Sulfuric Acid and Gibberellic Acid (GA$_3$) Treatment Combined with Exposure to Cold Temperature Modulates Seed Proteins during Breaking of Dormancy to Germination in *Tilia miqueliana*

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Abstract

Tilia miqueliana produces woody seeds that exhibit deep dormancy. In this study, we used cell biology methods, including Paraffin section determination and Coomassie brilliant blue staining, as well as proteomics-based methods, including two-dimensional electrophoresis with matrix-assisted laser desorption/ionisation tandem time-of-flight mass spectrometry (2DE–MALDI–TOF/TOF), to examine the effects of H$_2$SO$_4$–GA$_3$ and cold stratification (3 °C) treatment on proteins during dormancy release and germination in *T. miqueliana* seeds. The results revealed that during cold stratification, the area and density of proteins in the endosperm cells of H$_2$SO$_4$–GA$_3$-treated seeds were significantly altered. Total protein content was continuously consumed and utilised. Storage proteins (albumin, globulin, prolamin, and glutelin) were degraded to varying degrees. Sixteen differential proteins were identified using mass spectrometry. Kyoto encyclopedia of genes (KEGG) pathway analysis revealed that the glycolysis/gluconeogenesis, secondary metabolite biosynthesis, glyoxylate and dicarboxylate metabolism, amino acid biosynthesis, and metabolic pathways were critical during dormancy release and germination. Gene ontology analysis and KEGG pathway annotation of differential proteins in the co-expression network indicated that the differential proteins are implicated in photosynthesis, glucose metabolism, biosynthesis of plant hormones, and glycolysis/gluconeogenesis. Synergistic interactions among these proteins accelerated dormancy release and germination. Therefore, H$_2$SO$_4$–GA$_3$ cold stratification treatment is the best method for achieving rapid dormancy release and increasing the germination rate of *T. miqueliana* seeds.

Keywords Dormancy release · Germination · H$_2$SO$_4$–GA$_3$ cold stratification treatment · Proteome · Storage protein · *Tilia miqueliana*

1 Introduction

Tilia miqueliana, a linden species unique to China, has declined sharply in abundance due to anthropogenic activities. Reproduction is hindered by the low filling rate (20–30%) and deep dormancy of the seeds, and the species is at risk of becoming endangered [1]. However, the cell biology processes involved in seed dormancy and its release remain poorly understood for this species. Molecular studies of protein types and functions can directly clarify changing mechanisms of physiological changes in living organisms [2]. In particular, proteomics analysis has been applied to elucidate the multi-step processes involved in seed germination in many species, through the investigation of expressional variation in groups of proteins [3].

In this study, we used cell biology methods including Paraffin section determination (PSD) and Coomassie brilliant blue (CBB) staining, as well as proteomics-based methods including two-dimensional electrophoresis matrix-assisted laser desorption/ionisation tandem time-of-flight mass spectrometry (2DE–MALDI–TOF/TOF), to examine the effects of H$_2$SO$_4$–GA$_3$ and cold stratification on proteins during dormancy release and germination in *T. miqueliana* seeds.

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PSD and CBB were applied to test protein morphology and content during cold stratification. Proteomics technology was used to detect differences in protein expression and identify the key metabolic pathways. This is the first study to examine the mechanism of seed dormancy release in *T. miqueliana* at both the cell biology and molecular levels. The results will enhance our understanding of woody plant seed proteomics.

2 Materials and Methods

2.1 Plant Material

Tilia miqueliana seeds were collected from the Huang Zangyu National Forest Park, China (34°03′N, 117°06′E) in November 2019. After mechanical peeling, seeds were dried under ambient temperature and humidity conditions until a moisture content of 9.74% (fresh weight basis) was achieved, after which they were incubated in plastic bags at 3 °C. The seeds were soaked in H_2SO_4 for 15 min and 500 mg/L GA_3 for 12 h. Next, the seeds were subjected to a cold-stratifying process at 3 °C (the temperature for dormancy breaking) for about 70 days in closed plastic bags filled with wet sand in darkness. The protein extraction tests consisted of three replicates (1200 seeds each).

2.2 Germination Test

Seeds were divided into four groups: control, H_2SO_4-treated, GA_3-treated, and $H_2SO_4+GA_3$-treated. Each group was replicated three times, with 100 seeds per replicate. Seeds in the control group were not subjected to any treatment. Seeds in the H_2SO_4 group were soaked in H_2SO_4 for 15 min, and then in deionised water for 12 h. Seeds in $H_2SO_4+GA_3$ group were soaked in H_2SO_4 for 15 min, and then in 500 mg/L GA_3 for 12 h. Seeds were then placed into closed plastic bags filled with wet sand, and subjected to cold stratification at 3 °C (the temperature for breaking dormancy) in the dark for approximately 70 days. After 15, 30, 35, 40, 45, 60, and 70 days of stratification, the seeds were placed on soaked cotton in a germination box and stored in a lightbox at a constant temperature of 25 °C for germination testing over a period of 4 weeks. When the radicle length reached 2 mm, the seeds were considered to have germinated. Three replicates of 100 seeds each were included in the control and H_2SO_4–GA_3-treated groups for each time point.

2.3 Paraffin Section Detection and Iodine/ Potassium Iodide Staining

Cell morphology of protein in endosperm was observed by PSD using a previously described iodine/potassium iodide staining method [4]. We observed five randomly selected seeds from each treatment group after 0, 15, 30, 35, 40, 45, 60, and 70 days. The seeds were cut lengthwise with a single blade and fixed in a 70% Formalin-acetic acid-alcohol (FAA) fixative solution (Servicebio, Wuhan, China) for 24 h. They were then dehydrated with the ethanol series and encased in a wax block. The wax blocks were sliced to a thickness of 4 μm using glass knives on an RM2016 ultramicrotome (Laika, Shanghai, China), and then stained with an iodine/potassium iodide dye solution for 2 min (G1070, Servicebio). Iodine can replace one or two of the hydrogen atoms on the benzene ring of tyrosine, on the protein peptide chain, converting it into a polypeptide chain containing iodinated tyrosine, which is yellow. Stained slices were observed under a light microscope (Eclipse Ci; Nikon, Tokyo, Japan). Images were analysed using CaseViewer software (https://www.3dhistech.com/caseviewer).

2.4 Determination of Total Protein, Albumin, Globulin, Prolamin, and Glutenin Content

Total protein, albumin, globulin, prolamin, and glutenin content in the endosperm were determined using a CBB colourimetry method described previously [6]. Sampling was performed at 0, 15, 30, 35, 40, 45, 60, and 70 days.

2.5 Protein Extraction

Seed samples were collected at 0, 15, 30, 35, 40, 45, 60, and 70 days in the stratification period. Three seed sample replicates (150 seeds each) were randomly taken at each time point. Subsequently, the endosperms of the seeds were removed and frozen in liquid nitrogen (− 80 °C), and then the endosperm protein was extracted using a phenol extraction method [5]. The protein concentration was determined using a standard Bradford assay [6].

2.6 Two-Dimensional Electrophoresis with Isoelectric Focusing/Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (IEF/SDS–PAGE)

2.6.1 Isoelectric Focusing

Proteins were separated using a horizontal two-dimensional electrophoresis instrument (Ettan IPGphor IEF). Non-linear adhesive gel strips with a length of 24 cm (Amersham Pharmacia Biosciences) were used with a linear pH gradient from 3 to 10 [7, 8]. For the first dimension separation, the analytical IPG strips were rehydrated for 13 h at 20 °C in 450 μL of rehydration buffer containing 1500 μg of sample proteins. The isoelectric focusing process was conducted at 20 °C using an Ettan IPGphor system (Cytiva, Little Chalfont,

UK), for 8 h at 50 V, 1 h at 100 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 1000–10,000 V (gradient), 1 h at 125,000 V, and 12 h at 500 V.

