顽拗性种子板栗脱水过程中蛋白质组分析

韩彪[1]，张铭佳[1,2]，崔程程[1]，仝伯强[1]，解孝满[1]

（1.暖温带林草种质资源保存与利用国家林业和草原局重点实验室，山东省林草种质资源中心，济南，250202；2. 北京林业大学生物科学与技术学院，北京，100083）

摘　要：【目的】研究顽拗性种子板栗脱水过程中蛋白质组和发芽能力的变化。【方法】以中国山东济南采集的成熟板栗种子为研究对象，测定不同含水量胚轴的蛋白质组变化和萌发情况。【结果】基于相对和绝对定量的蛋白质组（iTRAQ）分析鉴定了 5201 个可信蛋白，20%含水量（MC）组具有最多的差异蛋白（DAP）和下调的差异蛋白，而 10%MC 组具有最多的上调差异蛋白。生物信息学分析表明，在 30%含水量时，与抗氧化系统和代谢系统相关的差异蛋白显着增加。在 20%MC 时，显著的差异蛋白主要与内质网中的核糖体和 RNA 相关。当胚胎轴脱水至 10%MC 时，“结构分子活性”的差异蛋白显著积累。【结论】板栗种子属于顽拗性种子，快速脱水过程对胚轴萌发产生严重影响。912 个差异蛋白的产生复杂的相互作用，特别是 563 个独特的差异，需要进一步的研究和功能验证。板栗种子 DAPs 可用于筛选不同脱水耐性的种质资源群体。

关键词：顽拗性种子；脱水敏感性；iTRAQ；蛋白质组

Proteome Analysis During Dehydration of Recalcitrant Seed *Castanea mollissima* Blume

Han Biao[1], Zhang Ming-jia[1,2], Cui Cheng-cheng[1], Tong bo-qiang[1], Xie Xiao-man[1]

（Key Laboratory of State Forestry and Grassland Administration Conservation and Utilization of Warm Temperate Zone Forest and Grass Germplasm Resources, Shandong Provincial Center of Forest and Grass Germplasm Resources, Jinan 250120, China；2. College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China）

Abstract:【Objective】In order to investigate proteome and germinability in response to the dehydration of recalcitrant seed *Castanea mollissima*.【Method】Mature seeds of *C. mollissima* were collected from a chestnut garden in Jinan city, Shandong province, China. the proteome and germination were measured in embryonic axes at different moisture contents.【Result】The results showed that the germination rate rapidly decreased when the embryo moisture content decreased to 20% or below. The isobaric tags for relative and absolute quantitation (iTRAQ)-based proteome analysis identified 5201 trusted proteins, the 20% moisture content (MC) group had the most differentially accumulated proteins (DAPs) and down-regulated DAPs, while the 10% MC group had the most up-regulated DAPs. Bioinformatic analysis showed that there was a significant increase in DAPs associated with the antioxidant system and metabolic system at 30% MC. At 20% MC, the significant DAPs were mainly associated with ribosome and RNA in the endoplasmic reticulum. When the embryonic axis was dehydrated to 10% MC, the DAPs of “structural molecule activity” accumulated significantly. The DAPs can be used for screening natural populations of *C. mollissima* for dehydration-tolerant germplasm resources.【Conclusion】The seeds of *C. mollissima* are recalcitrant, and an MC of 20% or less had a serious impact on embryonic axis germination during rapid desiccation. The complex interactions of 912 DAPs, particularly 563 unique DAPs, need further investigation and experimental validation. The DAPs can be used to screen natural populations of *C. mollissima*, with the aim of discovering dehydration-tolerant germplasm resources.

Key words: recalcitrant seed; desiccation sensitive; iTRAQ; proteome.

Based on the response to dehydration, seeds can be divided into orthodox seeds, intermediate seeds and recalcitrant seeds. The mature recalcitrant seeds possess high moisture contents and cannot tolerate removal cellular water without viability loss, and thus they cannot be dried and stored for long periods (Li and Pritchard, 2009; Xia *et al.*, 2012; Walters *et al.*, 2013). Recalcitrant seeds pose a significant challenge to seed cryopreservation, and thus the associated desiccation-sensitive molecular mechanisms need to be studied. Seeds have been used as models to understand desiccation tolerance by "omics" approaches, and some proteomic, metabolomic, and transcriptomic studies have been reported to describe changes in seeds (Leprince *et al*., 2010). These studies have led to a significant increase in the knowledge of seed-related processes, such as dormancy, germination, and tolerance to abiotic stress (Soeda *et al*., 2005; Su *et al.*, 2019).

Proteomics be used to evaluate the response of proteins to a particular treatment, allowing for protein quantity and protein–protein interactions to be revealed concurrently（Ji *et al.*，2021）. The *Medicago truncatula* Gaertn. seeds was used to analyze the heat-stable proteome of the radicles, identifying the late embryogenesis proteins associated with desiccation tolerance (Boudet *et al*., 2006). Proteome changes in several fruit species have also been used to describe changes associated with chilling injury or oxidative stress during cold storage (Molassiotis *et al*., 2013). Therefore, proteomic research can be used to identify desiccation sensitivity-related protein changes in recalcitrant seeds.

Castanea mollissima Blume is a widely distributed and important economic forest tree species in China, and many farmers rely on chestnuts as their main economic source in mountainous areas (Zou *et al.*, 2015). However, *C. mollissima* has desiccation-sensitive seeds, which makes seed preservation difficult to achieve (Zong *et al.*, 2010). An iTRAQ proteomic analysis approach was used in the present study to compare the differentially accumulated proteins (DAPs) of *C. mollissima* embryonic axes under different moisture contents. Desiccation sensitivity-related proteins were discovered and analyzed through bioinformatics analysis.

