-PCR detection was standardized using the expression level of the *18S rRNA* gene. All experiments were conducted in three replicates (n=3). Expression levels were calculated using the Livak calculation method ($2^{-\Delta\Delta Ct}$) [22]. The gene specific primers used are listed in table 1.

Table 1 Primer names and sequences in experiments of gene expression

Primer names	Sequences (5'-3')	Lengths (bp)
qPc(S)-GA2ox-F	TAATGGTGATGTTGGTTGGA	20
qPc(S)-GA2ox-R	TAAAGAGGTGGTGTGTGTAGA	21
qPc(S)-GA3ox-F	GGTAAGTTCCGACCCGCA	18
qPc(S)-GA3ox-R	AATACGAGGAGCACAAAGCC	20
qPc(S)-ABI5-F	CGCAGGAATCGTAAGTGAGC	20
qPc(S)-ABI5-R	CGAAGAAAACCAAGCCGTC	19
qPc(S)-THBO-F	TCTGTTGCGGTCCGATTTC	19
qPc(S)-THBO-R	ATAACCCAAGGTATGCCCA	19
18S rRNA-F	GTGTTGCTTACCCACGAAA	19
18S rRNA-R	AAGGGCACAAGGCGGAT	17

Proteomics analysis

The proteins from the collected seeds of *Phellodendron chinense* Schneid were homogenized in PBS (pH 7.6) containing 65 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl, and 3 mM NaN_3 (Sinopharm, CHN) by using a glass pestle and mortar. Subsequently, the proteins were separated using two-dimensional electrophoresis and identified by mass spectrometry referred to published method [23].

Data analysis

The Origin 2018 and IBM SPSS Statistics 22 softwares were used to perform data analyses.

Results

Exogenous GA_3 promotes seed germination

With the prolongation of culturing periods, the germination rates of the *Phellodendron chinense* Schneid seeds treated with H_2O and exogenous GA_3 increased gradually (Fig. 1I). The seeds treated with H_2O (control) began to germinate on the third day, and had few hypocotyls (Fig. 1B). By the 6^{th} day of culturing, most of the seeds had germinated, and had many short hypocotyls (Fig. 1C). Furthermore, the cotyledons were observed on the 9^{th} day; however they were short (Fig. 1D). The germination rates of the seeds under H_2O treatment were 0%, 13.27%, 52.33%, 73.62% at 0, 3, 6, 9 days, respectively. The seeds treated with GA_3 also began to germinate on 3^{th} day, and the germination rate was 21.63%, i.e., GA_3 enhanced germination by 61.37% compared with control (Fig. 1F). The seeds under the 6-day culturing, exhibited a germination rate of 75.26%, hence GA_3 increased germination by 22.93%, and the hypocotyls were longer compared with control (Fig. 1G). On 9^{th} day of culturing, the seed germination rate was 83.36%, GA_3 improving germination by 9.75% compared with control at the same period (Fig. 1H). Furthermore, the cotyledons and hypocotyls under GA_3 treatment were larger and longer than in the control group. These results showed that exogenous GA_3 promoted the germination of *Phellodendron chinense* Schneid seeds, and elevated its germination rates.

Exogenous GA_3 enhances enzyme activities

During the culturing times of 0-9 days, the SOD activities in the *Phellodendron chinense* Schneid seeds treated with H_2O and exogenous GA_3 all gradually increased, and then began to decrease after reaching the maximum on the 6^{th} day (Fig. 2A). Culturing on the 6^{th} day, the SOD activity in seeds under H_2O treatment was 6394.08 $U{\cdot}min^{-1}{\cdot}g^{-1}FW$, which was 1.47-fold compared with that of the culturing at the initial stage. On the 9^{th} day, the SOD activity began to decrease, and it was 1.34-fold compared with that of the initial period culturing. The highest peak of SOD activity of *Phellodendron chinense* Schneid seeds treated with GA_3 also appeared on the 6^{th} day. The activity was 8239.37 $U{\cdot}min^{-1}{\cdot}g^{-1}FW$, which was 1.39-fold and 1.29-fold compared with that of the initial stage and control group at the same stage, respectively. Culturing on the 9^{th} day, the SOD activity began to decrease, reducing by 42.38% compared with that of the initial period, and decreased by 41.69% compared with control group on 9^{th} day. Notably, the SOD activities in the seeds treated with GA_3 were higher compared with control group during the culturing period of 0-6 days.

During the entire culture periods, the POD activities in the *Phellodendron chinense* Schneid seeds treated with H_2O and exogenous GA_3 increased gradually, and reached the peak on the 9^{th} day (Fig. 2B). In the control group, culturing for 0, 3, 6 and 9 days the POD activities in the seeds were 2058.05, 26532.20, 50190.86 and 60533.00 $U{\cdot}min^{-1}{\cdot}g^{-1}FW$, respectively. Moreover, the POD activities at 3, 6, 9 days were 12.89-, 24.39-, and 29.41-fold, respectively compared with that at 0 day. When the seeds were treated with

GA_3, the POD activities were 23303.05, 64946.67, and 77722.91 $U \cdot min^{-1} \cdot g^{-1}FW$ at 3, 6, 9 days, respectively, which increased by 30.63-, 87.16-, and 104.50-fold compared with that at 0 day. Besides, the POD activities in the experimental group seeds at 0, 3, 6 and 9 days were 35.79%, 87.83%, 129.40% and 128.40%, respectively, compared with that of the control group over the same culturing times. Furthermore, the result showed that the POD activity in seeds under GA_3 treatment were higher than the control during late germination (6-9 days).

