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ARTICLE

Analysis of Seed Phenotypic and Metabolic Characteristics of Diploid and Tetraploid Tartary Buckwheat

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ABSTRACT

Polyploid plants grow well, are stress tolerant, and are rich in nutrients and bioactive compounds. Thus, they are useful for improving crop quality and yield. In this study, we compared the seed characteristics and metabolite profiles of diploid and tetraploid tartary buckwheat, which was developed via an artificially induced chromosome doubling event. The length, width, area, and thousand-grain weight were greater for the tetraploid seeds than for the diploid seeds. However, the germination rate decreased for the tetraploid seeds. Additionally, there was a gap between the shell and kernel of the tetraploid seeds. Moreover, the water absorption rate was higher for the tetraploid than for the diploid seeds. Chromosome doubling increased the seed total flavonoid content and deepened the seed color. A principal component analysis of the ultrahigh-pressure liquid chromatography-high resolution mass spectrometry data revealed the clear separation between the diploid and tetraploid samples. An orthogonal partial least squares-discriminant analysis and other multivariate statistical analyses identified 83 differentially abundant compounds, with most of the flavonoid metabolites more abundant in the tetraploid than in the diploid seeds. Research on tartary buckwheat polyploidy may result in enhanced germplasm resources and may clarify the mechanism underlying the biosynthesis of bioactive compounds.

KEYWORDS

Tartary buckwheat; diploid; tetraploid; seed

1 Introduction

Buckwheat is an annual dicotyledonous plant belonging to the genus Fagopyrum in the family Polygonaceae. Although it is globally distributed, buckwheat originated in China, with *Fagopyrum tataricum* (tartary buckwheat) and *Fagopyrum esculentum* (common buckwheat) representing the two main cultivated species [1]. Buckwheat is a highly adaptable species that is resistant to various stresses (e.g., barren conditions and drought) [2]. There is considerably more interest in tartary buckwheat than in common buckwheat because of its greater flavonoid content [3,4]. Tartary buckwheat also contains phenolic acids, polysaccharides, peptides, D-chiral inositol, and other biologically active ingredients [5–7]. Numerous studies proved that tartary buckwheat has anti-bacterial, anti-oxidative, and anti-aging effects and may be useful for preventing and treating hypertension, hyperglycemia, hyperlipidemia,

obesity, and coronary heart disease as well as for enhancing the human immune system [8–10]. Seed is the major exploitive part of tartaty buckwheat for humans, and has been widely used to develop products such as noodles, nutrient powder, moon cakes, cakes, tea, and wine [11,12]. Because of the recent increasing interest in tartary buckwheat and its products, breeders have focused on developing new varieties to satisfy the market demand [11,13,14]. Therefore, breeding programs have attempted to generate high-yielding tartary buckwheat varieties with large grains and high flavonoid contents. However, because of the frequent occurrence of extreme weather conditions, the increasing importance of diseases and insect pests, and the degeneration of varieties, the selection and breeding of stress-resistant tartary buckwheat varieties is also necessary.

Polyploidization is an important factor driving the evolution of higher plants as well as speciation. Plant polyploidy is a common phenomenon, with more than 70% of angiosperms having undergone at least one chromosome doubling event during evolution, which is considered to be an important mechanism for plant adaptive evolution and speciation [15–17]. Chromosome doubling events have occurred during the evolution of the food crops rice, corn, and wheat. These events result in changes to the genomic structure of polyploid plants, which lead to substantial increases in the genetic diversity and adaptability of species [18]. Another consequence of chromosome duplication is the increase in cell size, which leads to the development of new plant characteristics, including strong growth potential, enlarged organs, enhanced stress resistance, and increased material synthesis [19]. Previous studies have confirmed that increasing seed production is an effective way to increase crop yield and quality. Regarding the polyploidy in tartary buckwheat, researchers have used chemical treatments to double the number of chromosomes in diploid tartary buckwheat varieties to obtain autotetraploid tartary buckwheat. Our research since the 1990s has involved tartary buckwheat chromosome doubling and variety selection. Previous investigations demonstrated that the chromosome doubling of tetraploid tartary buckwheat leads to increased yield potential and adaptability [20]. Previous studies have proved that compared with diploid tartary buckwheat, tetraploid tartary buckwheat has higher growth potential, biological yield, and stress resistance [21–23]. There are currently only a few studies on polyploid tartary buckwheat, and most of them are focused on agronomic characteristics, morphology, physiology, and yield. There is relatively little research on tartary buckwheat grain quality and the mechanism underlying tartary buckwheat grain formation. Unlike wheat, rice, and other crops, there is increasing interest in the active ingredients (e.g., flavonoids) of tartary buckwheat. Whether ploidy changes affect the tartary buckwheat seed traits and components remains to be determined. In this study, previously developed polyploid tartary buckwheat materials were analyzed. Specifically, seed phenotypes and quality characteristics were comprehensively investigated to provide the theoretical basis and practical guidance for optimizing the use of polyploid tartary buckwheat germplasm resources.