1 Materials and Methods

1.1 Seed materials

Mature seeds of *C. mollissima* were collected from a chestnut garden in Jinan city, Shandong province, China. All the seeds were collected from the same clone in order to ensure consistency. After harvesting, the seeds were sealed in plastic bags and stored at 2°C until further germination tests and iTRAQ-based proteomic analysis.

1.2 Desiccation method and moisture content test

For the desiccation experiments, 20 embryonic axes were placed in an oven at 103°C for 17 h to determine the initial moisture content, with three replicates. Some embryonic axes were then dried by placing them on an open Petri dish (7 cm in diameter) in a drying chamber containing silica gel, with three fans used to enhance dry air circulation in the chamber. The desired moisture contents (50%, 40%, 30%, 20%, 10%) of the embryonic axes were gravimetrically determined by comparison with the initial moisture content.

1.3 Germination test

For the germination test, 30 embryonic axes of different moisture contents were surface-sterilized. The embryonic axes were then cultured in tissue-culture bottles containing modified Murashige & Skoog (MS) medium [half-strength nitrates, 0.5 mg/L 6-benzylaminopurine (6-BA), 30 g/L sucrose, 1 mL/L plant preservative mixture, 5 g/L agar, pH=5.6] (Corredoira *et al.*, 2004). The embryonic axes were cultured in the dark for 2 weeks and then transferred to a 12/12 h light-dark photoperiod for 2 weeks at 25°C, following which germination was scored. The germination test was repeated three times.

1.4 Protein extraction

Every moisture content group used 10 embryonic axis for protein extraction and repeated two times. Equal amounts of embryonic axis samples were ground into a fine powder in liquid nitrogen. Then, 1 mL extraction buffer was added, and the mixtures were combined with Tris-phenol buffer and mixed for 30 min at 4°C. The mixtures were then centrifuged at 7100 *g* for 10 min at 4°C to collect the phenol supernatant, to which five volumes of 0.1

M pre-cooled ammonium acetate-methanol buffer was added, and the mixture precipitated overnight at -20°C. The samples were then centrifuged at 12000 *g* for 10 min to collect the precipitate, which was washed with five volumes of pre-cooled methanol. The samples were centrifuged at 12,000 *g* for 10 min at 4°C, following which the precipitates were dried at room temperature and then dissolved in lysis buffer for 3 h. Finally, the samples were centrifuged at 12,000 *g* for 10 min, and the supernatant collected. Protein concentration was determined using the Bradford assay, and the samples were stored at -80°C.

1.5 Filter-Aided Sample Preparation (FASP) digestion and iTRAQ labeling

The 120 μL buffer [100 mM TEAB, 10 mM DTT, 8 M urea, pH=8.0] and 100 μg protein extract were mixed in an ultrafiltration tube and incubated for 1 h (60°C). Indoleacetic acid (IAA) was added to a final concentration of 50 mM and incubated in the dark for 40 min. The solutions were centrifuged at 12,000 *g* for 20 min (4°C), and the flow-through was discarded. We then added 100 μL TEAB (300 mM) to the solutions and centrifuged them at 12,000 *g* for 20 min. The filter units were transferred into new collection tubes, to which 100 μL TEAB and 2 μL sequencing-grade trypsin (1 μg/μL) were added. The solutions were incubated at 37°C for 12 h. The digested peptides were collected and centrifuged at 12,000 *g* for 20 min. We then placed 50 μL TEAB (200 mM) in the tube and centrifuged it once again, and the solutions were collected and lyophilized (Wisniewski *et al.*, 2009).

For iTRAQ labeling, the lyophilized samples were resuspended in 100 μL TEAB (200 mM), following which 40 μL of the sample was transferred into 1.5-mL Eppendorf tubes for labeling. We then added 200 μL isopropanol to the iTRAQ reagent vial, followed by eddy current blending and centrifugation. Next, 100 μL iTRAQ label reagent was added into the sample and incubated at room temperature for 2 h. Finally, 200 μL high-performance liquid chromatography (HPLC)-grade water was added to the sample and incubated for 30 min in order to terminate the reaction. The labeling peptides solutions were lyophilized and stored at -80°C. Reversed-phase liquid chromatography (RPLC) separation was performed on an Agilent Zorbax Extend-C18 column (Agilent). Mobile phases A (ACN-H_2O, 2:98) and B (ACN-H_2O, 90:10) were used for the RP gradient. (Lin *et al.*, 2017).

1.6 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

With 100% solvent A (water/acetonitrile/formic acid=98/2/0.1), the trapping and desalting procedure were carried out at a flow rate of 4 μL/min for 5 min. Next, an elution gradient of 5%–85% solvent B (water/acetonitrile/formic acid=5/95/0.1) over 67 min (followed by 95% A over 3 min) was used on an analytical column. The ion spray voltage was set at 2.4 kV, the nebulizer gas was 12 PSI, the curtain gas was 40 PSI, and the interface heater temperature was 150°C. Survey scans were acquired in 250 ms, and up to 40 product-ion scans (50 ms) were collected if exceeding a threshold of 260 cps with a charge state of 2–4. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set at 14 s (Wisniewski *et al.*, 2009).

1.7 Bioinformatics analysis

The selected differentially accumulated proteins were analyzed on the OmicsBean database platform for Gene Ontology (GO) functional annotation and enrichment analysis. The software was used to identify homologous sequences from which the functional annotation could be transferred to the studied sequences. After the annotation steps, the differentially accumulated proteins were studied using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways to analyze the metabolic pathways of the proteins in the cells. Finally, protein interactions between DAPs and KEGG pathways were analyzed and revealed using the OmicsBean database platform.