With the prolongation of the incubation time, the CAT activities of *Phellodendron chinense* Schneid seeds treated with H_2O and GA_3 also increased gradually, and reached the peal on the 9^{th} day (Fig. 2C). During culture period, the CAT activities in the seeds under H_2O treatment were, 1139.67, 1360.54 and 2058.58 $U \cdot min^{-1} \cdot g^{-1}FW$ at 3, 6, 9 day, which were 2.21-, 2.64- and 3.99-fold, respectively, compared with that at 0 day. After GA_3 treatment, the CAT activities in the seeds were 1580.42, 2668.22 and 2774.72 $U \cdot min^{-1} \cdot g^{-1}FW$ at 3, 6, 9 day, which were 2.30-, 3.88- and 4.04-fold, respectively, compared with initial stage. Compared with control group, the CAT activities were 1.33-, 1.39-, 1.96- and 1.35-fold at the same stage, respectively. The result indicated that the CAT activities of *Phellodendron chinense* Schneid seeds treated with GA_3 were higher than those in the control during in all the culture periods.

Exogenous GA_3 promotes the accumulation of berberine

During times of germination, the levels of berberine in the *Phellodendron chinense* Schneid seeds treated with H_2O reached their peak at the 3^{rd} day (19.87 $ng \cdot g^{-1}DW$), which was 2.24-fold compared with the levels in the initial stage, then began to decrease gradually (Fig. 3). Culturing for 9 days, the berberine level was 15.89 $ng \cdot g^{-1}DW$. This was 1.95-fold and 0.80-fold compared with that at 0 day and 3 day, respectively. Culturing at the 3^{rd} day, the content of berberine in seeds treated with GA_3 reached the maximum, which was 2.24-fold higher compared with that at 0 day, and then began to reduce gradually. Germination on the 9^{th} day, the berberine content was 2.15-fold compared with that of initial stage, but it was higher than that of control group at the same time. The contents of phellodendrine in the seeds treated with H_2O increased gradually, and reached the peak at the 9^{th} day. The phellodendrine content under the H_2O treatment was 2.61-, 1.70- and 1.52-fold on the 9^{th} day compared with that at 0, 3, 6 day, respectively (Fig. 3). The contents of phellodendrine in seeds under GA_3 treatment improved gradually, and reached its peak on the 9^{th} day, which was 3.35-, 1.26- and 1.26-fold compared with culturing for 0, 3, 6 days; however, the level of phellodendrine was lower than that of control group at the same culturing periods.

Exogenous GA_3 enhances the content of endogenous GA_3

The results showed that the contents of endogenous GA_3 in seeds under H_2O and GA_3 treatment were 882.27 $ng \cdot g^{-1}FW$ and 14927.81 $ng \cdot g^{-1}FW$ at 24 h; the latter was enhanced 14.92-fold compared with former (Fig. 4A). Under the same conditions, the contents of ABA in seeds were 6.94 $ng \cdot g^{-1}FW$ and 7.18

ng·g^{-1}FW, with no significant difference reported (Fig. 4B). These results indicated that exogenous GA_3 significantly enhanced the contents of endogenous GA_3 during seed germination.

Exogenous GA_3 regulates gene expression

During 0-6 day, the expression levels of *Pc(S)-GA2ox* in the seeds under GA_3 treatment gradually increased, which were 10.88-, 29.31-, 139.89-fold compared with those under the H_2O treatment, and were 2.69- and 12.86-fold, respectively, compared with those of the GA_3 treatment at 0 day. Then, the expression level of *Pc(S)-GA2ox* was reduced at 9 day, but it was obviously higher than in the control. During the entire periods, the expression levels of *Pc(S)-GA3ox* gradually increased, and reached the peak at 9 day, which were 438.96-fold compared with that under the H_2O treatment, and were 141.60-fold compared with that under the GA_3 treatment at 0 day. The expression levels of *Pc(S)-ABI5* in the seeds treated with GA_3 were maximum and minimum on the 3rd day and 9th day, respectively, which were increased by 9.14-fold and reduced by 2.11-fold compared with the seeds in the GA_3 treatment at 0 day; however, they were higher than the H_2O treatment seeds at 0 day. During 0-6 day, the expression levels of *Pc(S)-THBO* in seeds under the GA_3 treatment gradually increased, and reached the maximum on the 6th day, which were enhanced by 1397.54-fold compared with the control (Fig. 5). These results indicated that exogenous GA_3 increased the expression levels of *Pc(S)-GA2ox, Pc(S)-GA3ox*, and *Pc(S)-THBO*, but inhibited the expression levels of *Pc(S)-ABI5*.

Proteomics analysis

The results showed that 22 different protein points were obtained in the H_2O treatment group, among which 14 proteins were related to energy metabolism and substance synthesis, and 3 proteins were related to resistance to virus invasion (Fig. 6 and Table 2). The expression levels of 13 identified proteins were down-regulated except for the up-regulated expression of protein TIC 214 on the 6th day in control group. However, among 3 proteins related to virus invasion, the expression level of ubiquitin folding modifier 1 was down-regulated on the 3rd day, while the expression level of antiviral protein S was continuously up-regulated on 3rd and 9th days. Furthermore, the expression level of the proteinase-chymotrypsin inhibitor CI-1A was up-regulated on the 6th day and down-regulated on the 9th day. Under GA_3 treatment, 6 different protein points were obtained, among which 3 proteins were related to substance synthesis, and 1 protein was related to substance metabolism. The coffee coenzyme A oxymethyl transferase was down regulated on the 3th day, but up-regulated on the 6th and 9th day. DNA guides RNA polymerase β'- subunit and 1, 4-alpha-glucan dismutase was identified on the 6th day. Except Coffee coenzyme A oxymethyl transferase, all of other 3 proteins were down regulated on the 9th day (Table 2).