2.6.2 Gel Strip Balance and SDS-PAGE Electrophoresis

Prior to the second dimension separation, the strips were equilibrated for 15 min in 10 mL of equilibration buffer I (1.5 mol/L Tris–HCl, pH 8.8, 6 mol/L urea, 2% SDS, 30% glycerol, 50 mg DTT, 0.002% [w/v] bromophenol blue), and then for 15 min in 10 mL of equilibrium buffer II (1.5 mol/L Tris–HCl, pH 8.8, 6 mol/L urea, 2% SDS, 30% glycerol, 200 mg IAA, 0.002% [w/v] bromophenol blue). For the second dimension separation, the strips were transferred onto sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) gels (12%) at 15 °C. A capping solution (0.002% bromophenol blue, 0.5% agarose, 192 mmol/L glycine, 25 mmol/L Tris–HCl) was used to fix the strips. Electrophoresis was conducted in the Ettan DALT-six system for 45 min at 100 V and 8 h at 200 V.

2.6.3 Gel Staining and Image Scanning Analysis

Upon the completion of electrophoresis, the gel was stained with a solution of 0.5 g Coomassie Brilliant Blue G250, 225 mL ethanol, 50 mL glacial acetic acid, and 225 mL ddH$_2$O for 12 h, and then decolourised in a solution of 10% ethanol and 10% glacial acetic acid, and finally washed until the background was clear. Digital images of the gels were acquired using an image scanner, and analysis was conducted using the PDQuest 8.0.1 software (Bio-Rad).

2.7 Mass Spectrometry Analysis and Identification of Differential Protein Spots

2.7.1 Treatment of Differential Protein Spots

The differential protein gel spots were placed in a 0.5 ml inlet centrifuge tube and rinsed twice with ultrapure water. Decolourising solution was added and gel spots were decolourised for 30 min, after which the solution was removed. We then added dehydrating liquid I and allowed gel spots to dehydrate for 30 min, and then removed the dehydrating liquid. The same procedure was repeated with dehydrating liquid II. We then added 10 µL of enzymatic hydrolysis working solution and imbibed for 30 min. Samples were enzymatically hydrolysed overnight (approximately 16 h) in 20 µL of enzymatic hydrolysis covering solution in a 37 °C water bath, after which the supernatant was transferred into a new centrifuge tube. We then added 50 µL of protein extract

to the remaining gel, incubated gels at 37 °C for 30 min, centrifuged them at 5000 g for 5 min, and then combined and freeze dried the supernatant.

2.7.2 Mass Spectrometry Detection and Protein Identification

The dry powder was dissolved in 5 µL of 0.1% Trifluoroacetic acid (TFA) solution, and then mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid containing 50% acetonitrile (ACN) and 1% TFA at a 1:1 ratio. We extracted a 1 µL sample for mass spectrometry point target identification using a ABI5800 tandem time-of-flight mass spectrometer (MALDI-TOF/TOF) (Applied Biosystems, Waltham, MA, USA), using the positive ion and automatic data acquisition modes for data collection. The mass spectrum scanning range of peptide mass fingerprinting was 800–500 Da. The 10 peaks with the highest intensity were selected for the secondary mass spectrum. We integrated the primary and secondary mass spectrometry data and used GPS 3.6 (Applied Biosystems) and Mascot 2.3 (Matrix Science, London, UK) to analyse data and identify proteins. Proteins were identified using the NCBI and Viridiplantae protein databases. A maximum of one mixed cut site was allowed. The fixed modification was Carbamidomethyl (C). Variable modifications were Acetyl (Protein N-term), Deamidated (NQ), Deoxidation (W), and Oxidation (M). MS tolerance was 100 ppm, and the MS/MS tolerance was 0.3 Da. A protein score confidence interval of > 95% indicated successful identification.

3 Results

3.1 Germination under Different Treatments

As shown in Fig. 1, the control seeds failed to germinate within 70 days, and the H$_2$SO$_4$-treated seeds failed to germinate within 45 days. The germination rate of the latter was $3.22 \pm 0.73\%$ after 60 days and $6.8 \pm 4.47\%$ after 70 days. The GA$_3$-treated seeds failed to germinate within 40 days, but achieved germination rates of $3.78 \pm 1.12\%$ after 45 days, $5.41 \pm 1.44\%$ after 60 days, and $8.13 \pm 1.46\%$ after 70 days. The dormancy of seeds treated with H$_2$SO$_4$–GA$_3$ began to break at 0–15 days; the germination rate was only $20 \pm 1\%$ at 15 days. Dormancy release continued during days 15–30. The germination rate reached $43 \pm 2\%$ at 30 days, exceeded 50% during days 35–45; late germination occurred at 60–70 days, with a germination rate of > 60%.

These results demonstrate that seeds treated with only H$_2$SO$_4$, GA$_3$, or cold stratification remained dormant after
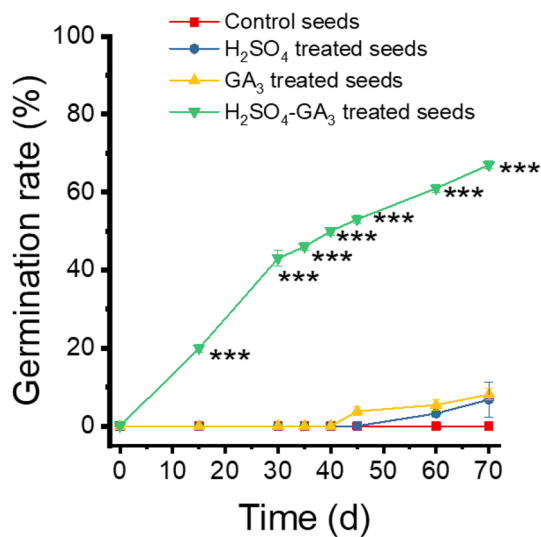
Fig. 1 Germination rates of control, H₂SO₄-treated, GA₃-treated, and H₂SO₄–GA₃-treated seeds during 0–70 days of cold stratification at 3 °C. Data are means ± standard error (SE) (n = 3)

70 days. Only $H_2SO_4 + GA_3 +$ cold stratification resulted in rapid dormancy release.

3.2 Protein Morphology and Total Protein Content Variation during Cold Stratification

The staining results revealed that the protein in the endosperm cells was stained light yellow at 0, 30, 35, 40, and 45 days, and deep yellow at 15, 60, and 70 days. The starch was dyed a dark brown-black colour. The area and density of the yellow-stained protein decreased at 15 days, followed by a spike at 30 days; thereafter, it decreased significantly at 35 days and gradually at 35–45 days. A final significant increase in protein density was observed at 60–70 days (Fig. 2).

From dormancy release to germination, total protein content exhibited a fluctuating downward trend (Fig. 3). Total protein content decreased at 0–30 days due to the consumption of a large amount of protein at dormancy release, followed by an upward trend at 30–45 days, perhaps due to the conversion of structural proteins and sugars into proteins. At 45–75 days, total protein content decreased due to protein hydrolysis during seed germination to provide nutrients for radicle growth.

3.3 Changes in Albumin, Globulin, Prolamin, and Glutelin Content during Cold Stratification

Protein content in seeds under cold stratification occurred in the order globulin > glutelin > albumin > prolamin (Fig. 4). At 0–15 days, albumin and globulin content decreased sharply, while that of prolamin and glutelin

increased. At 15–35 days, globulin, glutelin, and prolamin content increased, while only albumin content decreased. At 35–45 days, glutelin and albumin decreased significantly, while globulin and prolamin content increased. At 45–70 days, albumin, globulin, prolamin, and glutelin content decreased significantly.

