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Proteomic characterization of the different stages of seed germination in *Cupressus gigantea*

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Abstract

Seed germination is a pivotal period of plant growth and development. This process can be divided into four major stages, swelling absorption, seed coat dehiscence, radicle emergence and radicle elongation. *Cupressus gigantea*, a tree native to Tibet, China, is characterized by its resistance to stresses such as cold, and drought, and has a high economic and ecological value. Nevertheless, given its unique geographic location, its seeds are difficult to germinate. Therefore, it is crucial to explore the mechanisms involved in seed germination in this species to improve the germination efficiency of its seeds, thereby protecting this high-quality resource. Here, our findings indicate that seed germination was enhanced when exposed to a 6-h/8-h light/dark photoperiod, coupled with a temperature of 20°C. Furthermore, the application of exogenous GA_3 (1 mg/ml, about 2.9 mM) stimulated the germination of *C. gigantea* seeds. Subsequently, proteomics was used to detect changes in protein expression during the four stages of seed germination. We identified 34 differentially expressed proteins (DEPs), including 13 at the radicle pre-emergence stage, and 17 at the radicle elongation stage. These DEPs were classified into eight functional groups, cytoskeletal proteins, energy metabolism, membrane transport, stress response, molecular chaperones, amino acid metabolism, antioxidant system and ABA signalling pathway. Most of them were found to be closely associated with amino acid metabolism. Combined, these findings indicate that, along with temperature and light, exogenous GA_3 can increase the germination efficiency of *C. gigantea* seeds. Our study also offers insights into the changes in protein expression patterns in *C. gigantea* seeds during germination.

Introduction

As an endemic species, *Cupressus gigantea* W. C. Cheng & L. K. Fu, commonly known as the 'God tree', is a rare tree species endemic to China (Liyuan et al., 2009). It has a narrow distribution because it is restricted to the dry valleys of Yarlung Tsangpo River and Nyang River (Fumei et al., 2017). It has high ecological and economic value because of its contribution to afforestation and timber production and also because it is the source of Tibetan incense (Yunxing and Xing, 1999). *C. gigantea* has been listed as a threatened species in the International Union for Conservation of Nature's Red List. The lifespan of *C. gigantea* is approximately 1000 years. However, this species is currently on the verge of extinction due to extreme climatic conditions, its dispersed distribution and slow natural renewal (Guo et al., 2020). Accordingly, the mechanisms involved in *C. gigantea* reproduction must be investigated in-depth to allow the development of efficient conservation strategies (Lei et al., 2022). Previous studies on seed germination and seed viability in *C. gigantea* have shown that mature *C. gigantea* are poor reproducers (Wang et al., 2005), with only 20–30% of trees bearing fruit, and their seed germination and survival rates are low. Moreover, the seeds are severely affected by disease, birds and mice. To date, relatively few studies have reported on the viability of *C. gigantea* seeds, and further research on the characteristics of seed germination in *C. gigantea* is needed to better understand and conserve this endangered species.

Seed germination initiates with the absorption of water by dry seeds, followed by embryonic expansion and rupture of the covering layer and the emergence of part of the embryo, the protrusion of the radicle is typically regarded as the completion of the germination process (Wang et al., 2005; Smolikova et al., 2022). Germination can be broadly divided into three phases, an

initial period of rapid water uptake, a plateau period and a period of further increased water uptake (Bewley, 1997; Santo Pereira et al., 2021). In the early phase of seed germination, seed dormancy is broken by the activation of physiological processes, seed respiration and various metabolic pathways (Han and Yang, 2015). During the stabilization phase, seed metabolism gradually intensifies and the embryo begins to grow (Oracz and Stawska, 2016). Gibberellic acid (GA), a key hormone in seed germination, is also synthesized and released during this phase, and promotes the decomposition of the endosperm (Ye-Hong and Hai-Ming, 2009; Miura et al., 2023). In the third stage, the seed coat ruptures and the radicle begins to elongate. At this stage, the seed rapidly absorbs water to meet the water requirements for germination and seedling growth (Srivastava et al., 2021). Studies have shown that the different stages of seed germination are regulated by distinct sets of proteins (Zaynab et al., 2019). For instance, during seed germination, storage proteins are degraded and functional proteins are synthesized, and processes such as starch hydrolysis, sucrose transport and lipid metabolism are activated (Nonogaki et al., 2010). Accordingly, it is necessary to explore in-depth the expression patterns of proteins that affect seed germination through proteomics. In addition, the vitality of germinating seeds and germination conditions are critical parameters for the development of practical production ecology as well as for forestry applications, as they determine the seed germination rate and the survival rate of emerging trees under complex environmental conditions, respectively (Galland et al., 2014). A comparative analysis of the germination rate and germination potential of *C. gigantea* under different treatment conditions showed that, under optimal conditions, the germination rate of cypress seeds reached 44%, whereas, under non-optimized conditions, it was less than 30%. Therefore, understanding the seed germination characteristics of *C. gigantea* in Tibet can also contribute to solving the problems of seed germination in other forest trees.

The genetic and molecular mechanisms involved in seed germination have been extensively studied in a wide range of plants (Osama et al., 2021); however, little is known about these mechanisms of in *C. gigantea*. Seed development from dormancy to germination results in complex physiological and biochemical changes, including the synthesis of reserve proteins and the production of antioxidant and other stress-related proteins (Sghaier-Hammami et al., 2020). Proteomics is an effective method for studying the mechanisms underlying seed germination, and many studies have employed this technique for large-scale protein expression analyses during this process (Li et al., 2018), especially in model species such as *Arabidopsis thaliana* or important crops (Xu et al., 2016; Sanit, 2021). Two -dimensional gel electrophoresis (2-DE) and gel-free liquid chromatography–tandem mass spectrometry (LC–MS/MS) are the most frequently used proteomics platforms in plant biological research. It has been reported that, although 2-DE and LC–MS/MS can identify similar numbers of proteins, experimental conditions (IPG strips, pH range and size and staining methods) can significantly influence outcomes; accordingly, 2-DE and LC–MS/MS have been proposed to be complementary methods for analysing samples with complex protein profiles (Sghaier-Hammami et al., 2021). 2-DE combines two different electrophoresis methods and separates molecules according to the isoelectric point and molecular mass (Meleady, 2018), achieving sensitive and accurate protein quantification. This technique has been used to characterize the proteins involved in metabolic, redox homeostasis and phytohormone regulation during seed germination (He et al., 2011; Ren et al., 2017). 2-DE and MS have been employed for protein identification in several plant species (Stergaard et al., 2004; Nakamoto et al., 2014). Proteomics combined with 2-DE technology is more effective at directly targeting differentially expressed proteins (DEPs), which not only reduces costs but also improves protein detection accuracy.

Relevant transcriptomic and proteomic analyses in many species have previously provided an overview of mRNA and protein accumulation patterns during seed germination, such as *Arabidopsis thaliana* (Bai et al., 2017; Sajeev et al. 2022), rice (He et al., 2011; Sano et al., 2022), maize (Dinkova et al., 2011), etc. Nevertheless, little is known about the mRNA and protein expression profiles in *C. gigantea* during this process. In this study, a 2-DE-based proteomic analysis was undertaken on the seeds of *C. gigantea* to identify DEPs during different stages of seed germination. Our findings provide relevant and reliable proteomic data on *C. gigantea* seed germination.