The grouping of gels cropped from different parts of the different gels, using clear delineation either with white space

Table 2 Identification of differential proteins during seeds germination under exogenous GA_3 treatment

Dispose	Number	Protein Name	Germination time/d			
			0	3	6	9
H_2O	**Energy**					
	1	Thylakoid body cavity 13.8kDa protein	●	↓	↓	↓
	2	Citrate dehydrogenase	●	↓	↓	↓
	7	Ctyochrome C	●	↓	×	×
	8	Phosphoenolpyruvate carboxylase	●	↓	×	×
	9	Protochlorophyllate reductase	●	↓	×	×
	13	Oxygen release enhances protein 3-2	●	↓	×	×
	14	Protein TIC 214	●	↓	↑	↓
	23	Chloroplast 50S ribosomal protein L14	×	×	×	●
	24	isocitrate dehydrogenase	×	×	×	●
	Anabolic metabolism					
	3	9-diethylene ether synthase（DES）	●	↓	↓	×
	4	L-alanine-D/L-isomerase	●	↓	↓	×
	10	chalcone synthase	●	↓	↓	↓
	11	30S ribosomal protein S18	●	↓	↓	↓
	16	13- hydroxyl lupine O- transferase	●	↓	×	×
	Virus/Defense					
	12	Ubiquitin folding modifier 1	●	↓	×	×
	17	Antiviral protein S	●	↑	↑	↑
	18	Corn subtilis proteinase-chymotrypsin inhibitor CI-1A	×	●	↑	↓
	unknown					
	15	Unknown mitochondrial protein ymf11	●	↓	↓	×
GA_3	**Anabolic metabolism**					
	5	DNA guides RNA polymerase β'-subunits	●	↑	↓	↓
	6	Coffee coenzyme A oxymethyl transferase	×	●	↑	↑
	20	DNA guides RNA polymerase β'- subunit	×	×	●	↓
	21	DNA guides RNA polymerase β'- subunit	●	×	↑	↓

	22	Histone H1	●	×	↑	↓
	19	1, 4-alpha-glucan dismutase	×	×	●	↓

The symbol "●" means detected protein, and the symbol "×" means no detected protein. The symbol "↑" means the protein content is increased, and the symbol "↓" means the protein content is reduced.

Discussion

Seed germination is a very important stage during the processes of growth and development in plants, and is accompanied by a series of physiological and molecular changes, including enzyme activities, endogenous phytohormone contents, gene expression levels, and protein types [24]. This process is affected by plant growth regulators, water, and temperature, among others [24–27]. The plant growth regulators are divided into five classes, one of them being Gibberellin (GA), which is a diterpenoid compound, and the most active artificially synthesized hormone [28, 29]. A previous studies showed that the germination rate of *Primula beesiana* seeds under 15/5℃ treatment was less than 10%, but exogenous GA_3 significantly increased seed germination [30]. In this study, exogenous GA_3 significantly promoted seed germination, enhanced germination rate, promoted the growth and elongation of the hypocotyl in *Phellodendron chinense* Schneid (Fig. 1). At the same time, exogenous GA_3 increased the enzyme activity levels of POD and CAT, but reduced the enzyme activity level of SOD in the seeds at 9 day (Fig. 2). The exogenous GA_3 increases the permeability of the seed coat and the membrane through osmosis, accelerates lipid peroxidation of the cell membrane [31], and enhances the antioxidant enzyme activities to reduce the damage caused by reactive oxygen species, explaining the enhanced seed germination in *Phellodendron chinense* Schneid seeds. Similarly, exogenous GA_3 could promote the transformation and utilization of starch and lipid through inducing or increasing the activities of amylase and other enzymes related to the glyoxylic acid cycle and tricarboxylic acid cycle [32, 33] further promoting seed germination. Furthermore, exogenous GA_3 increased the content of berberine through elevating the expression level of *Pc(S)-THBO*, but reduced the phellodendrine content in seeds (Fig. 3). These results indicated that exogenous GA_3 promoted seed germination in *Phellodendron chinense* Schneid.

Seed germination is a complex and ordered reaction process, which is regulated by endogenous phytohormones, and the phytohormone contents are controlled by key synthesis enzymes and genes [34, 35]. The ratio of GA_3 and ABA in seeds is an important factor in seed germination, and their synthesis and accumulation are regulated by genes [31, 36]. Previous studies have shown that exogenous GA_3 promotes seed germination in Fraxinus by increasing the content of endogenous GA_3, reducing the content of ABA, and regulating the expression levels of genes related to the synthesis of GA_3 and ABA [10]. In the present study, the content of GA_3 in seeds under exogenous GA_3 treatment was significantly higher than the control group, but there was no significant change in the content of ABA (Fig. 4). The contents of GA_3 and ABA are related to the expression levels of *Pc(S)-GA2ox*, *Pc(S)-GA3ox*, and *Pc(S)-*

ABI5 genes. Our results showed that exogenous GA_3 obviously enhanced the expression levels of *Pc(S)-GA2ox* and *Pc(S)-GA3ox*, but significantly reduced the expression level of *Pc(S)-ABI5*, consistent with the contents of endogenous GA_3 and ABA in seeds under the GA_3 treatment (Fig. 5), as well as with the findings of a previous study [37]. It was further confirmed that exogenous GA_3 promotes seed germination by increasing the levels of endogenous GA_3 and the expression levels of *Pc(S)-GA2ox* and *Pc(S)-GA3ox*.