1.8 Statistical analysis

The data were analyzed using Microsoft Excel 2013 and SPSS 18.0 software (SPSS Inc., Chicago, USA). The results were expressed as the mean ± standard error. Proteins were identified and analyzed using ProteinPilot 5.0 (SCIEX company, USA) software. Only proteins identified with a false discovery rate (FDR) ≤1% and unique peptides ≥1 were considered for the protein lists. Proteins with *P*-values ≤0.05 were considered as differentially accumulated.

2 Results

2.1 Embryonic axis germination test

The initial moisture content of the fresh C. *mollissima* embryonic axis was 63%. During desiccation, the MC of the embryonic axis decreased from 63% to 30%, whereas the germination rate and normal seedling rate did not decrease. When the embryo MC decreased to 20%, the germination percentage decreased to 68.9%, and the normal seedling percentage was 51.4%. When the embryo MC declined to 10%, the percentages of germinated were only 15.7%. Dehydration affects germination percentage of *C. mollissima*. The data indicated that C. *mollissima* has desiccation-sensitive seeds and is a recalcitrant seed.

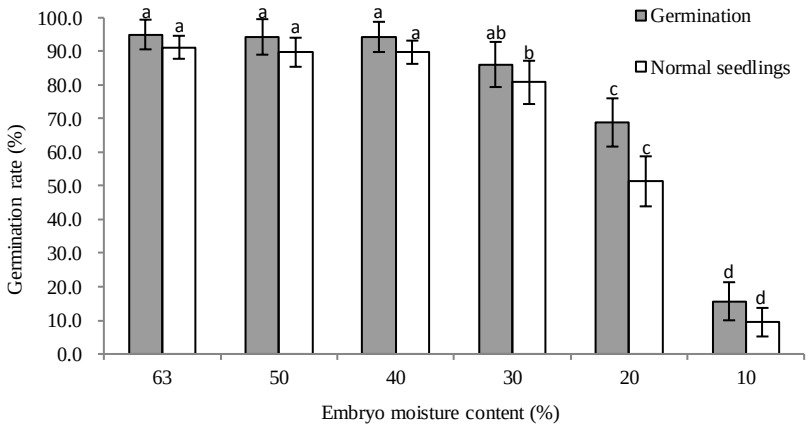
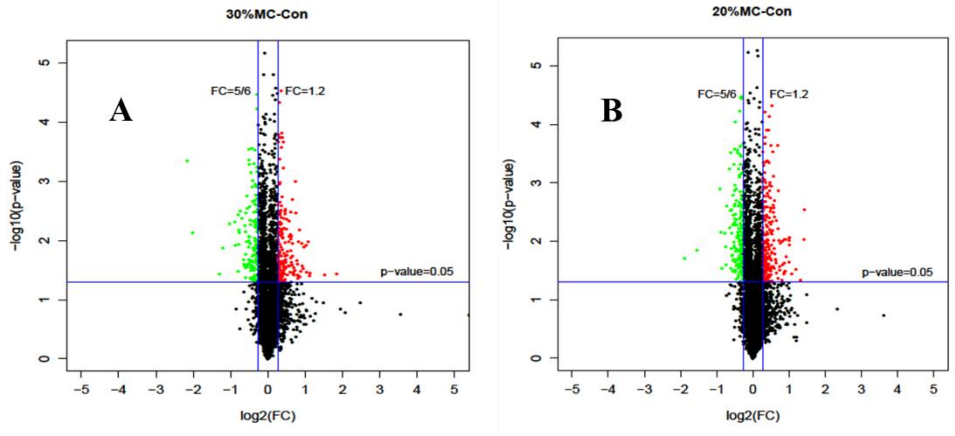


Fig.1 Germination and normal seedling rate of the embryonic axis of *C. mollissima* at different moisture contents.

Data are the means of three replicates ± SD, and bars labeled with the same lower-case letter indicate no significant difference at the 0.05 probability level.

2.2 Differentially accumulated proteins(DAPs) identification

Based on an Unused Cutoff >1.3 and peptides ≥1, 5201 trusted proteins were identified from the iTRAQ analysis using the embryonic axis of different moisture content. The trusted proteins were screened at P-values < 0.05 and FC values >1.2 or < 5/6, differentially accumulated proteins (DAPs) of each comparable group were found. The 20% MC group had the most DAPs (394), more than 10% MC group DAPs (370) and The 30% MC group (307). The 20% MC group also had the most down-regulated DAPs (192), while the 10% MC group had the most up-regulated DAPs (225).