Materials and methods

Seed germination

C. gigantea seeds were collected from the coastal areas of the Brahmaputra River in Tibet, China in November 2018. After drying, the seeds were sealed and stored at 0–8°C for 1 year. In this study, 200 seeds of average fullness and size were selected as experimental materials. The seeds were placed on moistened gauze in Petri dishes (8 cm diameter), stratified at 4°C for 2 days and then placed in a lighting incubator and rinsed with water three times a day. During stratification, the seeds were treated with different concentrations of GA_3 (0 [control], 1, 1.5, 2 and 2.5 mg/ml). Seeds were transferred to a lighting incubator with the light duration set to 0, 8 and 16 h and the incubation temperature set to 10, 15, 20, 25 and 30°C. Three biological replicates were set up and 100–150 seeds were cultured each time. Seeds were photographed at four different stages according to days after germination. The germination time was divided into four stages, namely, Stage A: day 1, the initiation stage of seed germination; Stage B: day 5, visible cracks in the seed coat; Stage C: day 9, completely broken seed coat; and Stage D: day 13, change in embryo colour.

Determination of seed length, protein content and fresh/dry weight

A Vernier calliper was used to determine seed length. Fresh and dry weights of the seeds were determined in 10 replicates. Fresh weight was measured immediately after harvest, and dry weight was measured after drying at 70°C for 24 h. Protein content was calculated using the Bradford method. Seeds were stored at −80°C for protein and RNA extraction.

Protein extraction

Two grams of seed were ground into a fine powder in liquid nitrogen and thoroughly mixed with TCA/acetone in 5 ml centrifuge tubes for 2 h. The homogenate was centrifuged at 13,500 rpm for 20 min at 4°C, and the precipitate was washed three times with 80% acetone. After discarding the supernatant, the precipitate was lyophilized dry, yielding crude protein. 0.1 g crude protein powder was extracted with 1 ml of water-saturated phenol

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containing 1 µl of 1 M DL-dithiothreitol (DTT) at room temperature for 1 h. After centrifugation at 13,500 rpm for 30 min at 4°C, the supernatant was transferred into a new tube. 4 ml of 1 M ammonium acetate in methanol containing 4 µl 1 M DTT were added to the supernatant overnight at −20°C. After centrifuging at 13,500 rpm for 5 min at 4°C, the precipitate was washed twice with 80 and 100% acetone, respectively, by centrifugation, and the sediment was dried to obtain the protein sample. The protein precipitate was dissolved in a buffer containing 0.1 M DTT, 10 mM EDTA and 1 M Tris-HCl (pH 7.5) for 1 h and centrifuged at 13,500 rpm for 30 min at 4°C. The resulting supernatant was the protein sample.

2-DE and image analysis

The total protein load from *C. gigantea* was 1.5 mg. First-dimension isoelectric focusing (IEF) separation was carried out on an IPGphor apparatus under the following conditions: 30 V for 13 h, 100 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 3,100 V for 1 h, 8,000 V for 1 h and finally, 8,000 V for 6 h. Subsequently, the IPG strips were equilibrated in 10 ml Buffer I, containing 0.1 g of DTT and 10 ml Buffer II, containing 0.15 g of iodoacetamide, respectively, for 15 min. Then, second-dimension separation of proteins was performed using 12.5% SDS–PAGE. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB). The samples were visualized using a GE Image Scanner III and analysed by Melanie 7.0 2-D gel analysis software. Protein spots showing 1.5-fold changes ($P < 0.05$) between samples were selected for further MS/MS analysis.

MALDI-TOF/TOF-MS and protein identification

The selected protein spots were excised and destained in 100 mM NH_4HCO_3 and acetonitrile, sequentially. The proteins were equilibrated in 10 ml buffer containing 25 mM NH_4HCO_3 at 37°C overnight. The next day, protein digestion was performed by adding 40 µl of trypsin solution (4 µg of trypsin in 40 µl of buffer) to the solution, the solution was shaken at 600 rpm for 1 min and then allowed to stand at 37°C for 16–18 h. The solution was transferred to a new collection tube and centrifuged at 14,000 × *g* for 15 min. Then, 40 µl of 10-fold diluted dissolution buffer was added, followed by centrifugation at 14,000 × *g* for 15 min, the addition of 40 µl of 10-fold diluted dissolution uffer and centrifugation at 14,000 × *g* for 15 min. Finally, the peptides were desalted using C18 ZipTips (Millipore, USA), lyophilized, redissolved in 40 µl of dissolution buffer and quantified (OD280). All samples were analysed in positive ion reflection mode on a 5800 MALDI-TOF/TOF mass spectrometer. The acquired mass spectra (*m/z* range: 700–4,000; resolution: 15,000–20,000) were processed using Flex Analysis v.2.4 software. To identify proteins, the mass signals were searched against the Swiss-Prot, NCBI and MSDB databases using the MASCOT search engine. The MS/MS fragment tolerance was set to ±0.5 Da. Cysteine aminomethylation was set as a fixed modification and methionine oxidation was set as a variable modification. Trypsin cleavage and K/R-P cleavage had a misidentification tolerance of 3. Confidence in matched peptide mass fingerprints ($P < 0.05$) was based on the MOWSE score. Sequences with more than two overlapping peptide fragments for a given feature were identified in the MOWSE score, which is considered homogeneous. The intensity values for the identified proteins were log10-transformed and compared based on the mean values of the groups. Hierarchical clustering of samples and proteins was performed using MeV 4.8 (http://www.tm4.org/mev.html). Proteins were functionally annotated and classified using the TargetP prediction tool.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Seed samples were ground to powder in liquid nitrogen and total RNA was isolated using the Omega Plant RNA Kit (Omega Genetics, Beijing, China) following the manufacturer's instructions. cDNA was prepared with the PrimeScript Reverse Transcriptase Kit (Takara, Japan). qPCR was performed in a LightCycler 480 System (Roche, USA) with LightCycler 480 SYBR Green I Master Mix and specific primers (Supplementary Table S1). The reactions were carried out in a 20 µl volume containing 2 µl of cDNA (50 ng/µl), 1 µl of primers (10 pmol), 10 µl of SYBR Green I Master Mix, and 6 µl of ddH_2O. Three biological replicates were used for each sample. The *18S rRNA* gene was used as a control. Relative gene expression levels were calculated using the $\Delta\Delta C_t$ method.

Data submission

MS data (identified spots) were translated to PRIDE XML using the PRIDE Submission Tool Version-2.7.3. A total of 34 PRIDE XML files were submitted to the ProteomeXchange repository following the ProteomeXchange submission guidelines (Simova-Stoilova et al. 2015). The data were deposited under the identifier PXD047123.

Statistical analysis

All data were analysed using SPSS 25 software. One-way analyses of variance (ANOVAs) with Duncan's multiple range tests ($P < 0.05$) and Tukey–Kramer Test were conducted in at least three independent experiments. Graphs were generated using GraphPad Prism 8.4 software.