The protein kinases [38, 39], protein phosphatases, and transcription factor [40, 41] classes of protein serve to regulate many physiological processes [42]. Seed germination marks the starting point of plant growth and development, which is regulated by some proteins [43]. These proteins regulate catabolism [44], energy metabolism [45], anabolism, and defense reactions [46]. A previous study showed that rate of seed germination in *Arabidopsis midasin1-1* (*mdn1-1*) was decreased by increasing the expression levels of SEED STORAGE ALBUMIN (SESA)1/3/4/5, EMBRYO SAC DEVELOPMENT ARREST4, and ABI5 compared with wild type (Col-0)[47]. In the present study, 17 proteins were identified in the *Phellodendron chinense* Schneid seeds during germination under normal conditions. The expression levels of most of these proteins were decreased, especially ctyochrome C, phosphoenolpyruvate carboxylase, 30S ribosomal protein S18, and chalcone synthase, which are related to oxidative phosphorylation, seed bloating, ribosome assembly, and flavonoid synthesis, further reducing the germination rate of *Phellodendron chinense* Schneid seeds. Under exogenous GA_3 treatment, 4 proteins were identified in *Phellodendron chinense* Schneid seeds. These proteins regulated the RNA synthesis, nucleosome assembly, and catabolism of lipids and sugars. These results indicated that exogenous GA_3 enhances the expression levels of key proteins during the processes of energy metabolism and RNA metabolism (Fig. 6 and Table 2). It was further confirmed that exogenous GA_3 promotes *Phellodendron chinense* Schneid seed germination through some key proteins that accelerate energy metabolism and RNA synthesis.

Conclusion

Herein, we demonstrated that exogenous GA_3 promotes seed germination in *Phellodendron chinense* Schneid by increasing endogenous GA_3 levels, increasing the expression levels of *Pc(S)-GA2ox* and *Pc(S)-GA3ox* and proteins related to energy metabolism and RNA synthesis. However, seed germination is a complex and orderly process, with the molecular mechanism underlying the regulation seed germination in *Phellodendron chinense* Schneid by exogenous GA_3 remaining unclear. Therefore, further study using transcriptomics and yeast two hybrid technologies should be conducted in future to elucidate this mechanism.

Abbreviations

ABA
abscisic acid; ABI5:ABSCISIC ACID-INSENSITIVE 5; ACN:acetonitrile; CAT:catalase; GA:Gibberellin;GA_3:gibberellin3; GA2ox:GA2-OXIDASE; GA3ox:GA3-OXIDASE; *mdn1-1*:*midasin1-1*;

PCR:polymerase chain reaction; POD:peroxidase; qRT-PCR:quantitative reverse transcription PCR; SESA:SEED STORAGE ALBUMIN; SOD:superoxide dismutase; THBO:TETRAHYDROPROTOBERBERINE OXIDASE; ZT:*trans*-Zetain.

Declarations

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Authors' contributions

Wenkai SUN and Xuejing ZHANG performed the experiments, wrote the first draft; Yahan WANG and Huanhuan LI analyzed the data, prepared the figures; Wende YAN, Hanjie HE, Lili CHEN and Gongxiu HE conceived and designed the experiments, reviewed and corrected the paper, approved the final draft. All authors read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are available by contacting the corresponding author (hejie224@163.com).

Ethics approval and consent to participate

The authors declare that the study was carried out following scientific ethics and conduct.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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Figures