2 Materials and Methods

2.1 Experimental Materials

Diploid tartary buckwheat seeds were obtained from the Key Laboratory of Coarse Cereal Processing, Chengdu University, China. Tetraploid tartary buckwheat was generated via a colchicine treatment and continuous selection for more than five generations. Samples were grown in an experimental field and subjected to normal field management practices.

2.2 Flow Cytometry

Leaves were collected and placed in a Petri dish. After adding 400 μL lysate, the leaves were chopped into smaller pieces using a blade for 30–60 s. Samples were filtered through a 30-μm membrane, after which 1,600 μL DAPI staining solution was added to the filtrate. The ploidy level was determined using the CyFlow® Ploidy Analyzer (Sysmex).

2.3 Seed Characteristics

Randomly selected diploid and tetraploid tartary buckwheat seeds were examined using a seed analyzer (Marvin GTA, Germany) to measure their length, width, and area. The thousand-grain weight was determined using an electronic balance. Whole seeds and seed cross-sections were examined using the SMZ745T stereomicroscope (Nikon). For the scanning electron microscopy analysis, seed cross-sections were prepared manually and then mounted on the sample table using double-sided adhesive tape. After the gold plating step, the seed cross-sections were examined and photographed using the Phenom instrument. Seed filling rate was also determined according to the previous report [22].

2.4 Color Assessment

The L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) parameters were measured using a colorimeter. The whiteness index (WI) was calculated according to the following equation: $WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$ [24].

2.5 Seed Water Absorption Rate

Before measuring their water absorption rate, seeds were weighed using an electronic scale. They were then placed in a beaker and immersed in water at 25°C. Seeds were collected every 2 h, blotted to remove moisture with filter paper, and weighed. The water absorption rate was calculated using the following equation: $[(M2 - M1)/M1] \times 100\%$, where M1 is the seed weight before the immersion in water and M2 is the seed weight after the immersion in water.

2.6 Seed Germination

Seeds were incubated in a darkened constant-temperature (25°C) phytotron. They were immersed in distilled water for 12 h and then placed in a Petri dish (covered with filter paper). Germination tests were conducted on day 0 (control group). The percentage of germinated seeds was used as the germination rate. The germination index was calculated using the following equation: $\sum(Gt/Tt)$, where Gt was the germination percentage on day t and Tt was the germination test day. Seeds were considered to have germinated if the coleoptile length was half the seed length.

2.7 Flavonoid Content and UHPLC-MS Analyses

The total flavonoid content was analyzed using aluminum chloride [25]. Briefly, 1 mL extract was mixed with 2 mL 0.1 M aluminum chloride and 3 mL 1 M potassium acetate. The absorbance of the resulting solution was measured at 415 nm using the Synergy HTX microplate spectrophotometer (BioTek). The total flavonoid content was calculated using rutin as the standard control.

Freeze-dried samples were dissolved in a 1,000 μL methanol: water (3:7) solution. The samples were incubated at 4°C for 12 h and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was passed through a 0.22-μm microfilter and collected in a fresh glass vial. The quality control sample comprised equal amounts of all samples.