Results

Optimization of seed germination conditions for C. gigantea

We first tested the effect of different incubation temperatures (10, 15, 20, 25 and 30°C) on seed germination under a uniform 16-h light/8-h dark photoperiod. Incubation at 20 and 25°C significantly accelerated the seed germination rate at early germination time compared to 10°C incubation, resulting **in** the seed germination rate reaching 93.51, 96.44 and 84.14% at 20, 25 and 10°C respectively at day 8 (Fig. 1A). Additionally, the germination speed and final germination rate were significantly inhibited in the 30°C incubation, suggesting that the optimum germination temperature for *C. gigantea* seeds is approximately 20°C. Next, the effects of incubation under different lighting conditions (0, 8 and 16 h light) on seed germination were tested when the temperature was kept constant at 20°C. The results indicated that the seed germination rate was most significantly enhanced under the 16-h/8-h light/dark photoperiod (Fig. 1B). During stratification at 4°C, seeds were treated with various concentrations of exogenous GA_3 (0, 1, 1.5, 2 and 2.5 mg/ml) followed by 16 h light/8 h dark incubation at 30°C to determine the optimal GA_3 concentration to break the germination inhibition caused by high temperature at 30°C. The seed treatment with 1.5 mg/mL GA_3 began to exert an inhibitory effect on seed

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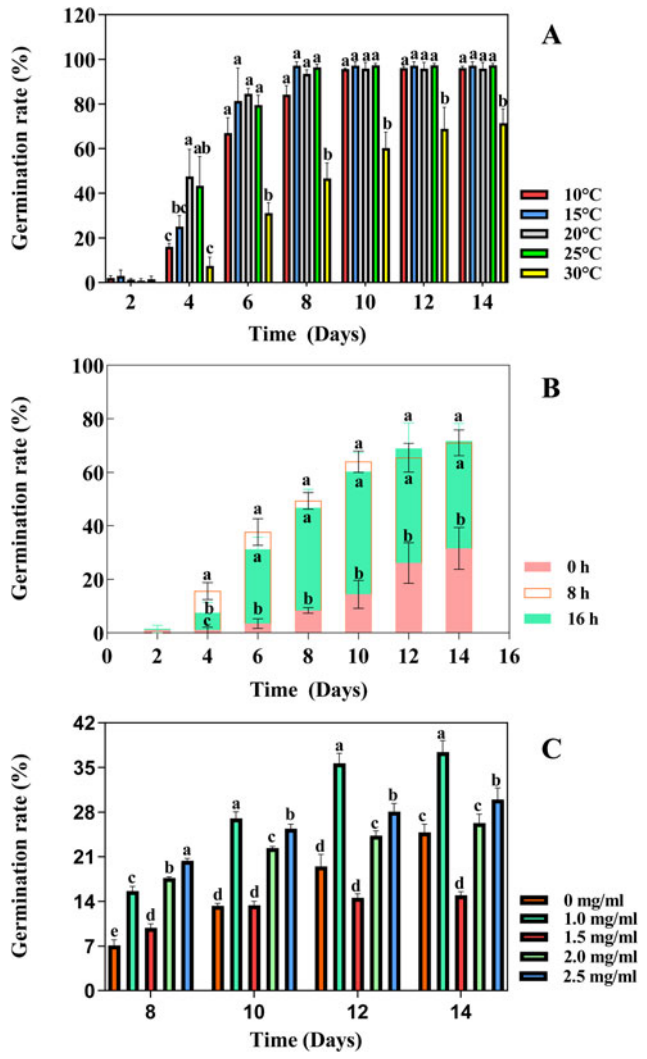

Figure 1. Effects of seed culture temperature, light duration and exogenous GA3 application on seed germination rate of *C. gigantea*. (A) Germination rate of *C. gigantea* seeds at different temperatures (10, 15, 20, 25 and 30°C) for different incubation times (2, 4, 6, 8, 10, 12 and 14 days). (B) Effect of light duration on seed germination of *C. gigantea*. (C) Impact of exogenous application of different concentrations of GA3 on germination rate of *C. gigantea* seeds. Data represent the mean ± SD of three independent biological replicates. Different letters indicate significant differences ($P < 0.05$, Tukey–Kramer tests).

germination after 14 days of incubation, with a germination rate of only 15.29% compared with 20.38% for non-treated control seeds. In contrast, seeds treated with 1 mg/ml GA_3 displayed the highest germination rate, reaching 38.69% at 14 days of incubation (Fig. 1C). In conclusion, in addition to altering the photoperiod to 16 h of light and 8 h of darkness, as well as maintaining a temperature of 20°C, the application of exogenous GA_3 (1 mg/ml, about 2.9 mM) was found to enhance the germination of *C. gigantea* seeds.

Morphological changes and biomass accumulation during seed germination

The morphological characteristics of seeds at the four stages of germination (Stage A–D) are shown in Fig. 2. In the early period (Stage A, swelling absorption, day 1) (Fig. 2A), seeds were transitioning from a dry to a swelling state due to rapid water uptake, indicating that they would soon sprout. In Stage B (seed coat dehiscence, day 5), cracks were visible on the seed coat surface, but the shape of the embryo and endosperm did not differ from that at Stage A (Fig. 2B). In Stage C (radicle emergence, day 9), seeds showed significant changes in morphology, with radicles breaking through the endosperm and cracking the seed coat (Fig. 2C). Elongated radicles showed red and embryonic axis showed green from bottom to top in Stage D (beginning of radicle elongation, day 13) (Fig. 2D). Radicle elongation indicated the completion of seed germination. The protein content decreased during germination, while seeds absorbed water and swelled, increasing in both volume and length (Figs. 3A, B). The fresh weight of the seeds increased whereas the dry weight of the seeds showed little change (Figs. 3C, D).

Identification of DEPs during seed germination

Sample collection is a critical step in the proteomic analysis of *C. gigantea* seeds. Because protein expression is time and space-dependent, collected tissue samples must be stored in a fresh, undamaged state to ensure the validity of the subsequent experimental data (Jolli and Ekbote, 2010). We compared two methods of protein precipitation (Supplementary Fig. S1). Phenol precipitation resulted in a marked loss in molecular mass, especially among the alkaline fragments, along with the presence of strong low-molecular-mass bands and poor separation of some protein spots (Supplementary Fig. S1A). The optimized TCA/acetone precipitation method was more effective in suppressing protease activity and diminishing sugar and salt interference (Supplementary Fig. S1B). Compared with phenol precipitation, TCA/acetone precipitation resulted in a significant increase in the number of protein spots on the protein map and more precise separation between protein spots, thereby providing images that were more suitable for the subsequent comparative analysis. This was consistent with the approaches used for *Cucumis sativus L* (He et al., 2012), *Elaeis guineensis* (Wang et al., 2014) and *Alexandrium* (Wang et al., 2009).

The results of 2-DE showed that the sample had a clear background and precise contours, and the protein spots were generally circular or elliptical, indicating that the 2-DE conditions were suitable for the separation of *C. gigantea* proteins (Fig. 4). The 2-DE images of all the protein spots were then compared, and spots with at least a 1.5-fold ($P < 0.05$) abundance difference were selected for analysis. The details of all the DEPs are shown in Supplementary Table S2. The molecular mass (*Mr*) of protein spots from seeds at the four germination stages ranged from 25 to 116 kDa and most had an isoelectric point at pH 4–7. Images of the distribution of all the protein spots are shown in Fig. 4. A total of 34 DEP spots were identified by 2-DE and were analysed by MALDI-TOF/TOF-MS. Among them, compared to Stage A, 31 DEPs were present at all four germination periods and three DEPs were only specific during germination, Of the 34 DEP spots, 21 were upregulated and 11 were downregulated.

Cluster analysis and functional distribution of the DEPs

Stage A as the starting expression level, the DEPs were found to group into three main expression stages (Stage B–D) as shown in Fig. 5A. Stage B contained 11 protein spots, all of which showed a trend towards upregulation of expression during the seed coat dehiscence stage. Stage C consisted of 13 protein spots that were downregulated during the radicle emergence stage. In Stage D

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Figure 2. Morphological characteristic of *C. gigantea* seeds during the four germination stages. (A) Seeds absorb water for 1 d (Stage A) (B) Seeds absorb water for 5 days (Stage B); (C) Seeds absorb water for 9 days (Stage C); (D) Seeds absorb water for 13 days (Stage D). Bar, 1 mm.