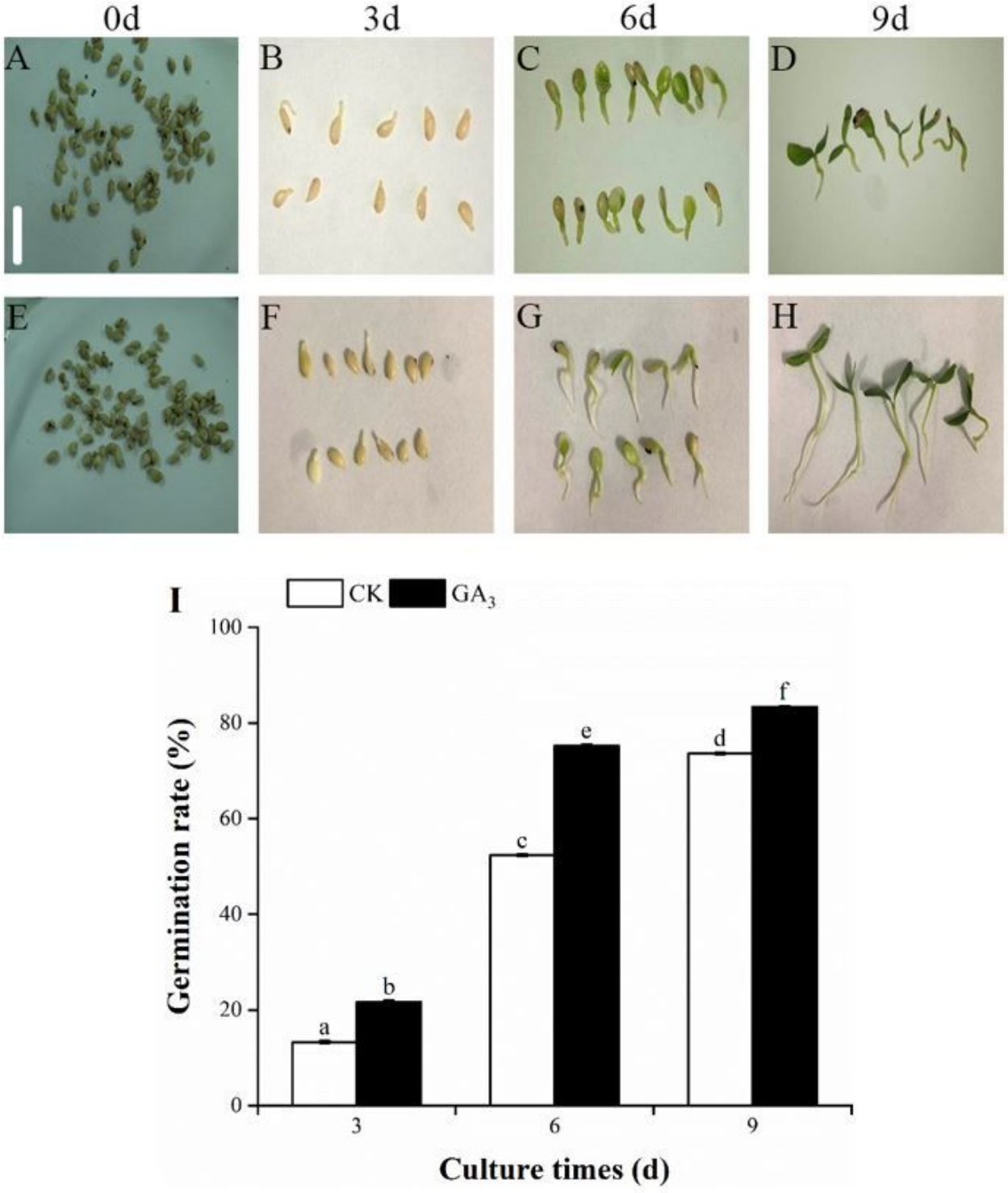

Figure 1

Germination rates of Phellodendron chinense Schneid seeds under H2O and GA3 treatment A, B, C and D: Seeds germination treatment with H2O at 0, 3, 6, 9 day. E, F, G and H: Seeds germination treatment with exogenous GA3 at 0, 3, 6, 9 day. Bar = 2 cm