3.4 Differentially Expressed Proteins (DEPs) in H₂SO₄–GA₃-Treated Seeds during Stratification

We detected 101 DEP spots exhibiting high reproducibility during stratification. Compared to 0, 64, 55, 68, 63, 66, 85 days, and 84 DEP spots were detected at 15, 30, 35, 40, 45, 60, and 70 days, respectively (Table 1). DEP expression spots exhibited an overall increasing trend, indicating that significant changes occurred from dormancy release to germination.

3.5 Functional Classification, Expression, and Metabolic Changes of DEPs during Stratification

We used DAVID 6.8 software (https://david.ncifcrf.gov/) to functionally annotate the DEPs according to Gene Ontology (GO) analysis [9]. Because *T. miqueliana* is a non-model species, its homology was mapped to the nearest model species, *Arabidopsis thaliana*.

3.5.1 Seed Storage Proteins (SSPs)

As shown in Fig. 5A, the levels of protein spots 2904 (At2g18540), 7202 (At2g28490), 8304 (At2g28490), 9701 (PAP85), and 8108 (CRD) fluctuated over 40 days. From 45 to 70 days, spots 8304 (At2g28490), 9701 (PAP85), and 8108 (CRD) were significantly upregulated, whereas 904 (At2g18540) and 7202 (At2g28490) were downregulated. Expression trends of protein spots 8304 and 7202 were completely different. Therefore, even for the same type of SSP, expression involves divergent characteristics, which degrade at different stages of dormancy release and germination. The results indicate that vicilin-like and 12S SSP were mobilised and utilised during stratification.

3.5.2 Programmed Cell Death (PCD) Proteins

Protein spots 402, 1405, and 6611 were identified as KDEL-tailed Cysteine endopeptidase1 (CEP1). This protein is associated with PCD in plants [10, 11]. As shown in Fig. 5B, after downregulation at 15 days, no significant change was identified in the level of protein spot 6611 at 15–70 days. No remarkable variation was reported in protein spots 402 and 1405 after 45 days of stratification, but
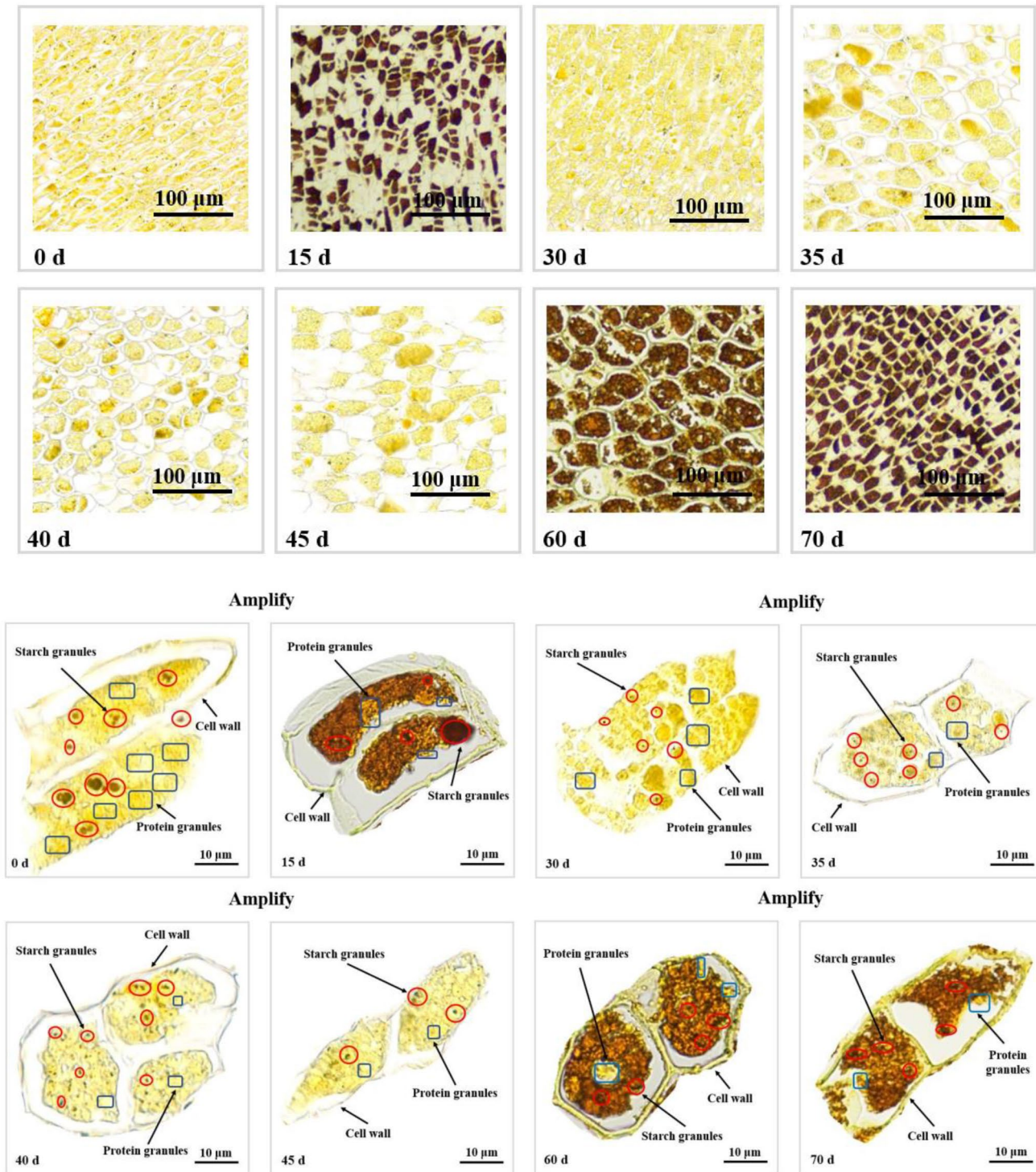
Fig. 2 Longitudinal section and amplified micrographs of *T. miqueliana* seeds treated with H_2SO_4–GA_3 and stained with iodine/potassium iodide during cold stratification. Yellow areas indicate protein. Dark brown-black areas indicate starch (Color figure online)

the spots were abundantly expressed during 60–70 days of stratification, indicating that PCD protein (CEP1) breaks down and mobilises endosperm reserves upon germination.

3.5.3 Defence Proteins

Protein spots 7804 and 8801 were identified as Catalase-2 (CAT2). CAT is one of the key enzymes in biological
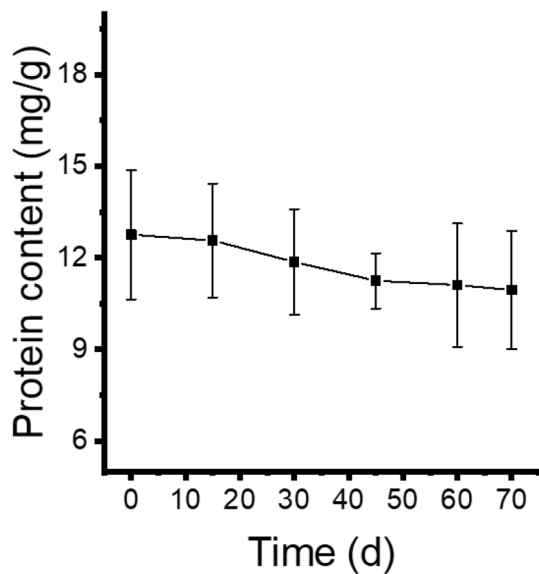
Fig. 3 Changes in the total protein content of H$_2$SO$_4$–GA$_3$-treated seeds during cold stratification. Data are means ± SE (n = 3)
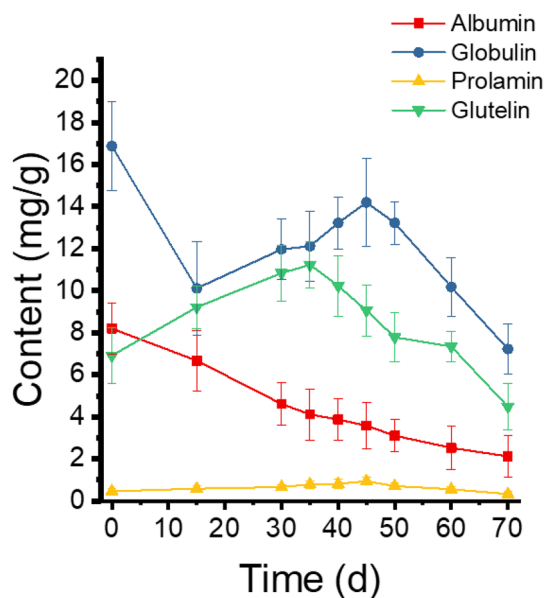