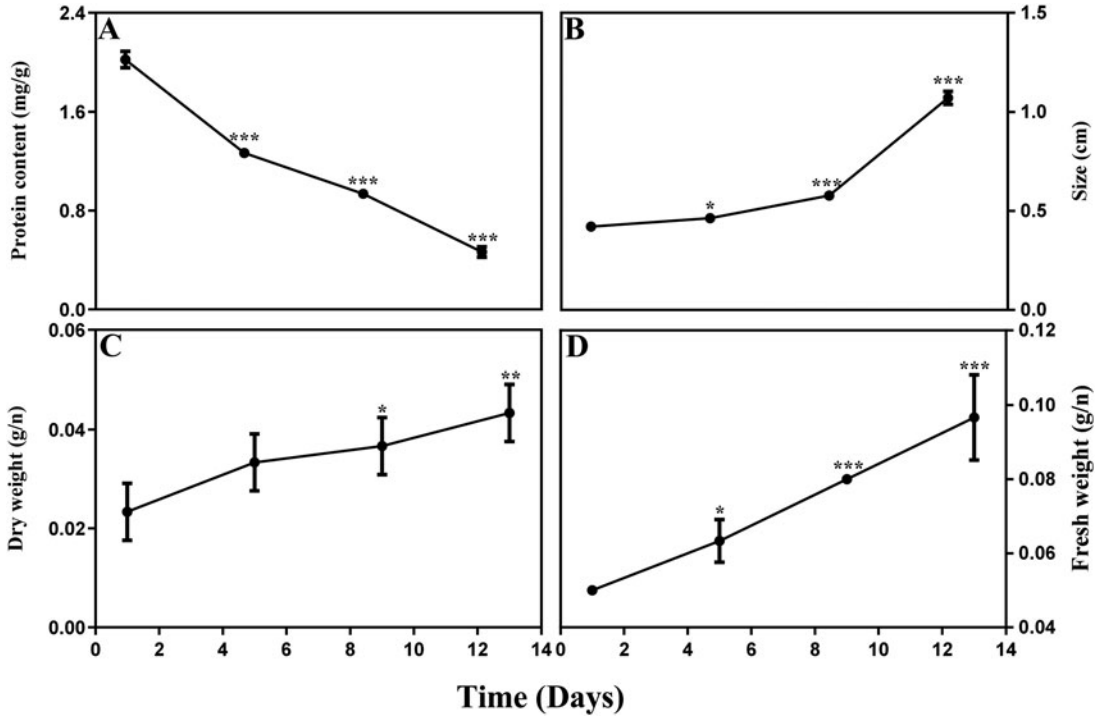

Figure 3. The changes of protein content (A), seed length (B), dry weight (C) and fresh weight (D) of seeds during seeds germination. The error bars represent ± SD of three biological replicates. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

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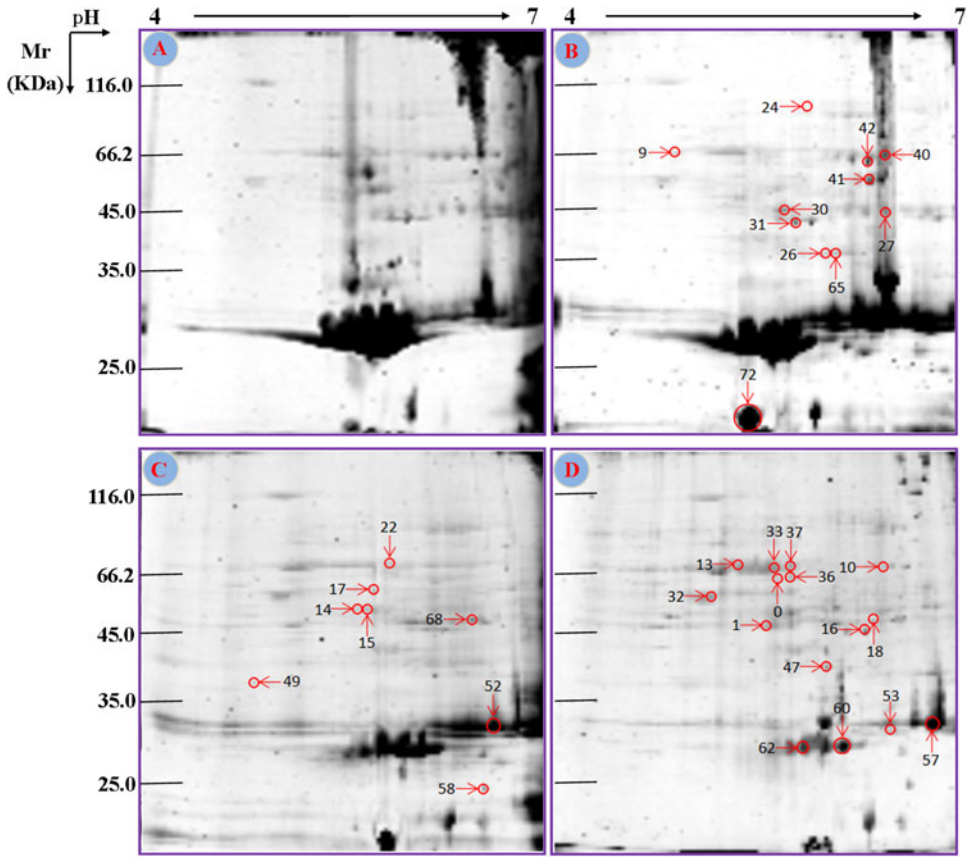

Figure 4. Proteome maps of *C. gigantea* seeds during four seeds germination periods by 2-DE. Protein 2-DE gel showing the accumulation of differential proteins in four periods of seed swelling (1 days) (A), germination (5 days) (B), emergence (9 days) (C) and radicle elongation (13 days) (D), respectively. All red markings are differential proteins by period, and the size of the circle markers is positively correlated with protein accumulation.

(10 protein spots), proteins showed a trend toward s upregulation of expression during the radicle elongation stage.

Based on the analysis of the MS data using the NCBI database and the results of MS identification, we classified the 34 DEP spots into 8 functional categories, including the cytoskeleton (10, 29.41%), energy metabolism (5, 14.71%), transport (5, 14.71%), stress response (5, 14.71%), molecular chaperones (3, 8.82%), amino acid metabolism (3, 8.82%), antioxidative enzymes (2, 5.88%) and ABA signalling pathway (1, 2.94%) (Fig. 5B).

Transcription patterns of nine key proteins at the seed germination stages

The transcriptional levels of nine DEPs during seed germination are shown in Fig. 6 and Supplementary Fig. S2. These nine key proteins were ATP synthase subunit beta (Spot 15), RuBisCO large subunit-binding protein subunit beta (Spot 17), enolase1 (Spot 18), heat shock protein 70 kDa family (Spot 22), heat shock protein 70 kDa family (Spot 33), RuBisCO large subunit-binding protein subunit alpha (Spot 32), thioredoxin, nucleoredoxin2 (Spot 36), 20 kDa chaperone protein, chloroplastic-like (Spot 58) and 11S-globulin (Spot 65). Two of them (Spots 17 and 38) showed transcription patterns corresponding to their protein expression trends (Fig. 6).