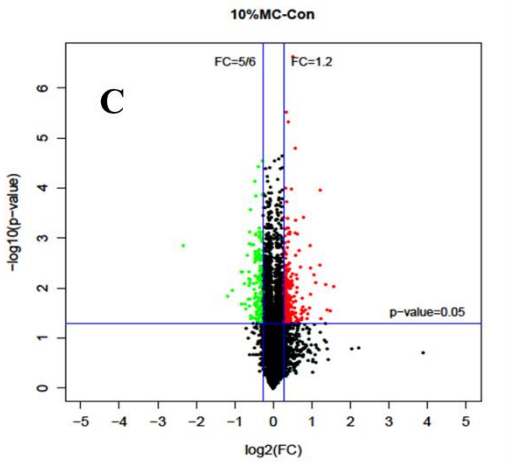


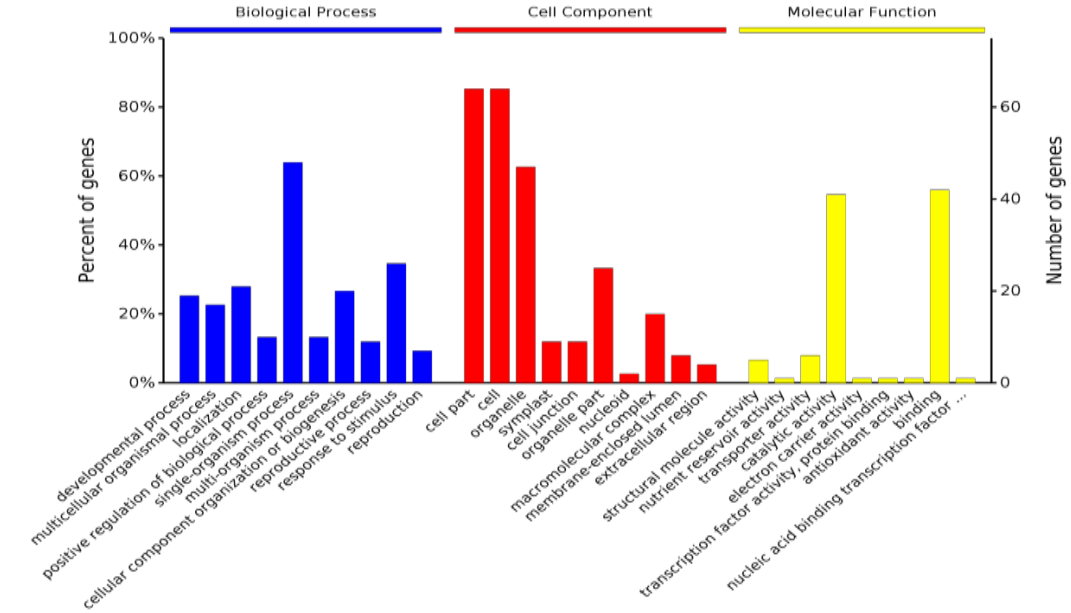
Fig. 2 Up-regulated and down-regulated differentially accumulated proteins between the embryonic axes of different moisture contents (MCs).

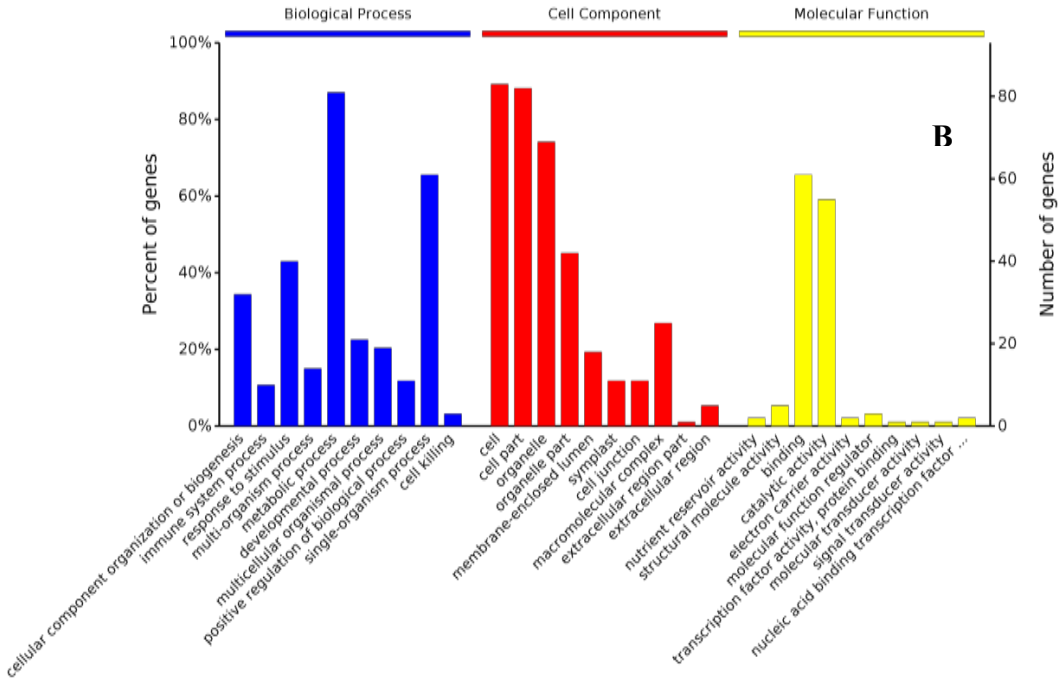
(A) Up-regulated and down-regulated DAPs in the 30% MC group. (B) Up-regulated and down-regulated DAPs in the 20% MC group. (C) Up-regulated and down-regulated DAPs in the 10% MC group. Note: green dots represent down-regulated DAPs, red dots represent up-regulated DAPs, and black dots represent non-significant DAPs.

2.3 Gene Ontology (GO) Annotation

Using the OmicsBean platform, the GO classifications of the DAPs were annotated. These GO terms were classified into Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The GO categories of the different groups are presented in figure 3 (A, B, C).

Based on a *P*-value < 0.01, the significantly different GO categories in the 30% MC group were (1) BP: developmental process, multicellular organismal process, localization, and positive regulation of the biological process. (2) CC: cell part, cell, organelle, symplast, cell junction, organelle part, and nucleoid. (3) MF: None (figure 3A). In the 20% MC group, the significantly different GO categories included (1) BP: cellular component organization or biogenesis, immune system process, response to stimulus, multi-organism process, metabolic process, and developmental process. (2) CC: cell, cell part, organelle, organelle part, membrane-enclosed lumen, symplast, cell junction, and macromolecular complex. (3) MF: None (figure 3B). In the 10% MC group, the significantly different GO categories were (1) BP: single-organism process, cellular component organization or biogenesis, developmental process, and growth. (2) CC: cell, cell part, organelle, organelle part, symplast, cell junction, membrane-enclosed lumen, and macromolecular complex. (3) MF: structural molecule activity (figure 3C).