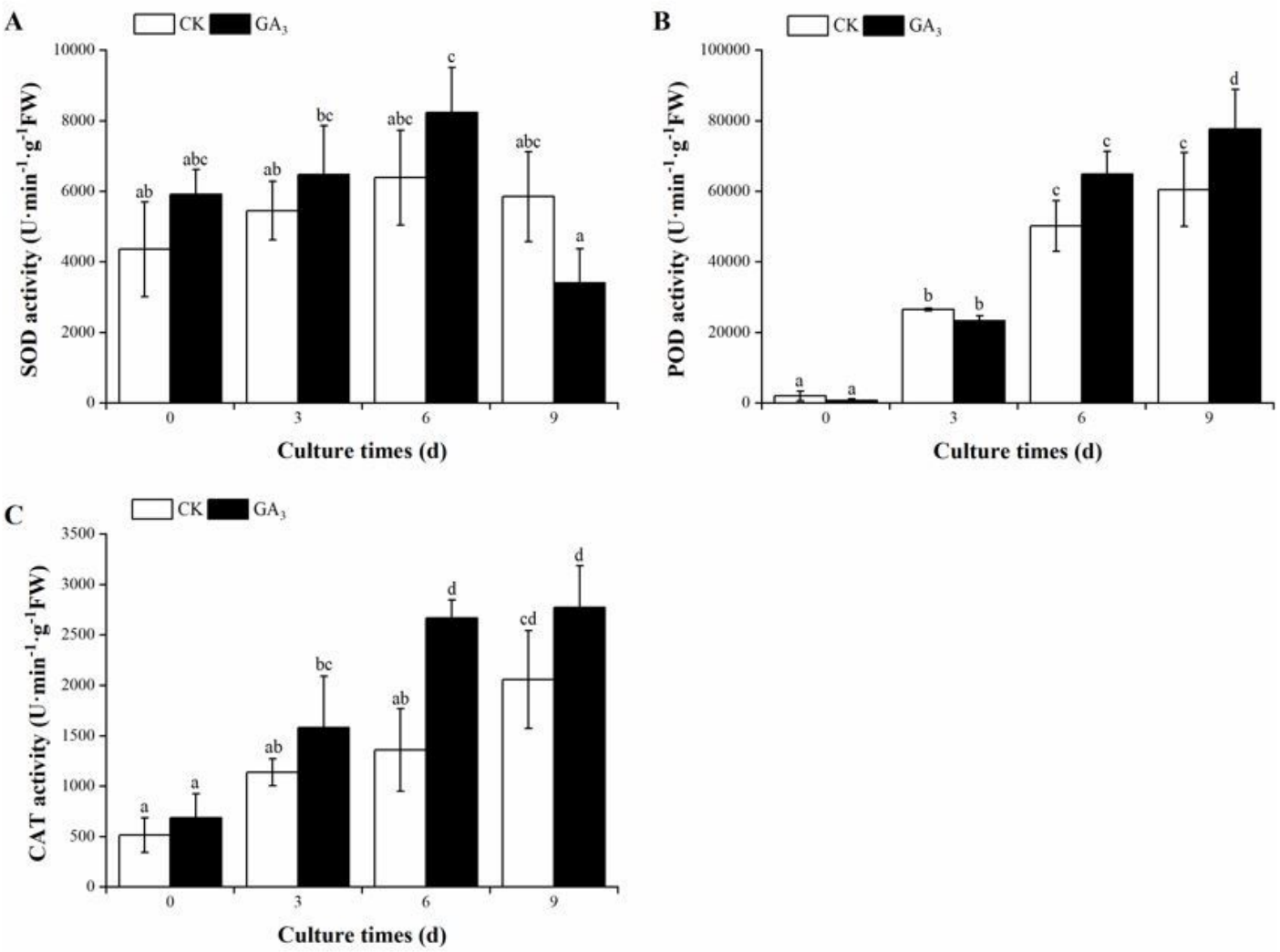

Figure 2

Enzyme activities of Phellodendron chinense Schneid seeds under H2O and GA3 treatment