Discussion

Seed germination necessitates coordination among multiple signal transduction pathways and physiological and biochemical processes, including responses to growth factors and activation of seed vigour-related properties. These events ultimately lead to seed growth, beginning with water uptake and ending with radicle elongation (Ma et al., 2022). GAs are positive regulators of seed germination (Moraes et al., 2021). We found that three factors were important for improving and accelerating the germination of *C. gigantea* seeds, namely, the photoperiod (hours of light), incubation temperature, and exogenous application of GA_3. The application of GA_3 (1 mg/ml, about 2.9 mM) during stratification effectively accelerated the seed germination rate. The concentration of exogenous GA_3 used varied among different plants, which may be influenced by the thickness of the seed coat. For *A. communis* par., the optimal concentration to promote seed germination was found to be 1 mg/l (Hou et al., 2005). In the case of populus seeds, a concentration of 10 mg/l GA_3 significantly increased the germination rate (Ji et al., 2003). Furthermore, the concentration of GA_3 used also depended on the duration of seed soaking in the experiment. For *Santalum album* seeds, the optimal concentration was determined to be 0.8 mg/ml (2.3 mM), with a soaking time of 6–24 h (Liu et al. 2010). On the other hand, the highest germination rate for *F. chinensis* seeds was achieved by soaking them in 0.2 mg/ml of GA_3 for 60 h (Li et al., 2012). In this study, seeds of *C. gigantea* were soaked for 48 h. Additionally, a constant incubation temperature of 20°C and a photoperiod of 16 h of light and 8 h of darkness were found to be optimal conditions for the promotion of seed germination under our experimental setup (Fig. 1).

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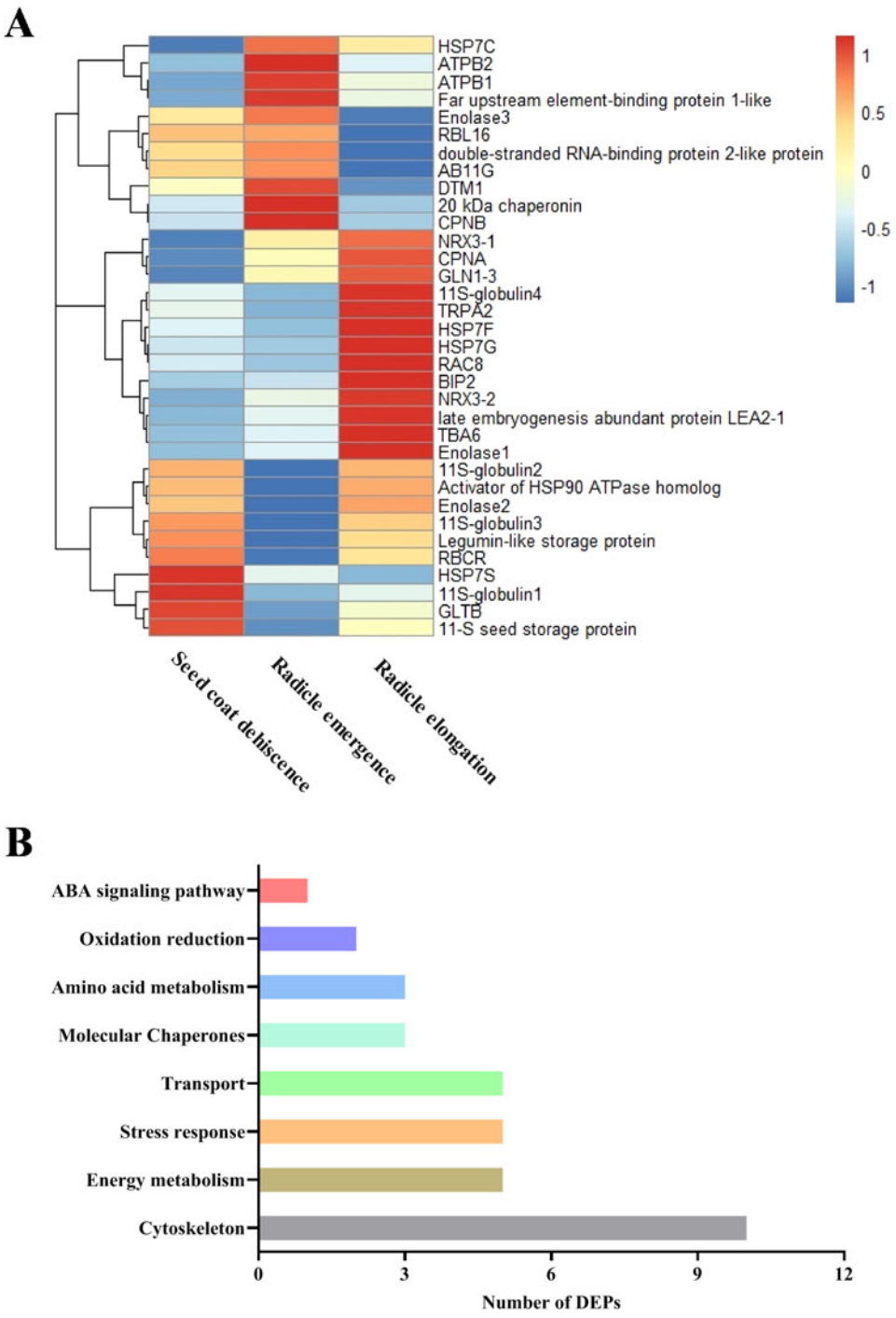

Figure 5. Classification of *C. gigantea* seed proteins. Hierarchical clustering analysis of the expression patterns of DEP spots (A) and functional classifications of DEPs identified (B) in *C. gigantea* during seed germination. Clustering was based on the protein expression levels during seed germination. Stage A was used as a control, and Stage B (seed coat dehiscence), Stage C (radicle emergence) and Stage D (radicle elongation) were included. Red colour indicates a positive abundance in protein spots and blue indicates a negative abundance in protein spots.

The expression patterns of the identified DEPs were also analysed at the transcriptional level, and Spots 17 and 38 showed the same expression pattern at both the protein and mRNA levels, indicating that the regulation of these proteins begins at the transcriptional stage. Most other proteins, such as Spot 22, Spot 18, had different mRNA and protein expression patterns, possibly because they are regulated post-transcriptionally or post-translationally (Kuhlemeier, 1992) (Fig. 6). Differences between transcript levels and protein abundance can also be due to the presence of several proteins under a single spot, which can happen when spots are particularly large.

Many of the DEPs detected in this study are involved in amino acid metabolism (Supplementary Fig. S2). Two proteins ferredoxin-dependent glutamate synthase (Spot 41) and glutamine synthetase cytosolic isoenzyme-like (Spot 47) have been implicated in glutamate metabolism. Enolase (Spots 18, 30 and 68) is