Biological Process
Cell Component
Molecular Function
Percent of genes
Number of genes
100%
80%
60%
40%
20%
0%
60
40
20
0
developmental process
multicellular organismal process
localization
positive regulation of biological process
single-organism process
multi-organism process
cellular component organization or biogenesis
reproductive process
response to stimulus
reproduction
cell part
cell
organelle
symplast
cell junction
organelle part
nucleoid
macromolecular complex
membrane-enclosed lumen
extracellular region
structural molecule activity
nutrient reservoir activity
transporter activity
catalytic activity
electron carrier activity
transcription factor activity, protein binding
antioxidant activity
binding
nucleic acid binding transcription factor ...


Biological Process
Cell Component
Molecular Function
B
Percent of genes
Number of genes
100%
80%
60%
40%
20%
0%
80
60
40
20
0
cellular component organization or biogenesis
immune system process
response to stimulus
multi-organism process
metabolic process
developmental process
multicellular organismal process
positive regulation of biological process
single-organism process
cell killing
cell
cell part
organelle
organelle part
membrane-enclosed lumen
symplast
cell junction
macromolecular complex
extracellular region part
extracellular region
nutrient reservoir activity
structural molecule activity
binding
catalytic activity
electron carrier activity
molecular function regulator
transcription factor activity, protein binding
molecular transducer activity
signal transducer activity
nucleic acid binding transcription factor ...

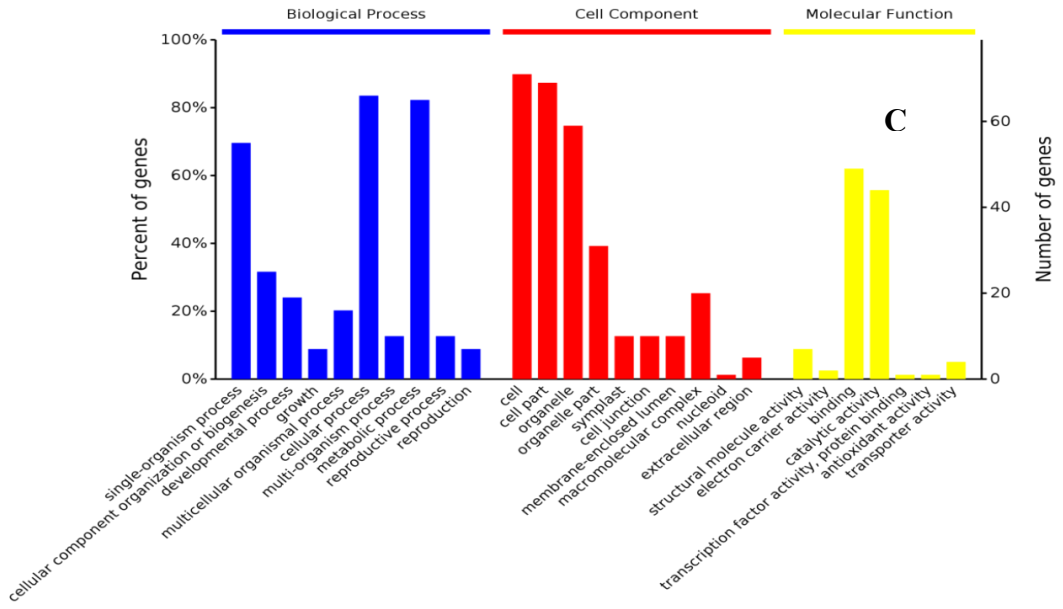


Fig. 3 Gene Ontology annotation of the DAPs.

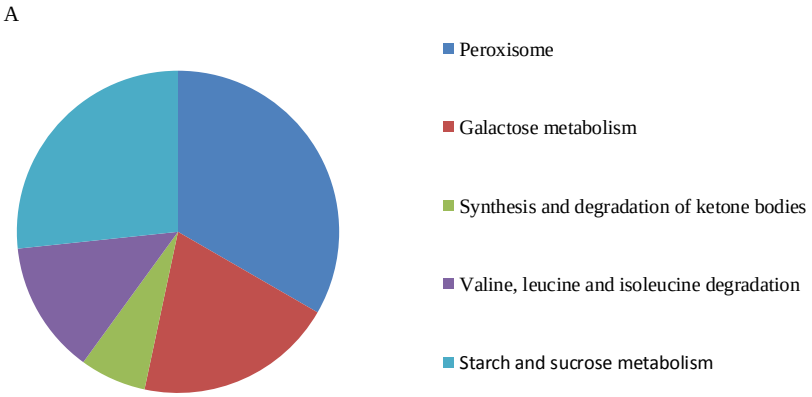
(A) GO categories of the 30% MC group. (B) GO categories of the 20% MC group. (C) GO categories of the 10% MC group.

2.4 KEGG pathway analysis

Based on the KEGG database, the biological functions of these proteins were classified into different pathways. In the 30% MC group, 40 pathways were identified in the KEGG database. Based on P<0.05, five significantly different pathways are indicated in figure 4A, “Peroxisome” and “Galactose metabolism” were the most represented pathways (P<0.01). The main pathways in the KEGG enrichment analysis were “Peroxisome,” “Starch and sucrose metabolism,” and “Galactose metabolism”.