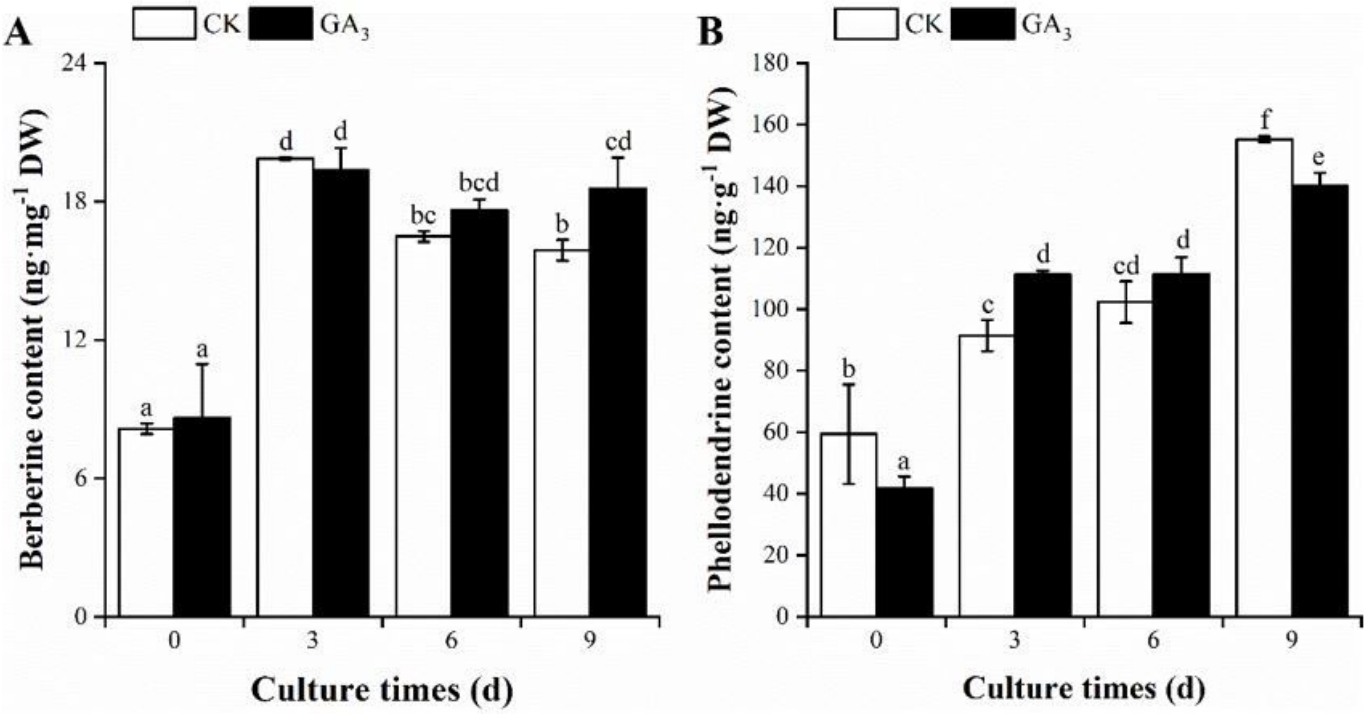

Figure 3

Contents of berberine and phellodendrine in seeds under H2O and GA3 treatment

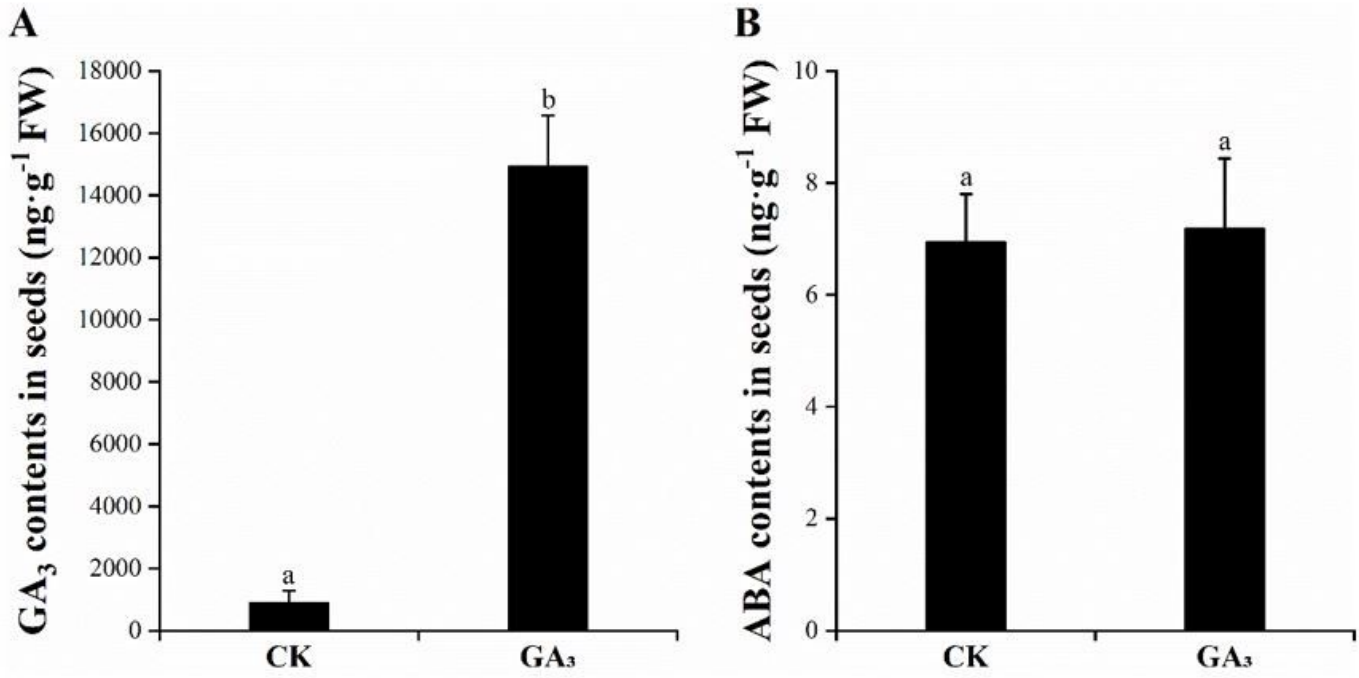

Figure 4

The contents of endogenous GA3 and ABA in seeds under H2O and exogenous GA3 treatment