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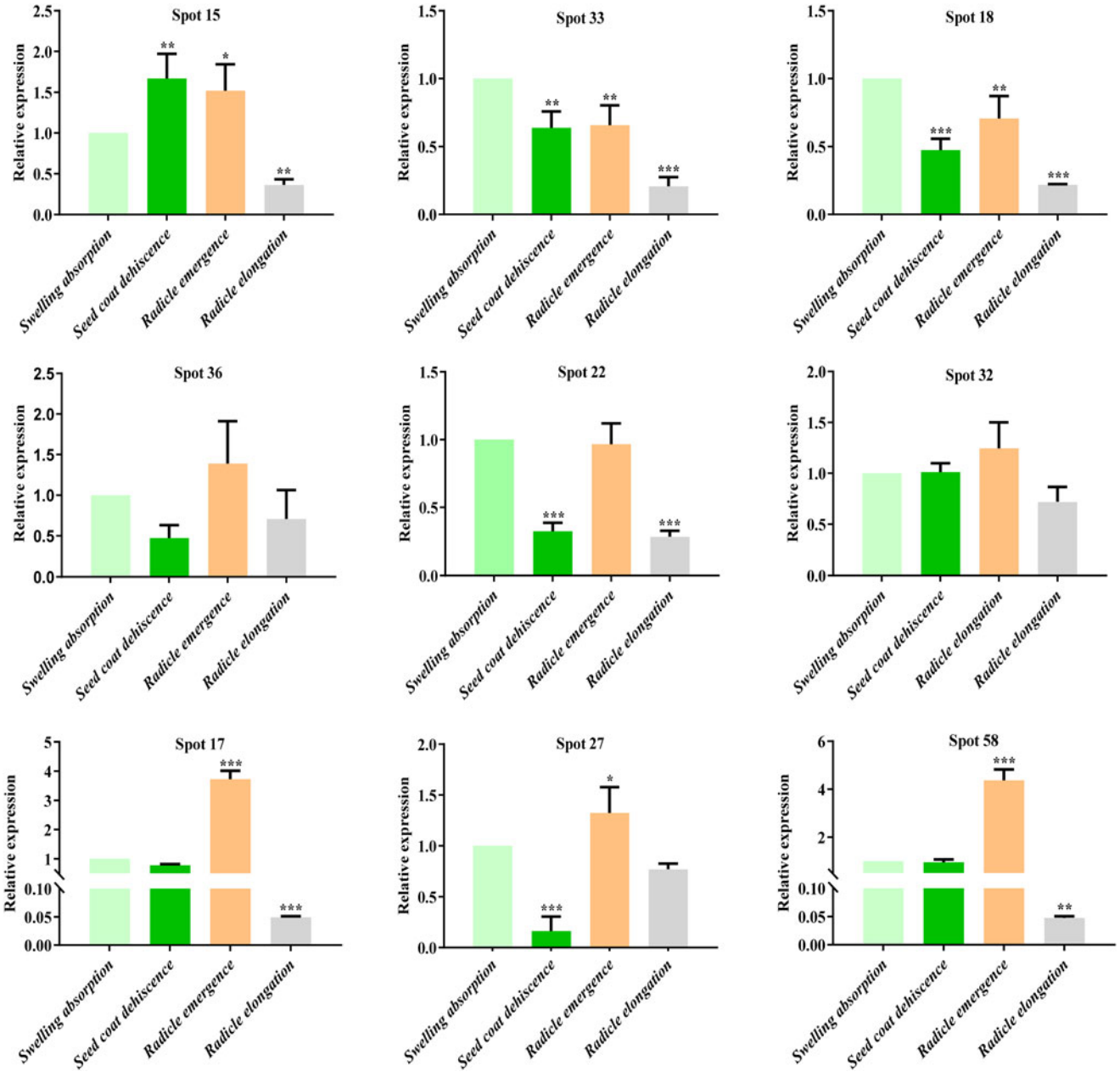

Figure 6. The relative gene expression changes of 9 DEPs during seed germination revealed by quantitative real-time PCR in *C. gigantea*. Expression of nine major differential protein sites at the bud stage (5 days), germination stage (9 days) and radicle elongation (13 days) of seeds, respectively. Spot 15 (ATP synthase subunit beta), Spot 18 (Enolase1), Spot 33 (Heat shock protein 70 family), Spot 22 (Heat shock protein 70 family), Spot 36 (Thioredoxin, nucleoredoxin2), Spot 32 (ruBisCO large subunit-binding protein subunit alpha), Spot 17 (ruBisCO large subunit-binding protein subunit beta), Spot 27 (11S-globulin) and Spot 58 (20 kDa chaperonin, chloroplastic-like). Data represent the mean ± SD of three independent biological replicates. Asterisks indicate significant differences (*$P \leq 0.05$, **$P \leq 0.01$, Tukey–Kramer tests).

known to play an important role in the glycolytic metabolic pathway, while thioredoxin (Spot 0 and Spot 36) is involved in the activation of enzymes in the pentose phosphate pathway. Furthermore, many of the identified DEPs act in the endoplasmic reticulum and are involved in transport, processing and splicing processes (Spots 1, 24 and 53) (Fig. S3). Luminal-binding protein (Spot 13) recognises mismatched RNA, which is then degraded by the degrading enzyme complex. The components of the degrading enzyme complex are related to stress response proteins (Spots 9, 26, 22, 33 and 37). DTM1 (Spot 60) is a signal peptidase complex-like protein located within the membrane of the endoplasmic reticulum (Supplementary Fig. S3).

In higher plants, the cytoskeleton plays fundamental roles in cell division, cell growth, cellular organization, intracellular transport and cell shape dynamics (Hasezawa and Kumagai, 2002). In our study, the expression of cytoskeletal proteins first decreased, and then increased during the process of seed germination (Supplementary Table S3). This suggested that the cytoskeleton is involved both at the early and late stages of seed germination in *C. gigantea* seeds. It has been reported that the cytoskeleton regulates cellular responses to biotic or abiotic stresses during germination under a variety of unfavourable external environments (Yemets et al., 2011). The functions of cytoskeletal proteins in higher plants under stress conditions have been comprehensively

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investigated (Rongqiu et al., 2011). For example, Protein blotting and immunocytochemical analyses revealed an increasing accumulation of microtubule proteins and microtubule cytoskeleton in *Jatropha curcas* seeds soaked in water for more than 48 h (de Brito et al., 2015). Additionally, under drought stress, a subset of genes associated with cytoskeleton organization was found to be dysregulated in developing wheat seeds, coincident with reduced seed size and delayed endosperm development (Begcy and Walia, 2015; Sanit, 2021).

Energy consumption is high throughout the seed germination process (Miladinov et al., 2014; Koo et al., 2015). In this work, two proteins involved in energy metabolism (ATP synthase subunit beta [Spots 14 and 15]) were identified among the DEPs. These two proteins, which were upregulated, are subunits of an enzyme that provides energy for *C. gigantea* seeds during germination (Supplementary Table S3). ATP synthase is required for energy production in the tricarboxylic acid (TCA) cycle (Chen et al., 2011). However, at the seed coat dehiscence stage, we found that the protein level of ATP synthase subunit beta was significantly decreased compared to Stage A. This result suggested that energy supply occurred late in seed germination. Notably, the glycolysis pathway generates most of the energy required for seed germination (Abokassem, 2005; Kadri et al., 2021). During germination, some proteins related to glycolysis (ferredoxin-dependent glutamate synthase [Spot 41] and glutamine synthetase cytosolic isozyme-like [Spot 47], which are involved in glutamate metabolism, and enolase [Spots 18, 30 and 68]) were the most prominently expressed. Taken together, these findings indicate that the TCA cycle and glycolysis are the primary energy sources for *C. gigantea* seed germination.

Most proteins involved in seed germination are membrane transport proteins. When seeds begin to germinate, they require the transportation of large amounts of compounds, such as water and nutrients, which are needed for the synthesis of numerous components. Transporters allow higher plants to uptake an abundance of compounds necessary for cellular component synthesis across specific cell membranes during germination (Routray et al., 2015). ABC transporters, for example, are critical for plant development, as they are involved in seed development, seed germination and secondary growth (Bruhn et al., 2020; Cai et al., 2021). In this study, several DEPs were associated with transport (Spots 13, 24, 42 and 53) and Spot 62 was an ABC transporter ATP-binding protein (Supplementary Table S2). Luminal-binding protein (Spot 13) participates in seed development, germination and early seedling growth (Forward, 2001). In our study, this protein was upregulated at radicle emergence and radicle elongation stages compared to Stage A (Table S3).