In group 20% MC, 46 pathways were identified in the KEGG database. Based on P<0.05, four significantly different pathways are indicated in figure 4B. “Protein processing in endoplasmic reticulum” was the most represented pathway (P<0.01). The main pathways in the KEGG enrichment analysis included “Protein processing in endoplasmic reticulum” and “Fatty acid metabolism”.

In the 10% MC group, 46 pathways were identified in the KEGG database. Based on P<0.05, nine significantly different pathways are indicated in figure 4C. The main pathways in the KEGG enrichment analysis included “Amino sugar and nucleotide sugar metabolism”, “Fatty acid metabolism,” and “Peroxisome”.



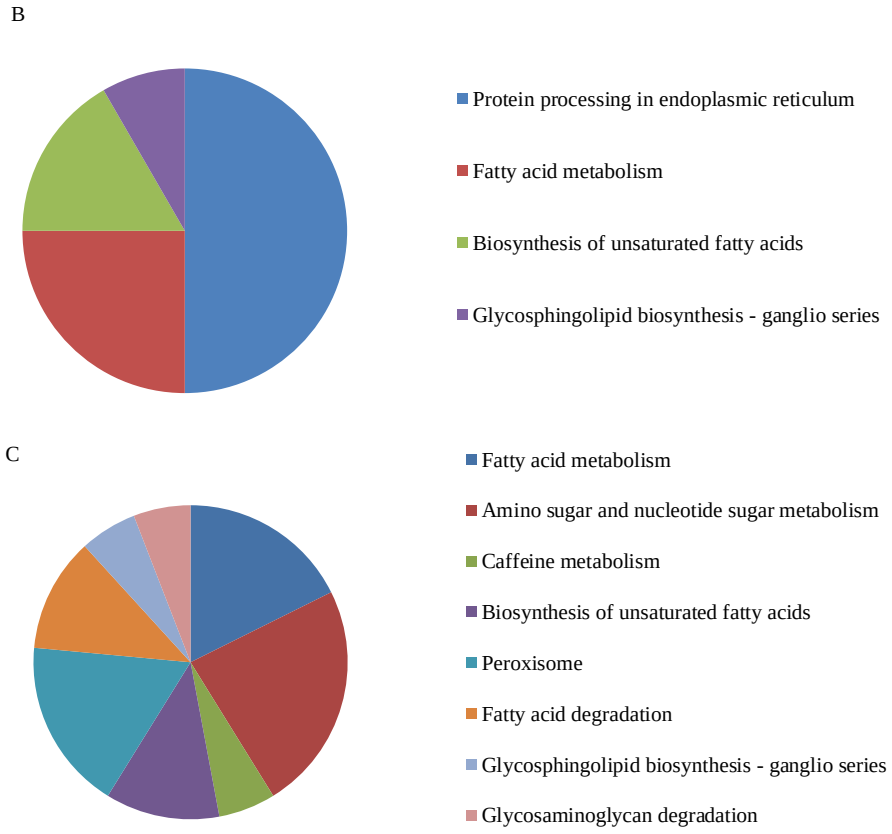


Fig. 4 Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the different groups.
(A) KEGG enrichment of the 30% MC group. (B) KEGG enrichment of the 20% MC group. (C) KEGG enrichment of the 10% MC group.

2.5 DAP-related gene analysis

In order to investigate the significant DAPs and their genes, KEGG-DAP gene interaction networks were built (figure 5). In the 30% MC group, significantly up-regulated DAP genes included ICDH, AGT1, and LACS1. These genes are associated with “Peroxisome” pathways (figure 5A). Significantly down-regulated DAP genes included At5g08180, MED37C, and At3g54440. At5g08180 regulates RNA and snoRNA binding, while MED37C is an RNA polymerase II-DAPendent gene. The At3g54440 gene is associated with “Galactose metabolism” pathways (figure 5A).

In the 20% MC group, significantly up-regulated DAP genes included RH16, MHF15.12, ILA, and ENO3. The RH16 gene functions in RNA helicase activity and rRNA processing. The MHF15.12 gene is associated with ribonucleoprotein and ribosomal protein, while the ILA gene functions in protein kinase binding and protein kinase regulator activity. The function of the ENO3 gene includes phosphopyruvate hydratase activity and glycolytic process. Significantly down-regulated genes included MED37E, PDIL1-1, At3g45770, RPL1, EIF(ISO)4G1, and At1g18270. MED37E, PDIL1-1, and RPL1 are associated with the “Protein processing in endoplasmic reticulum” pathway. At3g45770 is associated with “Fatty acid metabolism”. EIF(ISO)4G1 is involved in mRNA binding and translation initiation factor activity. At1g18270 functions in NAD and NADP binding and oxidoreductase activity (figure 5B) .

In the 10% MC group, significantly up-regulated DAP genes included MHF15.12, LACS1, and rpoB. MHF15.12 was also significantly up-regulated in the 20% MC group. The LACS1 gene was also significantly up-regulated in the 30% MC group. The rpoB gene functions in DNA-dependent RNA polymerase and catalyzes the transcription of DNA into RNA and is associated with “Pyrimidine metabolism” pathways. Significantly down-regulated DAP genes included At4g02470, At5g08180, RPL1, and MED37C. The At5g08180 and MED37C genes were also significantly down-regulated in the 30% MC group, while the RPL1 gene was also significantly down-regulated in the 20% MC group. At4g02470 functions in ATP binding (figure 5C).

Fig.5 Key differentially accumulated protein gene and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway interaction networks of (A) 30% MC group, (B) 20% MC group, (C) 10% MC group.