Fig. 4 Changes in albumin, globulin, prolamin, and glutelin content in H$_2$SO$_4$–GA$_3$-treated seeds during cold stratification. Data are means ± standard deviation (SD) (n = 3)

defence systems [12]. Figure 5C shows that the levels of spots 7804 and 8801 fluctuated at 60 days, and were upregulated rapidly during 60–70 days. Protein spots 4401, 4402, 4403 were identified as 1-Cys peroxiredoxin (PER1). Peroxiredoxins (Prxs) are involved in antioxidant defence in photosynthesis, respiration, and stress responses [13, 14]. As shown in Fig. 5D, the levels of spot 4401 differed at 45 days, and exhibited a rapid downregulation during 45–60 days and

a rapid upregulation during 60–70 days. Spots 4402 and 4403 exhibited an unstable trend. Protein spots 5403 and 5404 were identified as short-chain dehydrogenase/reductase (SDRs). SDRs are the most extensively distributed oxidoreductases [43]. As shown in Fig. 5E, spots 5403 and 5404 were significantly downregulated at 30 days and then fluctuated over 30–70 days. Protein spot 2905 was reported as a probable mediator of RNA polymerase II transcription subunit 37c (MED37C). It is also known as (HSP70). Figure 5F shows that spot 2905 was upregulated to a peak at 30 days and downregulated over 30–70 days. This implies that the defence protein CAT2 enhanced seed defence functions during germination, whereas large-scale breakdown of other defence proteins (PER1, SDRs, and MED37C) promoted dormancy release and germination.

3.5.4 Energy Metabolism Proteins

Protein spot 8502 was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH/GAPC2). GAPDH critically affects the carbon metabolism of cells; it acts as the key Enzyme of glycolysis (EMP) [15]. As shown in Fig. 5G, spot 8502 was significantly upregulated at 15 days, fluctuated over 15–45 days, and was then upregulated dramatically over 45–70 days. Spot 5702 was identified as bifunctional enolase 2/transcriptional activator (ENO2). ENO2 is a rate-limiting enzyme in glycolysis [44]. Figure 5G shows that spot 5702 was downregulated dramatically at 15 days, and then fluctuated over 15–70 days. This indicates that the EMP pathway was the dominant respiratory pathway during dormancy, weakening upon dormancy release and once again becoming dominant during germination.

3.5.5 Kinesin-Like Protein

Protein spots 102, 106, 202, 204, 212, 302, 2107, 2403, 5602, and 6701 were identified as a Kinesin-like protein (KIN-14). Kinesin is a typical molecular motor protein that transports various membrane organelles and protein complexes along the microtubule orientation [16]. As shown in Fig. 5H, levels of the 10 protein spots were constantly fluctuating. Peaks in the levels of spots 106 and 6701 appeared at 40 and 60 days, respectively, whereas levels of the other eight protein spots peaked at 30 and 45 days. This indicates that Kinesin-like protein (KIN-14) accelerated substance transport in endosperm cells.

3.5.6 MuDR Family Transposase

Protein spot 6201 was identified as a MuDR family transposase (At1g64255). Transposons are mobile DNA molecules that exist extensively in the genome of higher plants. Mutator (MU) refers to the most active transposon. The autonomy

Table 1 Variation in protein expression during cold stratification

Time(days)	Number of differential protein spots	Down-regulated expression (number)	Up-regulation of expression (number)	Up-regulated protein proportion (%)
8.04 (15)	64	24	40	62.50
8.20 (30)	55	32	23	41.82
8.24 (35)	68	37	31	45.59
8.29 (40)	63	29	34	53.97
9.03 (45)	66	33	33	50.00
9.18 (60)	85	23	62	72.94
9.28 (70)	84	24	60	71.43

factor of the Mu transposon is MuDR [17]. MuDR consists of mudrA and mudrB; mudrA transcribes a 120-kDa transposase, and mudrB encodes a 23-kDa MURB protein [18]. Figure 5I suggests that the levels of spot 6201 decreased to a nadir at 40 days and then fluctuated over 40–70 days. This implies that large-scale degradation of MuDR family transposase promoted dormancy release and germination.

3.5.7 Cation/Calcium Exchanger

Protein spot 6507 was identified as cation/calcium exchanger 1 (CCX1). The Ca^{2+}/Cation antiporter (CaCA) superfamily proteins transport cytosolic Ca^{2+} or other cations across membranes against their electrochemical gradient by exploiting the downhill gradients of H^+, Na^+, or K^+ that are generated by primary transporters [19]. As shown in Fig. 5J, spot 6507 was upregulated to the peak value at 30 days, and was then downregulated until disappearing over 30–70 days This indicates that the cation/calcium exchanger CCX1 probably accelerated the transport of sodium and potassium ions in endosperm cells.

3.5.8 Sucrose Phosphate Synthase

Protein spot 6507 was identified as Sucrose phosphate synthase1 (SPS1). Sucrose phosphate synthase (SPS) is a critical enzyme in sucrose biosynthesis in plants and microbes [20]. Three SPS gene families have been reported in dicotyledonous plants: A, B, and C [21]. As shown in Fig. 5K, spot 6507 continued to be downregulated until 70 days. Sucrose content exhibited an upward trend (Supplementary data Fig. 2). These results indicate that SPS1 participates in and regulates the synthesis of sucrose, which provides nutrition for dormant and germinating seeds.

3.5.9 P-Loop-Containing NTP Hydrolase Superfamily Protein

Protein spots 7501 and 7507 were identified as a P-loop-containing NTP hydrolase superfamily protein (At3g12020).

NTP refers to a vital region of the protein, covering two distinct motifs: "Walker A" and "Walker B" [22]. Domains from this superfamily are capable of hydrolysing Adenosine triphosphate (ATP) or guanosine triphosphate (GTP); they can also act as kinases and transferases on their own or in combination with other superfamilies [23]. At3g12020 can catalyse the hydrolysis of NTP and use its energy to generate power [24]. At3g12020 has ATPase activity and can be combined with ATP, and participates in the microtubule-based process and microtubule-based movement (Table 3). The energy generated by ATP/GTP can be used to catalyse the movement of microtubules to the positive end [25]. As shown in Fig. 5L, spots 7501 and 7502 were upregulated at 30 days and then downregulated irregularly over 30–70 days. This implies that NTP hydrolysis was catalysed to provide power for microtubule movement, thus accelerating dormancy release.