Conclusion

Preliminary studies can contribute to the understanding of the expression patterns of proteins essential for seed germination in higher plants, including *C. gigantea*. In this study, in addition to altering the photoperiod to 16 h of light and 8 h of darkness, as well as maintaining a temperature of 20°C, the application of exogenous GA_3 (1 mg/ml, about 2.9 mM) was found to enhance the germination of *C. gigantea* seeds. Then, using a combination of 2-DE-based protein identification and proteomics, we identified 34 proteins that were differentially expressed, 13 at the radicle pre-emergence stage, and 17 at the radicle elongation stage. Many of the identified DEP spots are closely related to amino acid metabolism. Additionally, the DEPs were found to be primarily involved in endoplasmic reticulum function. The TCA cycle and glycolysis were the primary energy sources for *C. gigantea* seed germination. Our study demonstrated that proteomics can provide reference information relating to multiple processes of seed germination and lays the foundation for understanding the characteristics of DEPs during *C. gigantea* seed germination.

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Data availability statement. All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

Competing interest. The authors declare that they have no conflict of interest. The present research did not involve human participants and/or animals. Informed consent was obtained from all individual participants included in the study.

References

Abokassem EM (2005) Activities of some enzymes during seed germination of some leafy vegetables under saline drainage water. *Biotechnology* **4**, 358–364.

Bai B, Peviani A, van der Horst S, Gamm M, Snel B, Bentsink L and Hanson J (2017) Extensive translational regulation during seed germination revealed by polysomal profiling. *New Phytologist* **214**, 233–244.

Begcy K and Walia H (2015) Drought stress delays endosperm development and misregulates genes associated with cytoskeleton organization and grain quality proteins in developing wheat seeds. *Plant Science* **240**, 109–119.

Bewley JD (1997) Seed germination and dormancy. *The Plant Cell* **9**, 1055–1066.

Bruhn O, Lindsay M, Wiebel F, Kaehler M, Nagel I, Böhm R, Röder C and Cascorbi I (2020) Alternative polyadenylation of ABC transporters of the C-Family (ABCC1, ABCC2, ABCC3) and implications on posttranscriptional micro-RNA regulation. *Molecular Pharmacology* **97**, 112–122.

Cai GQ, Wang GL, Kim SC, Li JW, Zhou YM and Wang XM (2021) Increased expression of fatty acid and ABC transporters enhances seed oil production in *Camelina*. *Biotechnology for Biofuels* **14**, 11.

Chen X, Alonso AP, Allen DK, Reed JL and Shachar-Hill Y (2011) Synergy between 13C-metabolic flux analysis and flux balance analysis for understanding metabolic adaption to anaerobiosis in *E. coli*. *Metabolic Engineering* **13**, 38–48.

de Brito CD, Loureiro MB, Santos Teles CA, Schuck MR, Fernandez LG and de Castro RD (2015) Behavior of *Jatropha curcas* L. seeds under osmotic stress: germination and cell cycle activity. *Acta Scientiarum-Agronomy* **37**, 279–287.

Dinkova TD, Márquez-Velázquez NA, Aguilar R, Lázaro-Mixteco PE and de Jiménez ES (2011) Tight translational control by the initiation factors eIF4E and eIF(iso)4E is required for maize seed germination. *Seed Science Research* **21**, 85–93.

Forward BS (2001) *Characterization and expression of the Douglas -fir luminal binding protein*. PhD thesis, University of Victoria, Canada.

Fumei X, Shiqiang R and Ciren P (2017) Effects of germination characters of *Cupressus gigantea* seed in different treatments. *Seed*.

Galland M, Huguet R, Arc E, Cue Ff G, Job D and Rajjou L (2014) Dynamic proteomics emphasizes the importance of selective mRNA translation and protein turnover during *Arabidopsis* seed germination. *Molecular & Cellular Proteomics* **13**, 252–268.

Guo S, Yang X, Zhang Z, Zhang F and Liu T (2020) Spatial distribution and temporal trend characteristics of agro-climatic resources and extreme climate events during the soybean growing season in Northeast China from 1981 to 2017. *Journal of Meteorology*; English version **34**, 15.

Han C and Yang PF (2015) Studies on the molecular mechanisms of seed germination. *Proteomics* **15**, 1671–1679.

Hasezawa S and Kumagai F (2002) Dynamic changes and the role of the cytoskeleton during the cell cycle in higher plant cells. *International Review of Cytology* **214**, 161–191.

He D, Han C, Yao J, Shen S and Yang P (2011) Constructing the metabolic and regulatory pathways in germinating rice seeds through proteomic approach. *Proteomics* **11**, 2693–2713.

He LZ, Lu XM, Tian J, Yang YJ, Li B, Li J and Guo SR (2012) Proteomic analysis of the effects of exogenous calcium on hypoxic-responsive proteins in cucumber roots. *Proteome Science* **10**, 15.

Hou J, Karim A and Gao Q (2005) Effect of different treatments on seed germination and seedling growth of almond seed. *Seed* **24**, 4.

Ji Q, Huang P, Men D and Wang Y (2003) Initial studies on increase germination percentage of *Populus euphratica* seeds. *Seed* (5), 7.

Jolli RB and Ekbote SD (2010) Influence of fruit maturity and post harvest ripening on seed quality of Chilli. *Karnataka Journal of Agricultural Sciences* **18**, 811–813.

Kadri S, Ayed ME, Kadri A, Limam F and Mokni M (2021) Protective effect of grape seed extract and orlistat co-treatment against stroke: effect on oxidative stress and energy failure. *Biomedicine & Pharmacotherapy* **136**, 111282.

Koo HJ, Park SM, Kim KP, Mi CS, Mi OL, Lee SK, Xia X and Hon CB (2015) Small heat shock proteins can release light dependence of tobacco seed during germination. *Plant Physiology* **167**, 1030–1038.

Kuhlemeier C (1992) Transcriptional and post-transcriptional regulation of gene expression in plants. *Plant Molecular Biology* **19**, 1–14.

Lei P, Liu Z, Li JX, Jin GZ, Xu LP, Ji XM, Zhao XY, Tao L and Meng FJ (2022) Integration of the physiology, transcriptome and proteome reveals the molecular mechanism of drought tolerance in *Cupressus gigantea*. *Forests* **13**, 16.

Li S, Mao Y and Jiang Y (2012) Detection method of seed germination percentage of *Fraxinus chinensis*. *Journal of North-East Forestry University* **40**, 1–4.

Li QF, Wang JD, Xiong M, Wei K, Zhou P, Huang LC, Zhang CQ, Fan XL and Liu QQ (2018) iTRAQ-based analysis of proteins co-regulated by brassinosteroids and gibberellins in rice embryos during seed germination. *International Journal of Molecular Sciences* **19**, 3460.

Liu X, Xu D, Zhang N, Xie Z and Chen H (2010) Effects of gibberellin on seed germination and seedling growth of sandalwood (*Santalum album* L). *Seed* **29**, 71–74.

Liyuan Z, Zengxian L, Li Z, Zhen X and Yujun Z (2009) Effects of tourist disturbance on giant cypress forest in Linzhi, Tibet. *Ecology and Environmental Sciences* **18**, 1413–1421.

Ma L, Wang C, Hu Y, Dai W, Liang Z, Zou C, Pan G, Lubberstedt T and Shen Y (2022) GWAS and transcriptome analysis reveal MADS26 involved in seed germination ability in maize. *Theoretical and Applied Genetics* **135**, 1717–1730.