Dots represent genes, red represents up-regulation, and green represents down-regulation. The rounded rectangles represent KEGG pathways, with blue representing higher significance, and yellow representing lower significance. The straight line represents the interaction, the solid lines represent gene–gene association, and the dotted lines represent KEGG–gene associations.

3 Discussion

Recalcitrant seeds are sensitive to drying and remain sensitive both during development and after they are harvested (Berjak and Pammenter, 2007). Although the degree of recalcitrance is hard to quantify, the relationship between moisture content and the germination of seeds or embryos is an important indicator (Walters *et al.*, 2013; Xia *et al.*, 2014). In this study, the harvest moisture content of the embryonic axis of *C. mollissima* was 63%. As this is a very high mature seed moisture content, we inferred that the *C. mollissima* seeds did not undergo dehydration during the seed maturation process. Germination rapidly decreased when the embryonic axis moisture content dropped to 20%, and at a 10% embryonic axis moisture content, there was only 15.7% germinated and 9.5% normal seedlings. The embryonic axis with 10% MC was able to germinate, possibly because the moisture content

of some of the embryonic axes may be higher than 10% or some embryonic axes are able to tolerate 10% moisture content. Feng *et al.* (2017) found that the recalcitrant seed of *Gingko biloba* was able to germinate at 15% moisture content. Xia *et al*. (2014) found that embryonic tissues following cryoexposure had greater tolerance to desiccation of the recalcitrant embryonic axes from temperate species. This phenomenon needs further investigation. Based on the changes in germination in the *C. mollissima* embryonic axis during desiccation, we suggest that *C. mollissima* seeds are recalcitrant and sensitive to desiccation.

The mechanisms by which recalcitrant seeds lose viability are various and complex. Preserving recalcitrant seeds poses a great challenge to seed banks, and embryonic axis cryopreservation is almost the only long-term and larger-scale *ex situ* conservation method for recalcitrant seeds (Daws *et al*., 2006; Li and Pritchard, 2009). Based on the iTRAQ analysis, we identified 912 DAP species, including 563 unique DAP species, from the different moisture contents embryo. The 20% MC group had the most DAP species (394 DAP) and unique DAP species (192 DAP), suggesting that 20% embryonic axis MC is associated with the most dramatic proteomic changes within the cell. If combined DAP significant changes with embryonic axis germination decrease, we found that dehydration to 20% MC could seriously affect embryonic axis germination and the cellular proteome of *C. mollissima*. Whether these significant changes in DAPs affect the germination of the embryonic axis requires further investigation, but these should nevertheless be correlated.

For desiccation-tolerant seeds, the acquisition of desiccation tolerance in germinated radicles and developing seeds is associated with the up-regulation of genes belonging to stress defense; for example, Late Embryo Abundant (LEA) proteins, antioxidants, and other metabolites (Boudet *et al*., 2006; Leprince *et al*., 2010). This study also found that the "Peroxisome," "Galactose metabolism," and "Starch and sucrose metabolism" were the main different pathways when the embryonic axis was dried to 30% MC, with the significantly up-regulated DAP genes being associated with antioxidant activity (ICDH) and metabolites (AGT1 and LACS1) (figure 5). However, we did not observe any significant changes in LEA proteins at different moisture contents. The lack of LEA proteins might be a reason for the desiccation sensitivity of the *C. mollissima* embryonic axis.

For some desiccation-sensitive seeds, such as *Avicennia marina* (Greggains *et al*., 2001), *Aesculus hippocastanum* (Obroucheva *et al*., 2016), *G. biloba* (Feng *et al*., 2017), *Camellia sinensis* (Chen *et al*., 2018), and *Quercus robur* (Ntuli *et al*., 2011), antioxidant system and lipid peroxidation are considered as important reasons for the viability loss of recalcitrant seeds during desiccation, and the accumulation of membrane lipid peroxides leads to decreased viability of recalcitrant seeds (Feng *et al*., 2017; Chen *et al*., 2018). Chen *et al*. (2018) further suggest that the change in phospholipase Dα1-mediated phosphatidic acid of the cytomembrane is the key determinant of desiccation-induced viability loss for seeds. Our study confirms that antioxidant systems and lipids have a significant role in desiccation-sensitive seeds during desiccation at the proteome level. Furthermore, the antioxidant system and lipid-related DAPs were not enriched in embryos at the same moisture content. The antioxidant system-related DAPs were significantly enriched at 30% MC (Figs. 5A), whereas the lipid-related DAPs were significantly enriched at 20% MC (Figs. 5B). The GO annotation also displayed significant changes in "membrane-enclosed lumen" at 20% MC and 10% MC, but not at 30% MC (figure 4).

Another interesting observation was that when we dehydrated the embryonic axes to 20% MC, the most significant and main pathway was "Protein processing in endoplasmic reticulum"(figure 5B), and further analysis revealed that ribosome and RNA-related DAPs exhibited the most significant change (figure 5A). Therefore, we consider that dehydration to 20% MC injured the ribosome and RNA in the endoplasmic reticulum and affected protein synthesis. Molecular Function (GO) did not change until the embryonic axis was dehydrated to 10% MC, at which point "structural molecule activity" the most significant (P-value<0.01) Molecular Function categories (figures 4C and 4D). Therefore, we suggest that dehydration to 10% MC destroyed the molecular functioning of the embryonic axis of *C. mollissima.*