3.5.10 Senescence-Associated Family Protein

Protein spots 5001, 6102, 7101, and 7102 were identified as senescence-associated family protein (DUF581, At4g17670). DUF581 contains a plant-specific Zf-FCS zinc finger domain that acts as an FCS-like zinc finger (FLZ), suggesting that the FLZ gene family plays a role in senescence [26]. As shown in Fig. 5M, the levels of spots 5001, 6102, 7101, and 7102 fluctuated at 45 days and were downregulated dramatically at 45–70 days. This indicates the degradation of senescence-associated proteins, which likely facilitated the recycling of nitrogen (N) and other nutrients, thus accelerating dormancy release.

3.6 Differential Proteins and Related Kyoto Encyclopedia of Genes Pathways in Different Stages of Dormancy Release and Germination

As shown in Fig. 5 and Table 2, the Kyoto encyclopedia of genes (KEGG) includes eight pathways (metabolic, secondary metabolic biosynthesis, carbon metabolism, peroxisome, glycolysis/gluconeogenesis, biosynthesis of amino
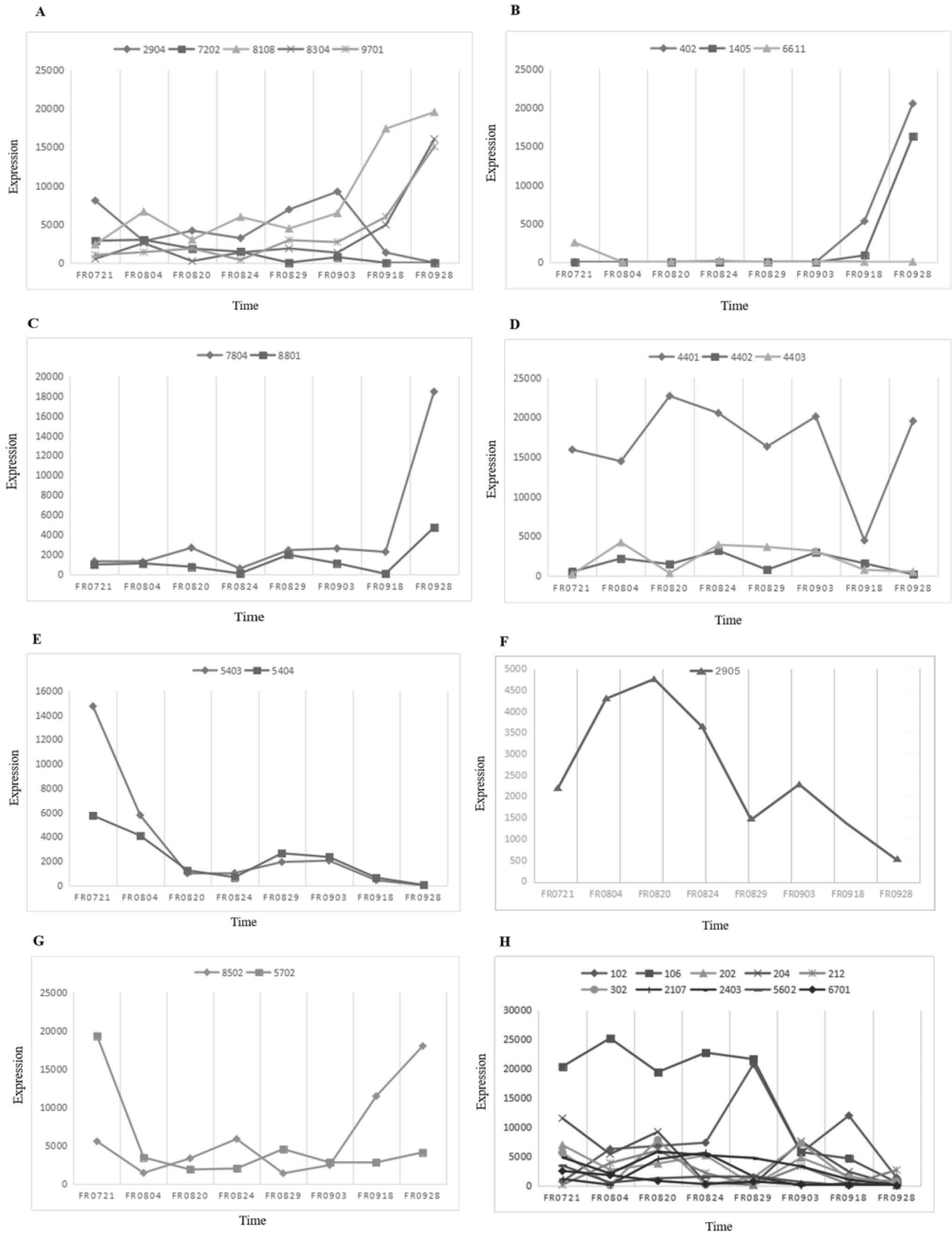
Fig. 5 Expression changes of **A** storage protein, **B** CEP1, **C** CAT2, **D** PER1, **E** SDRS, **F** MED37C, **G** GAPC2 ENO2, **H** KIN14B, **I** MuDR, **J** CCX1, **K** SPS1, **L** NTP, and **M** DUF581 at 0 (FR0721), 15 (FR0804), 30 (FR0820), 35 (FR0824), 40 (FR0829), 45 (FR0903), 60 (FR0918d), and 70 days (FR0928)
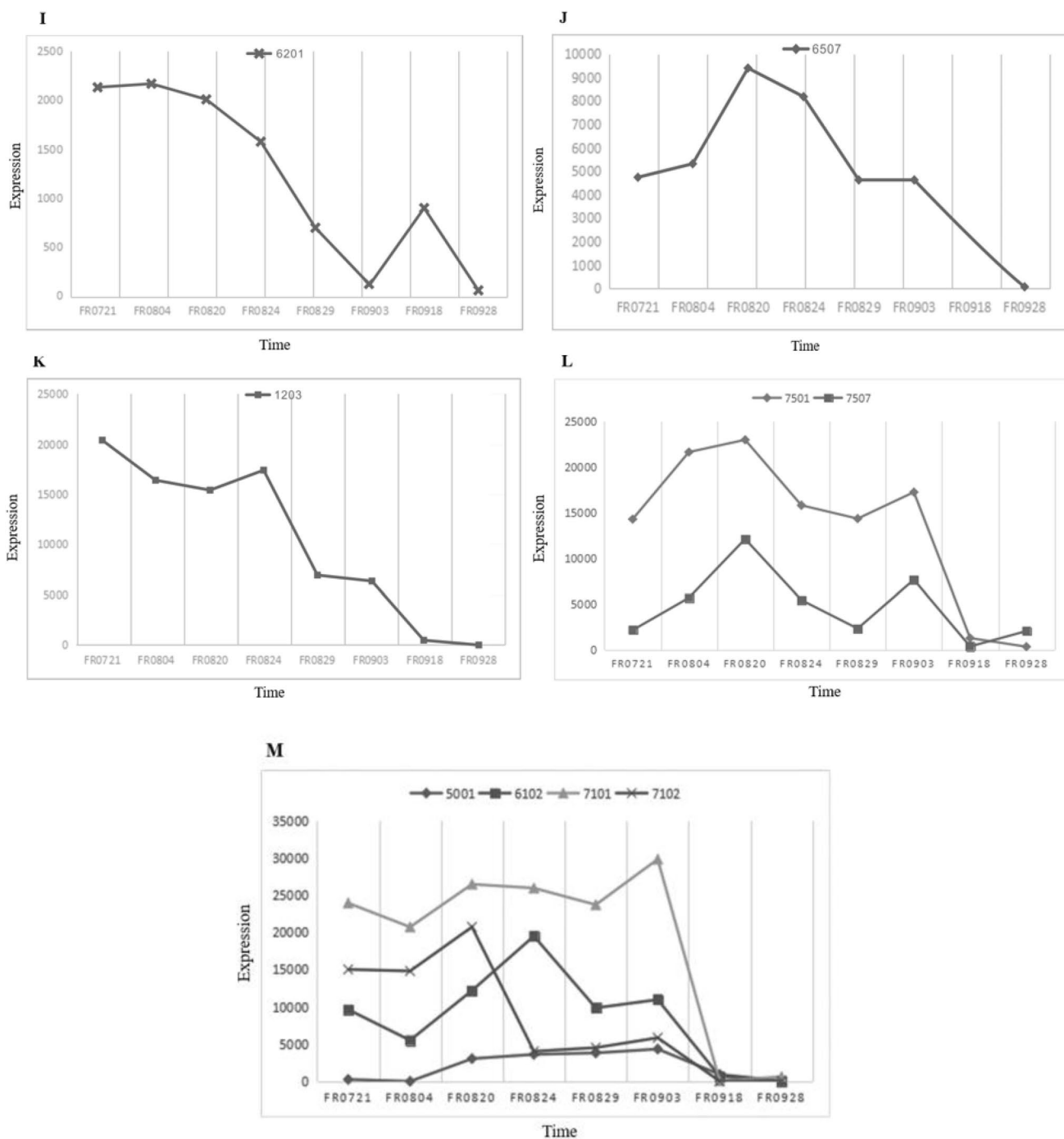
Fig. 5 (continued)