Meleady P (2018) Two-dimensional gel electrophoresis and 2D-DIGE. *Methods in Molecular Biology (Clifton, N.J.)* **1664**, 3–14.

Miladinov Z, Radic V, Miklic V, Crnobarac J and Mrdja J (2014) Effect of biostimulators on sunflower germination energy and seed germination. *Field & Vegetable Crops Research* **51**, 29–37.

Miura C, Furui Y, Yamamoto T, Kanno Y, Honjo M, Yamaguchi K, Suetsugu K, Yagame T, Seo M, Shigenobu S, Yamato M and Kaminaka H (2023) Autoactivation of mycorrhizal symbiosis signaling through gibberellin deactivation in orchid seed germination. *Plant Physiology* **18**, 546–563.

Moraes LD, Andrade FLD, Gomes RF and Santos LD (2021) Could the umbel order selection and GA3 treatment improve seed germination in Amazon chicory species? *Bragantia* **80**, 11.

Nakamoto A, Sato T, Hirosawa N, Nakamoto N, Enoki Y, Chida D, Usui M, Takeda S, Nagai T and Sasaki A (2014) Proteomics-based identification of novel proteins in temporal tendons of patients with masticatory muscle tendon–aponeurosis hyperplasia. *International Journal of Oral & Maxillofacial Surgery* **43**, 113–119.

Nonogaki H, Bassel GW and Bewley JD (2010) Germination – still a mystery. *Plant Science* **179**, 574–581.

Oracz K and Stawska M (2016) Cellular recycling of proteins in seed dormancy alleviation and germination. *Frontiers in Plant Science* **7**, 8.

Osama SK, Kerr ED, Yousif AM, Phung TK, Kelly AM, Fox GP and Schulz BL (2021) Proteomics reveals commitment to germination in barley seeds is marked by loss of stress response proteins and mobilisation of nutrient reservoirs. *Journal of Proteomics* **242**, 10.

Ren XX, Xue JQ, Wang SL, Xue YQ, Zhang P, Jiang HD and Zhang XX (2017) Proteomic analysis of tree peony (*Paeonia ostii* 'Feng Dan') seed germination affected by low temperature. *Journal of Plant Physiology* **224-225**, 56–67.

Rongqiu Z, Yingjun W, Min X, Gaoneng W and Weili Z (2011) Effects of α-1 ipoic acid on the expressions of cytoskeleton proteins in the oxidative stress-induced sertoli cells. *Chinese Journal of Andrology* **25**, 17–22.

Routray P, Masalkar PD and Roberts DM (2015) Nodulin intrinsic proteins: facilitators of water and ammonia transport across the symbiosome membrane. In de Bruijn FJ (ed.), *Biological Nitrogen Fixation*. John Wiley & Sons, Inc., pp. 695–704. doi:10.1002/9781119053095.ch69.

Sajeev N, Baral A, America AHP, Willems LAJ, Merret R and Bentsink L (2022) The mRNA-binding proteome of a critical phase transition during Arabidopsis seed germination. *New Phytologist* **233**, 251–264.

Sanit S (2021) Induction on seed germination and seeedling performances against sunflower (*Helainthus annuus* L.) and Castor bean (*Ricinus communis* L.) as influenced by different water stress treatments. *International Journal of Sciences* **10**, 41–50.

Sano N, Lounifi I, Cueff G, Collet B, Clément G, Balzergue S, Huguet S, Valot B, Galland M and Rajjou L (2022) Multi-omics approaches unravel specific features of embryo and endosperm in rice seed germination. *Frontiers in Plant Science* **13**, 20.

Santo Pereira AD, Oliveira HC, Fraceto LF and Santaella C (2021) Nanotechnology potential in seed priming for sustainable agriculture. *Nanomaterials* **11**, 28.

Sghaier-Hammami B, Hammami SBM, Baazaoui N, Gómez-Díaz C and Jorrin-Novo JV (2020) Dissecting the seed maturation and germination processes in the non-orthodox *Quercus ilex* species based on protein signatures as revealed by 2-DE coupled to MALDI-TOF/TOF proteomics strategy. *International Journal of Molecular Sciences* **21**, 26.

Sghaier-Hammami B, Castillejo MA, Baazaoui N, Jorrin-Novo JV and Escandón M (2021) GeLC-Orbitrap/MS and 2-DE-MALDI-TOF/TOF comparative proteomics analysis of seed cotyledons from the non-orthodox *Quercus ilex* tree species. *Journal of Proteomics* **233**, 6.

Simova-Stoilova LP, Romero-Rodríguez MC, Sánchez-Lucas R, Navarro-Cerrillo RM, Medina-Aunon JA and Jorrín-Novo JV (2015) 2-DE proteomics analysis of drought treated seedlings of *Quercus ilex* supports a root active strategy for metabolic adaptation in response to water shortage. *Frontiers in Plant Science* **6**, 16.

Smolikova G, Strygina K, Krylova E, Vikhorev A, Bilova T, Frolov A, Khlestkina E and Medvedev S (2022) Seed-to-seedling transition in *Pisum sativum* L.: a transcriptomic approach. *Plants (Basel)* **11**, 1686.

Srivastava AK, Kumar JS and Suprasanna P (2021) Seed 'primeomics': plants memorize their germination under stress. *Biological Reviews* **96**, 1723–1743.

Stergaard O, Finnie C, Laugesen S, Roepstorff P and Svennson B (2004) Proteome analysis of barley seeds: identification of major proteins from two-dimensional gels (pI 4–7). *PROTEOMICS* **4**, 2437–2447.

Wang J, Zheng W and Pan G (2005) Relation between being endangered and seed vigor about *Cupressus gigantea* in Tibet. *Scientia Silvae Sinicae* **41**, 37–41.

Wang D-Z, Lin L, Chan LL and Hong H-S (2009) Comparative studies of four protein preparation methods for proteomic study of the dinoflagellate *Alexandrium* sp using two-dimensional electrophoresis. *Harmful Algae* **8**, 685–691.

Wang Y, Li J, Wu Y, Zhang D and Lei X (2014) Establishment of the proteomics research system for *Elaeis guineensis* embryos. *Guangdong Agricultural Sciences* **41**, 126–129.

https://doi.org/10.1017/S0960258524000126 Published online by Cambridge University Press

Xu HH, Liu SJ, Song SH, Wang RX, Wang WQ and Song SQ (2016) Proteomics analysis reveals distinct involvement of embryo and endosperm proteins during seed germination in dormant and non-dormant rice seeds. *Plant Physiology & Biochemistry* **103**, 219–242.

Ye-Hong GUO and Hai-Ming LIN (2009) Regulatory effect of GA and KT on seeds germination of *Nitraria tangutorum* Bobr. *Chinese Journal of Eco-Agriculture* **17**, 1196–1199.

Yemets AI, Krasylenko YA, Lytvyn DI, Sheremet YA and Blume YB (2011) Nitric oxide signalling via cytoskeleton in plants. *Plant Science* **181**, 545–554.

Yunxing L and Xing T (1999) Study on the introduction of Cupressus species. *Forest Research* **12**, 304–309.

Zaynab M, Pan D, Fatima M, Noman A, Chen S and Chen W (2019) Proteomic analysis reveals key proteins in seed germination of *Cyclobalnopsis gilva*. *Biochemical Systematics and Ecology* **83**, 106–111.

https://doi.org/10.1017/S0960258524000126 Published online by Cambridge University Press