The UPLC-Q Exactive orbitrap high-resolution mass spectrometry was used in this study. The H3 column was selected and the column temperature was 40°C. The sample injection volume was 3 μL and the auto-sampler temperature was set at 4°C. The mobile phase consisted of acetonitrile (A) and 0.1% formic acid water (B) with a gradient program: 0–3 min, 95% (B); 3–4 min, 95%–70% (B); 4–12 min, 70%–30% (B); 12–13 min, 30%–5% (B); 13–16 min, 5% (B). The MS conditions were as follows: scanning mode: full MS/dd-MS; mass spectrum parameters: spray voltage, 3,500 V; capillary temperature, 320°C; probe heater temperature, 350°C; sheath gas flow rate, 40 Arb; aux gas flow rate, 10 Arb; mass

range (m/z), 66.70–1,000; full ms resolution, 70,000; MS/MS resolution, 17,500. Mass spectrometry data were processed using the Compound Discoverer 3.0 software.

2.8 Statistical Analyses

Data were processed using Microsoft Excel 2010 and presented as mean ± standard deviation. The significance of any differences was determined using SIMCA and MetaboAnalyst 5.0. Principal component analysis (PCA) in multivariate statistic process was first used to compare the metabolic profiles with 95% confidence interval. Partial least squares discriminant analysis (PLS-DA) and Orthogonal partial least squares discriminant analysis (OPLS-DA) were carried out to discriminate between different groups. The significant differences of the metabolites identified by variable importance in projection (VIP) with >2 and P-value < 0.01.

3 Results

3.1 Tetraploid Identification and Seed Phenotype Analysis

Flow cytometry was used to analyze the DNA content in the leaf cells of diploid tartary buckwheat and colchicine-induced materials to confirm that a stable tetraploid tartary buckwheat material was obtained. The peak signal of the control sample (2 N) was 13,536.88, whereas the peak signal of the sample after chromosome doubling was 26,416.93, which reflected the successful generation of tetraploid tartary buckwheat (Fig. 1a).

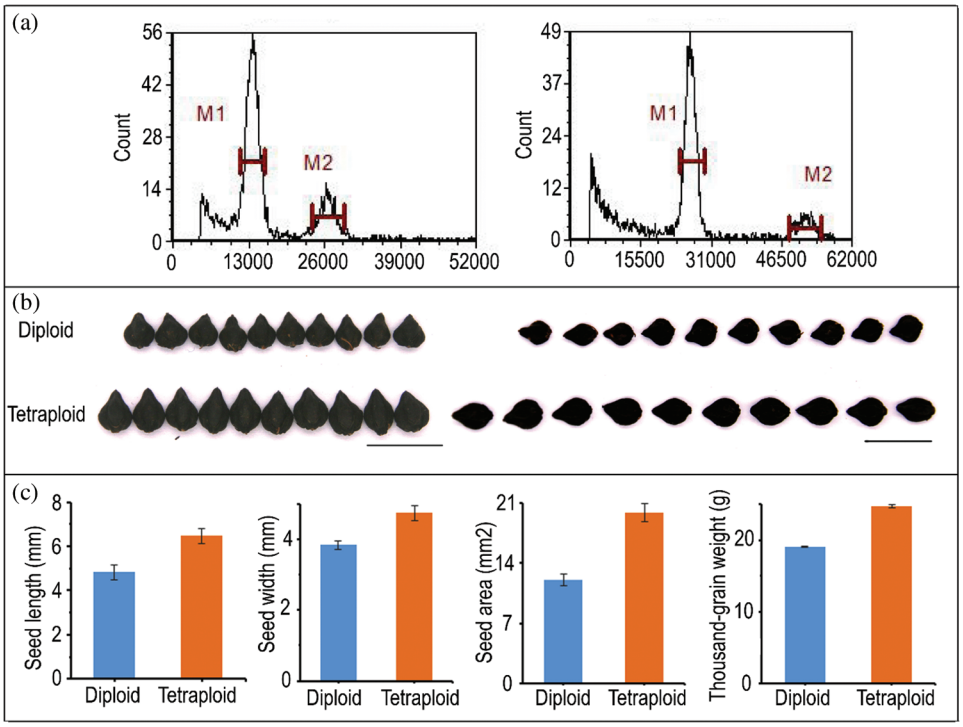

Figure 1: Relative fluorescence intensity of diploid and tetraploid (a); Seed morphology (b); Comparison of seed length, width, area and a thousand-grain weight (c); Data represent mean values ± SE. Bar: 1 cm