acids, tryptophan metabolism, glyoxylate, and dicarboxy-late metabolism) and six differential proteins (CAT2, ENO2, GAPC2, PER1, SDRA, and SPS1) that are involved in the process of dormancy release and germination. The carbon metabolism and peroxisome pathways appear at all stages, and involve defence proteins (CAT2, SDRA) and energy proteins (ENO2, GAPC2). The number of KEGG pathways increased to seven at the critical stage of dormancy release

(35 days). The newly added pathways include glycolysis/gluconeogenesis, secondary metabolic biosynthesis, amino acid biosynthesis, and glyoxylic acid and glyoxylic acid metabolism. Proteins implicated in these pathways include defence proteins (CAT2, PER1, SDRA) and energy proteins (ENO2, GAPC2). Two defence proteins (CAT2, PER1) and one energy protein (ENO2) exhibited fluctuating trends. One energy protein (GAPC2) exhibited a significant downward

Table 2 Kyoto encyclopedia of genes and genomes (KEGG) pathways associated with differentially expressed proteins at Casaletto 30 (FR0820), 35 (FR0824), 40 (FR0829), 45 (FR0903), 60 (FR0918d), and 70 days (FR0928)

Time	Pathway Name	Pathway ID	Pvalue	Genes
FR0820 30d	Peroxisome	ath04146	3.11E-03	SDRS, CAT2
	Carbon metabolism	ath01200	2.64E-02	ENO2, CAT2
	Tryptophan metabolism	ath00380	4.69E-02	CAT2
FR0824 35d	Peroxisome	ath04146	0.00011	SDRS, CAT2
	Carbon metabolism	ath01200	0.000118	ENO2, CAT2, GAPC2
	Tryptophan metabolism	ath00380	0.00131	CAT2
	Biosynthesis of secondary metabolites	ath01110	0.00273	PER1, ENO2, CAT2, GAPC2
	Glyoxylate and dicarboxylate metabolism	ath00630	0.00336	CAT2
	Glycolysis/Gluconeogenesis	ath00010	0.0077	ENO2, GAPC2
	Biosynthesis of amino acids	ath01230	0.0364	ENO2, GAPC2
FR0829 40d	Peroxisome	ath04146	3.11E-03	SDRS, CAT2
	Carbon metabolism	ath01200	2.64E-02	ENO2, CAT2
	Tryptophan metabolism	ath00380	4.69E-02	CAT2
FR0903 45d	Peroxisome	ath04146	3.11E-03	SDRS, CAT2
	Carbon metabolism	ath01200	2.64E-02	ENO2, CAT2
	Tryptophan metabolism	ath00380	4.69E-02	CAT2
FR0918 60d	Carbon metabolism	ath01200	2.82E-03	ENO2, GAPC2, CAT2
	Peroxisome	ath04146	4.62E-03	SDRS, CAT2
	Glycolysis/Gluconeogenesis	ath00010	7.70E-03	ENO2, GAPC2
	Biosynthesis of secondary metabolites	ath01110	2.53E-02	PER1, ENO2, GAPC2, CAT2
	Biosynthesis of amino acids	ath01230	3.64E-02	ENO2, GAPC2
	Metabolic pathways	ath01100	3.95E-02	SPS1, PER1, SDRS, ENO2, GAPC2
FR0928 70d	Peroxisome	ath04146	2.99E-04	SDRS, CAT2
	Carbon metabolism	ath01200	5.04E-04	ENO2, CAT2, GAPC2
	Tryptophan metabolism	ath00380	2.41E-03	CAT2
	Glyoxylate and dicarboxylate metabolism	ath00630	6.14E-03	CAT2
	Glycolysis/Gluconeogenesis	ath00010	1.39E-02	ENO2, GAPC2
	Biosynthesis of secondary metabolites	ath01110	1.69E-02	PER1, ENO2, CAT2, GAPC2

Box refers to the new pathway in FR0824 (35d) and FR0918 (60d)

trend. There are six KEGG pathways at the germination stage (60 days), and the metabolic pathway is the unique pathway during this stage. Proteins involved in this stage are SPS1, PER1, SDRA, ENO2, and GAPC2. Sucrose phosphate synthase protein (SPS) exhibited a significant downward trend.

3.7 Construction of a Protein Co-Expression Network during Seed Germination

Based on the adjacency coefficient relationship between proteins, which in turn was determined using a Weighted gene co-expression network analysis (WGCNA) algorithm, proteins with adjacency coefficients of > 0.6 were retained

to build a protein co-expression network. The network was visualised using Cytoscape 3.3 and included 16 nodes and 60 connection edges, with 28 negative correlation connections and 35 positive correlation connections (Fig. 6). Table 3 lists annotated GO functions and pathways of proteins in the co-expression network. The proteins in the co-expression network are associated with photosynthesis, glucose metabolism, and responses to metal ions; certain proteins also participate in hormone synthesis, sugar biosynthesis, and other pathways. The GO functions and pathways involved proteins At2g28490, At3g12020, GAPC2, PAP85, SPS1, At1g64255, and At2g18540.

Dormancy release and early stages of germination involved storage proteins (vicilin-like, 12S), PCD protein (CEP1), defence proteins (CAT2, PER1, SDRA, MED37C), energy proteins (ENO2, GAPC2), kinesin-like protein
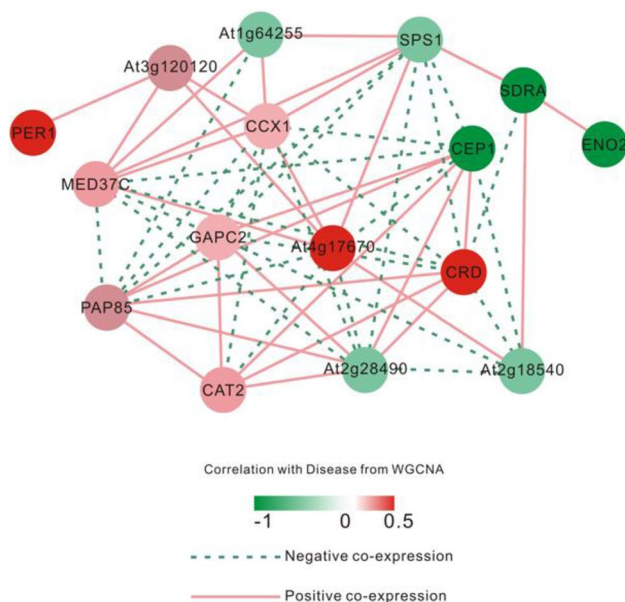
Fig. 6 Protein co-expression network diagram. We detected significant correlations at 16 nodes (PER1, At3g120120, At1g64255, SPS1, SDRA, ENO2, CCX1, CEP1, MED37C, GAPC2, At4g17670, CRD, PAP85, CAT2, At2g28940, and At2g18540) and 60 connecting edges, among which 28 were negative and 35 were positive. Nodes indicate correlation coefficients between proteins and time points. A change in node colour indicates change in regulation; red indicates up-regulation and green indicates down-regulation. The dotted green line indicates a negative correlation between proteins, whereas the solid red line indicates a positive correlation (Color figure online)

(KIN14B), senescence-associated family protein (DUF581), MuDR family transposase (At1g64255), cation/calcium exchanger protein (CCX1), sucrose phosphate synthase protein (SPS), and P-loop-containing NTP hydrolase superfamily protein (At3g12020).