4 Conclusions

The seeds of *C. mollissima* are recalcitrant, and an MC of 20% or less had a serious impact on embryonic axis germination during rapid desiccation. When we dehydrated the embryonic axis to 30% MC, the stress defense systems of antioxidants and metabolites successfully avoided dehydration injury and ensured the germination of the

embryonic axis. At this stage, the main metabolic process was the degradation of polysaccharides ("Galactose metabolism" and "Starch and sucrose metabolism"). However, perhaps the lack of LEA proteins or factors relating to membrane lipids can account for the limited desiccation protection at 20% MC. At this moisture content, desiccation stress had injured the ribosome and RNA in the endoplasmic reticulum, further affecting protein synthesis. The obvious result of this injury was that more DAPs were identified, and the embryonic axis germination rate reduced rapidly. When we dehydrated the embryonic axis to 10% MC, desiccation damaged the "structural molecule activity" and "binding" of the embryonic axis. Comparing the 10% MC embryonic axis with the 30% MC embryonic axis, the genes associated with "Metabolic pathways" were abundant and significantly down-regulated. The complex interactions of 912 DAPs, particularly 563 unique DAPs, need further investigation and experimental validation. The DAPs can be used to screen natural populations of *C. mollissima*, with the aim of discovering dehydration-tolerant germplasm resources.

References

Berjak P, Pammenter N W. 2007. From *Avicennia to Zizania*: seed recalcitrance in perspective. Annals of Botany. 101, 213–228.

Boudet J, Buitink J, Hoekstra F A, *et al*. 2006. Comparative analysis of the heat stable proteome of radicles of medicago truncatula seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. Plant Physiology, 140, 1418-1436.

Chen H Y, Yu X M, Zhang X D, *et al*. 2018. Phospholipase Dα1-mediated phosphatidic acid change is a key determinant of desiccation-induced viability lossin seeds. *Plant Cell and Environment*, 2018, 50–63.

Chen Q, Yang L, Ahmad P, *et al*. 2011. Proteomic profiling and redox status alteration of recalcitrant tea (*Camellia sinensis*) seed in response to desiccation. Planta. 233, 583–592.

Corredoira E, San-Jose M C, Ballester A, *et al*. 2004. Cryopreseravtion of zygotic embryo axes and somatic embryos of European chestnut. CryoLetters, 25, 33-42.

Daws M I, Garwood N C, Pritchard H W. 2006. Prediction of desiccation sensitivity in seeds of woody species: A probabilistic model based on two seed traits and 104 species. Annals of Botany, 97, 667–674.

Feng J, Shen Y B, Shi F H, *et al*. 2017. Changes in seed germination ability, lipid peroxidation and antioxidant enzyme activities of *Ginkgo biloba* seed during desiccation. Forests, 2017, 286.

Greggains V, Finch-Savage W E, Atherton N M, *et al*. (2001). Viability loss and free radical processes during desiccation of recalcitrant *Avicennia marina* seeds. Seed Science Research, 11, 235–242.

Ji M C, Fu B, Zhang Y J. 2021. Recent Progress of Analytical Methods of ProteomicsBased on Mass Spectrometry. Journal of Chinese Mass Spectrometry Society, 42(05): 862-877.

Leprince O, Buitink J. 2010. Desiccation tolerance: From genomics to the field. *Plant Science*, 179, 554-564.

Li D Z, Pritchard H W. 2009. The science and economics of ex situ plant conservation. *Trends in Plant Science,* 14, 614-621.

Lin M, Fang J, Qi X, *et al*. 2017. Itraq-based quantitative proteomic analysis reveals alterations in the metabolism of actinidia arguta. Scientific Reports, 7, 5670.

Molassiotis A, Tanou G, Filippou P, *et al*. 2013. Proteomics in the fruit tree science arena: new insights into fruit defense, development, and ripening. Proteomics, 13, 1871-1884.

Ntuli T M, Finch-Savage W E, Berjak P, *et al*. 2011. Increased drying rate lowers the critical water content for survival in embryonic axes of English oak (*Quercus robur* L.) seeds. Journal of Integrative Plant Biology, 2011, 270–280.

Obroucheva N, Sinkevich I, Lityagina S. 2016. Physiological aspects of seed recalcitrance: A case study on the tree *Aesculus hippocastanum*. Tree Physiology, 36, 1127–1150.

Soeda Y, Konings M C J M, Vorst O, *et al*. 2005. Gene expression programs during Brassica oleracea seed maturation, osmopriming,and germination are indicators of progression of the germination process and the stress tolerance level. Plant Physiology, 137, 354–368.

Su C, Jiang Y, Yang Y, *et al*. 2019. Responses of duckweed (*Lemna minor* L.) to aluminum stress: physiological and proteomics analyses. Ecotoxicology and Environmental Safety, 170, 127-140.

Walters C, Berjak P, Pammenter N, *et al*. 2013. Preservation of recalcitrant seeds. Science, 339, 915-916.

Wisniewski J R, Zougman A, Nagaraj N, *et al*. 2009. Universal sample preparation method for proteome analysis. Nature Methods, 6, 359–362.

Xia K, Daws M I, Stuppy W, *et al*. 2012. Rates of water loss and uptake in recalcitrant fruits of *Quercus* species are determined

by pericarp anatomy. Plos One, 7, e47368.

Xia K, Hill L M, Li D Z, *et al*. 2014. Factors affecting stress tolerance in recalcitrant embryonic axes from seeds of four *Quercus* (Fagaceae) species native to the USA or China. Annals of Botany, 114, 1747–1759.

Zong M, Cai Y P. 2010. Desiccation Sensitivity of *C.mollissima* Seeds at Different Developmental Stages. Journal of Northeast Forestry University, 38, 21-23.

Zou F, Guo S J, Xiong H, *et al*. 2015. Effects of Different Pollination Treatments on Nutrition Changes of the Ovary in Chinese Chestnut (*C.mollissima* Blume). Advance Journal of Food Science and Technology. 8, 157-162.