Seed size differed significantly between the tetraploid and diploid tartary buckwheat samples. More specifically, the length, width, and area were respectively 4.82 ± 0.34 mm, 3.84 ± 0.12 mm, and 12.04 ± 0.71 mm^2 for the diploid tartary buckwheat seeds, whereas they were respectively

6.46 ± 0.34 mm, 4.74 ± 0.20 mm, and 19.92 ± 1.07 mm^2 for the tetraploid tartary buckwheat seeds (Figs. 1b, 1c). The thousand-grain weight of tetraploid tartary buckwheat was 1.3-times greater than that of diploid tartary buckwheat. Therefore, doubling the number of chromosomes increased the tartary buckwheat seed size.

Compared with mature diploid tartary buckwheat seeds, more mature tetraploid tartary buckwheat seeds were detected at the water surface (Fig. 2a). Additionally, the seed filling rate of tetraploid tartary buckwheat was 50% lower than that of diploid tartary buckwheat. These findings indicated that doubling the number of chromosomes did not improve the tartary buckwheat seed filling rate. Scanning electron microscopy was used to examine the internal structures of the seed cross-sections (Fig. 2b). The examination revealed the effects of a ploidy level change on the internal microstructures of tartary buckwheat seeds. Compared with the diploid tartary buckwheat seeds, the tetraploid tartary buckwheat seeds had smaller starch grains, larger spaces between starch grains, and a more dispersed distribution of starch grains. Moreover, the tetraploid tartary buckwheat shell and kernel were clearly separated. The seed husk–kernel gap is an important characteristic for the selection of tartary buckwheat germplasm resources because it influences tartary buckwheat dehulling. Whether chromosome doubling affects the dehulling of tartary buckwheat seeds will need to be more thoroughly investigated.

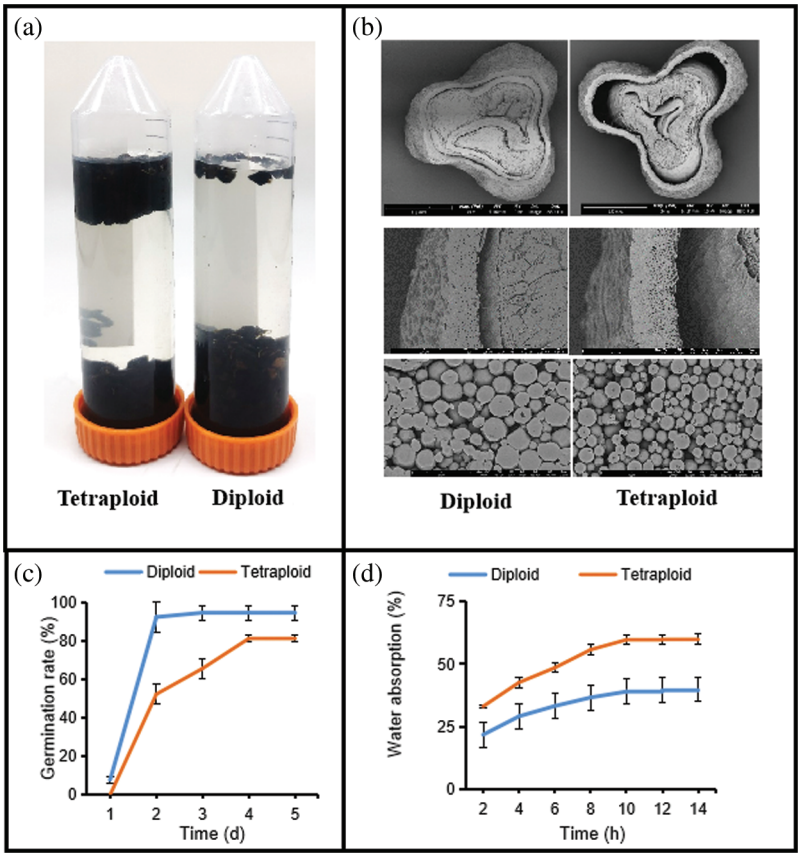

Figure 2: Representative images of seeds after water uptake (a); SEM observation results of the seed morphology (b); Germination rate (c); Water absorption (d)

3.2 Water Absorption and Germination Characteristics

The germination rate of the diploid seeds reached 92.22% on day 2, which was higher than the germination rate of the tetraploid seeds (52.22%) (Fig. 2c). The germination rate peaked on day 3 for the diploid tartary buckwheat seeds (94.45%), whereas it peaked on day 4 for the tetraploid seeds (81.11%). Thus, the tetraploid seeds tended to germinate more poorly than the diploid seeds.