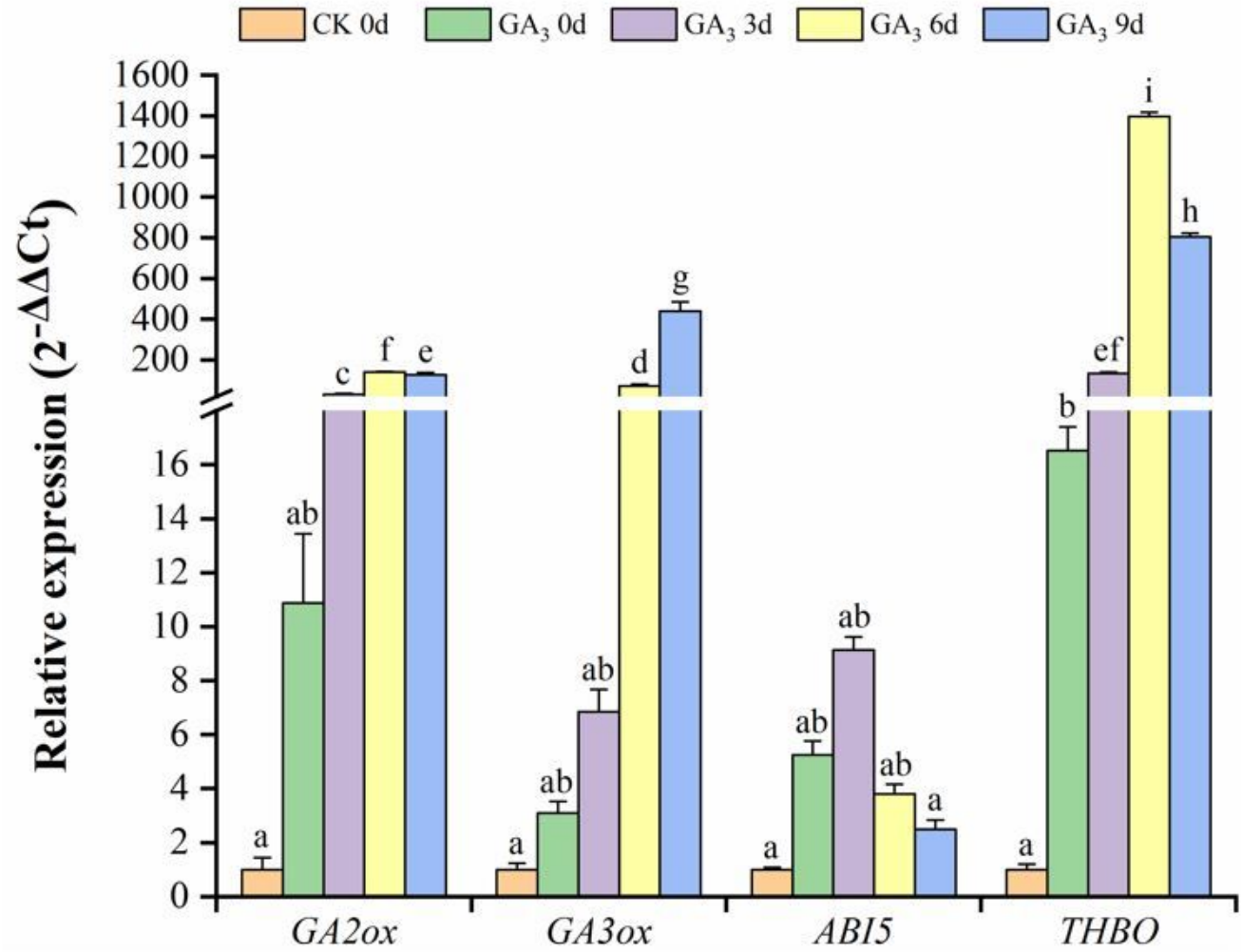

Figure 5

Relative expression levels of Pc(S)-GA2ox, Pc(S)-GA3ox, Pc(S)-ABI5 and Pc(S)-THBO in seeds under H2O and GA3 treatment

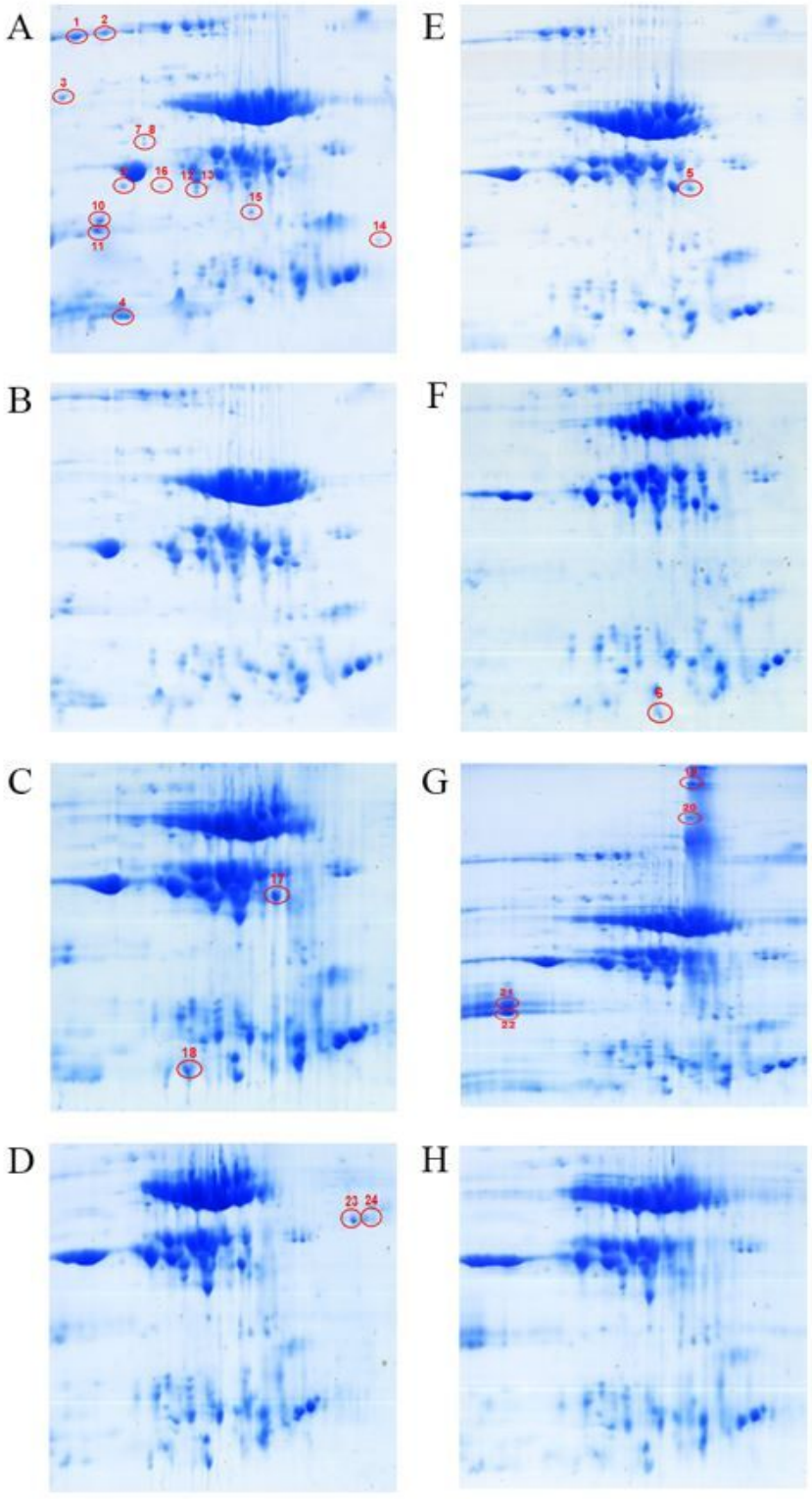

Figure 6

Proteomics analysis of Phellodendron chinense Schneid seeds under GA3 treatment A, B, C and D: proteomics analysis of Phellodendron chinense Schneid seeds under water treatment at 0 d, 3 d, 6 d and 9 d; E, F, G and H: proteomics analysis of Phellodendron chinense Schneid seeds under GA3 treatment at 0 d, 3 d, 6 d and 9 d

Supplementary Files

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