Storage proteins (vicilin-like, 12S), defence proteins (CAT2, PER1), energy protein (ENO2), kinesin-like protein (KIN14B), and senescence-associated protein (DUF581) all exhibited fluctuating trends. KEGG pathway analysis indicated that the defence protein CAT2 was involved in peroxisome, carbon metabolism, tryptophan metabolism, dicarboxylate metabolism, and biosynthesis of the secondary metabolite glyoxylate. The defence protein PER1 was also involved in glyoxylate biosynthesis. PCD protein (CEP1), defence protein (SDRA), energy protein (GAPC2), and MuDR family transposase (At1g64255) all exhibited significant downward trends. KEGG pathway analysis indicated that the defence protein SDRA was involved in peroxisome and metabolic pathways, whereas the energy protein GAPC2 was involved in carbon metabolism, glycolysis/gluconeogenesis, and biosynthesis of glyoxylate and amino acids. Defence protein (MED37C), cation/calcium exchanger protein (CCX1), sucrose phosphate synthase protein (SPS),

and P-loop-containing NTP hydrolase superfamily protein (At3g12020) all exhibited significant fluctuations.

The later stages of germination involved storage proteins (vicilin-like, 12S), PCD protein (CEP1), defence proteins (CAT2, PER1, SDRA, MED37C), MuDR family transposase (At1g64255), cation/calcium exchanger protein (CCX1), sucrose phosphate synthase protein (SPS), and P-loop-containing NTP hydrolase superfamily protein (At3g12020). Storage proteins (vicilin-like, 12S), PCD protein (CEP1), defence proteins (CAT2, PER1), and energy protein (GAPC2) all exhibited significant upward trends. KEGG pathway analysis indicated that the specific type of defence protein (CAT2) involved in the pathway remained unchanged, but the defence protein (PER1) became newly involved in metabolic pathways. Defence proteins (SDRA, MED37C), MuDR family transposase (At1g64255), cation/calcium exchanger protein (CCX1), sucrose phosphate synthase protein (SPS), and P-loop-containing NTP hydrolase superfamily protein (At3g12020) all exhibited significant downward trends. KEGG pathway analysis indicated that the defence protein SDRA became newly involved in metabolic pathways.

4 Discussion

4.1 Effects of Treatments on Dormancy Release and Germination

The germination test revealed that untreated seeds remained dormant, whereas seeds treated using a single method (H_2SO_4, GA_3, or cold stratification) remained dormant after 70 days. Dormancy of seeds treated with the combination of $H_2SO_4 + GA_3 +$ cold stratification was broken at 45 days. This is consistent with another study, which found treatment of *Tilia amurensis* seeds achieved 60% germination with hulling + acid etching (98% H_2SO_4 for 20–25 min) + GA_3 soaking (48 h) + cold stratification (4 °C for 20–25 days) [27]. Dormancy was released in H_2SO_4-treated seeds following 100 days of cold stratification. Seeds treated with $GA_3 +$ cold stratification or cold stratification alone remained dormant (Supplementary data Fig. 1). Therefore, GA_3 cannot enter the seed to break dormancy unless the seed is treated with H_2SO_4. This indicates that the seed coat hinders the absorption and utilisation of GA_3, which is consistent with studies indicating that GA_3 had little effect on germination of un-hulled pear seeds, while hulling + cold stratification significantly improved germination [28]. Thus, elimination of the mechanical constraints and permeability barriers in the seed coat with H_2SO_4 is the first step in breaking the dormancy of *T. miqueliana* seeds, whereas GA_3 and cold stratification accelerate dormancy release.

Table 3 Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway annotation of DEPs in the co-expression network

Category	Term	Protein
Biology process	G0:0046165~alcohol biosynthetic process	GAPC2
	GO:0010038~response to metal ion	GAPC2
	GO:0015979~photosyntbesis	SPSI, GAPC2
	G0:0007017~ microtubule-based process	AT3G12020
	G0:0007018~microtubule-based movement	AT3G12020
	G0:0046686~response to cadmium ion	GAPC2
	G0:0006006~glucose metabo lic process	GAPC2
Cellular component	G0:0005618~cell wall	GAPC2, PAP85
	G0:0030312~ external encapsulating stmcture	GAPC2, PAP85
	G0:0043228~non-membrane-bounded organelle	GAPC2, AT3G12020
	G0:0009536~plastid	GAPC2, SPSl
	GO:0012505~ endomem brane system	AT2G28490, AT2G I 8540, AT2GJ8540
Molecular function	G0:0045735~nutrient reservoir activity	AT2G 18540, PAP85
	G0:0000166~nucleotide binding	GAPC2, AT3G12020
	G0:0046914~transition metal ion binding	AT1G64255
	G0:0043167~ ion binding	AT1G64255
	G0:0043169~cation binding	AT1G64255
	G0:000827Q~zinc ion binding	AT1G64255
	G0:0046872~ metal ion binding	AT1G64255
KEGG pathway	ath01070:Biosynthesis of plant hormones	GAPC2
	ath01062:Biosynthesis of terpenoids and steroids	GAPC2
	ath00010:Glycolysisl gluconeogenesis	GAPC2
	ath01061: Biosynthesis of phenylpropanoids	GAPC2
	ath00900:Terpenoid backbone biosynthesis	SPS1
	ath01065: Biosynthesis of alkaloids deri ved from histidine and purine	GAPC2
	ath01063: Biosynthesis of alkaloids derived from shikimate pathway	GAPC2

4.2 Effects of H₂SO₄–GA₃ Treatment on Protein Morphology, Total Protein Content, and Storage Protein during Stratification

During cold stratification, total protein content decreased significantly during dormancy release (0–35 days), and increased significantly during germination (60–70 days). This finding is consistent with a previous report of protein trends during *Ginkgo biloba* seed germination [29]. Thus, H₂SO₄–GA₃ treatment accelerates dormancy release and promotes germination.

Storage protein is an important component of seed storage materials; it provides nutrients and energy for seed germination and seedling growth [30]. The main storage proteins contained in seeds are globulin, albumin, prolamin, and glutenin [31]. In this study, the order of protein content was globulin > glutelin > albumin > prolamin; the degree of their degradation varied. The content of albumin, globulin, and glutelin, which may be the main nitrogen sources for embryo and bud growth, consistently decreased. Prolamin content was lowest and did not change significantly over time. We

speculate that prolamin may not be a nutritional protein for *T. miqueliana*; instead, it may be a functional or structural protein in seeds of this species. Changes in albumin, globulin, and glutelin content were consistent with those of *Stylosanthes* SW. seeds during germination [32], which indicate that storage protein decomposes continuously during cold stratification after H₂SO₄–GA₃ treatment, to provide energy for seed germination.

4.3 Proteomics Analysis of H₂SO₄-GA₃-Treated Seeds during Stratification

4.3.1 Screening and Annotation of DEPs

Seed storage proteins are used as carbon and nitrogen sources during seed germination; therefore, they play an important role in seedling formation [33]. In this study, protein spots 2904 (At2g18540), 7202 (At2g28490), 8304 (At2g28490), and 9701 (PAP85) were identified as vicilin-like SSPs. Protein spot 8108 (CRD) was identified as a 12S SSP. SSPs are components of a

seed storage substance that provides nutrients and energy for seed germination and early seedling growth [34]. The expression of vicilin-like SSPs and the 12S SSP exhibited a fluctuating trend, indicating that they were mobilised from dormancy release to germination. This finding is consistent with the trend in 12S SSP expression during dormancy release reported in *P. amurense* Rupr. seeds [2].

PCD is an active and orderly process controlled by specific genes [35]. Protein spots 402, 1405, and 6611 were identified as CEP1, which is associated with PCD in plants. After seed germination, protein spots 402 and 1405 were expressed in large quantities, which is consistent with PCD during seed germination. A previous study found that if PCD did not occur in the aleurone layer during cereal seed germination, the embryo would cease activity due to a lack of nutrient supply, terminating the germination process [36].

The process of dormancy release and germination was accompanied by redox reactions in proteins. Defence proteins prevent reduced proteins from being oxidised [37]. Protein spot 2905 was identified as a probable mediator of RNA polymerase II transcription subunit 37c (MED37C), a heat shock protein (HSP; heat shock 70 kDa protein 4) belonging to HSP70. HSPs are sensitive to high temperatures, improving cell defence [38]. The most well understood HSPs are those with molecular masses of 60, 70, 90, and 110 kDa [39]. Extensive research indicates that a major function of some HSP70s is to act as a molecular chaperone. Although the production of HSPs has frequently been described under heat stress, their expression and function have been also confirmed during fundamental processes such as seed development. Generally, HSPs are highly expressed in mature, dry seeds, e.g. in the late stages of seed development, but tend to disappear during germination [40]. The expression level of spot 2905 peaked during the dormant stage (0–30 days) and declined significantly during the critical phase of dormancy release (30–40 days), reaching its lowest value during the germination stage (60–70 days). This is consistent with studies reporting that HSP is the essential protein for maintaining dormancy in *Arabidopsis* seeds [25]. HSP may induce germination in tobacco. Ectopically expressed NtHSPs in the cytoplasm interact with proteins involved in seed germination and change the status of the proteins, such that they are ready to support seed germination without additional stimulus [40].

Energy metabolism is crucial to seed germination, and is characterised by the mobilisation of starch, fat, and protein [41]. In this study, protein spot 8502 was identified as GAPDH/GAPC2. GAPDH critically affects the carbon metabolism of cells, acting as the key enzyme of glycolysis. ENO2 is a rate-limiting enzyme in glycolysis. The trends in GAPDH and ENO2 expression observed in this study were consistent with those reported for the three key enzymes K7, K15, and K26 in the EMP pathway of maize [42].

4.3.2 Screening of DEPs and Construction of the Co-Expression Network

WGCNA screening revealed that the proteins SPS1, SDRS, ENO2, At1g64255, At4g17670, and At2g28490 were significantly associated with seed dormancy. SPS is a vital enzyme in sucrose biosynthesis in plants and microbes [20]. SDRs are the most extensively distributed oxidoreductases, regulating the physiological and biochemical reactions of plants by participating in a variety of primary and secondary metabolic reactions [43]. ENO2 acts as a rate-limiting enzyme in glycolysis, capable of catalysing the conversion of 2-phosphate-D-glycerate (2-PGA) to phosphoenolpyruvate (PEP) in the glycolysis pathway [44]. At1g64255 is a MUDR family transposase, responding toQuery developmentally regulated signals [45]. At4g17670 is the senescence protein DUF581, which contains an FCS-like zinc finger (FLZ). He's research group reported that FLZ genes are associated with senescence and ABA mediated seed dormancy in A. thaliana [46, 47]. Laxmi identified transcript accumulation in senescing leaves compared with rosette leaves, suggesting a role for the FLZ gene family in senescence [48]. At2g28490 is a storage protein, a vital component of substance storage in seeds that provides nutrients and energy for seed germination and early growth of seedlings [34].

GO function and KEGG pathway annotation analysis of proteins in the co-expression network revealed that DEPs were are largely associated with photosynthesis, the glucose metabolic process, and responses to metal ions, and are involved in the plant hormone and glycolysis/glycogenesis biosynthesis pathways. These findings are consistent with a report that DEPs of *Davidia involucrate* seeds are mainly involved in the photosynthesis and EMP pathways during germination [49].

5 Conclusion

We used PSD, CBB, and 2DE–MALDI–TOF/TOF to investigate the effects of H_2SO_4–GA_3 and cold stratification on proteins during dormancy release and germination in *T. miqueliana* seeds. Our results indicated that H_2SO_4–GA_3, combined with cold stratification, enhanced dormancy release. The morphology of the protein changed significantly, and the degradation and utilisation of albumin, globulin, prolamin, and glutelin in endosperm cells were accelerated. Sixteen differential proteins were identified through mass spectrometry, including storage proteins, PCD protein, defence proteins, energy proteins, kinesin-like protein, senescence-associated family protein, MuDR family transposase, cation/calcium exchanger protein, sucrose phosphate synthase protein, and P-loop-containing NTP hydrolase superfamily protein. KEGG

pathway annotation results indicated that the different proteins at all time points were involved in the carbon metabolism and peroxisome pathways. Seven KEGG pathways participated in the critical stage of dormancy release (30–35 days). Pathways added were biosynthesis of secondary metabolites, glyoxylate and dicarboxylate metabolism, glycolysis/gluconeogenesis, and biosynthesis of amino acids. Metabolic pathways were the unique pathway that participated in the early stage of germination (60 days), and which enriched the most differential proteins. The proteins in the co-expression network were mainly related to biological processes such as responses to metal ions, photosynthesis, the microtubule-based process, microtubule-based movement, responses to cadmium ions, and the glucose metabolic process. They were involved in biosynthesis of hormones, phenylpropanoids, terpenoids, and steroids, glycolysis/gluconeogenesis, terpenoid backbone biosynthesis, and biosynthesis of alkaloids derived from histidine, purine, and the shikimate pathway. They act synergistically and participate in the physiological and molecular processes of protein metabolism and degradation, which accelerate dormancy release and germination.

Author Contributions YBS conceived the original screening and research plans; YW performed the experiments; YW designed the experiments and analyzed the data; YW conceived the project and wrote the article with contributions of all the authors. YW agrees to serve as the author responsible for contact and ensures communication. All authors have read and approved the manuscript.

Data Availability All data generated or analysed during this study are included in this published article.

Code Availability The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021999. *Submission details*: (1) Project Name: Key protein variations during seeds germination in *Tilia miqueliana*, (2) Project accession: PXD021999, (3) Project DOI: Not applicable. *Reviewer account details*: (1) Username: reviewer_pxd021999@ebi.ac.uk, (2) Password: DIyJPxwv.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical Approval We strictly comply with the Convention on the Trade in Endangered Species of Wild Fauna and Flora, and also abide by Law of the People's Republic of China on Wildlife Protection. Research permission on *Tilia miqueliana* tree has been obtained from Jiangsu Wildlife Protection Station. The collection of experimental intact seeds was approved by the Management Office of Huang Zangyu National Forest Park in Anhui Province, China.

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