The water absorption rate of the diploid and tetraploid tartary buckwheat seeds gradually increased for the first 10 h of the water immersion treatment and then stabilized (Fig. 2d). At 10 h, the water absorption rate of tetraploid tartary buckwheat seeds was 1.5-fold higher than that of the diploid seeds. The peak water absorption rate was significantly higher for the tetraploid tartary buckwheat seeds than for the diploid tartary buckwheat seeds. The morphological and structural changes that occurred in the tetraploid seeds may have increased the internal space, which increased the water absorption capacity.

3.3 Coloration and Total Flavonoid Content

There were obvious differences in the coloration of the diploid and tetraploid tartary buckwheat samples (i.e., visible to the naked eye). The seed colors were analyzed using a colorimeter. In this color model, L* represents white and black, whereas a* represents red and green and b* represents yellow and blue. The L*, b*, and WI values of the tetraploid tartary buckwheat seeds decreased, which reflected an increase in the darkness and a decrease in the brightness of the seed color (Figs. 3a, 3b). The flavonoid content of the tetraploid tartary buckwheat seeds was 1.2-times higher than that of the diploid tartary buckwheat seeds (Figs. 3c, 3d), which was an extremely significant difference. Hence, chromosome doubling increased the total flavonoid content of tartary buckwheat seeds. The new germplasm developed in this study may be useful for breeding new varieties with enhanced qualities (e.g., increased accumulation of bioactive compounds).

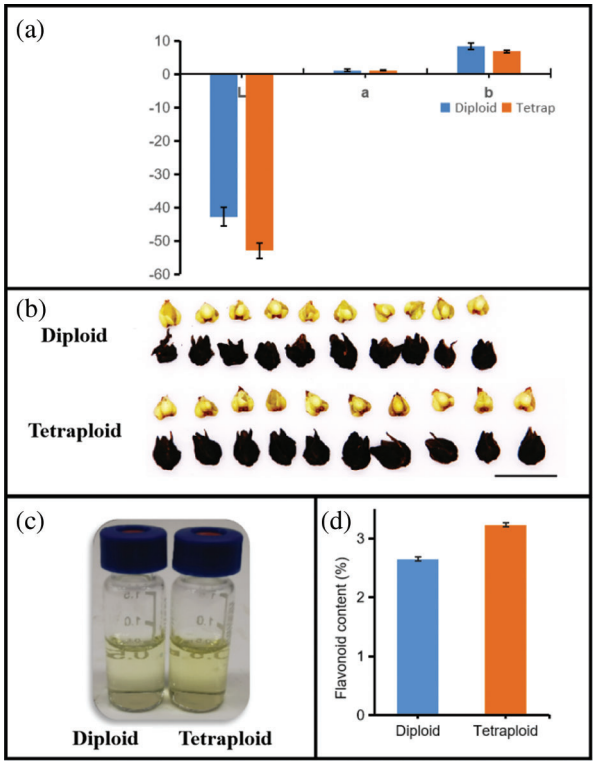

Figure 3: Value of L*, a* and b* (a); The representative images of samples used in this study (b); Extracting solution of buckwheat seed (c); Total flavonoid contents (d)

3.4 Metabolite Analysis

The metabolites in the diploid and tetraploid tartary buckwheat samples were detected and screened by UHPLC-MS. Main peaks were identified by matching with the spectral characteristics from previous studies and online databases. Metabolites in samples were identified according to Compound Discoverer software. The outline of metabolite peaks was very similar among different groups, but some distinct differences could be observed between these common peaks. Generally, metabolites were participants in primary metabolism and secondary metabolism, which may reflect the nutritional value of seeds.

A principal component analysis (PCA) can reveal the separation between sample groups and present the overall differences between samples. In the PCA score plot (Fig. 4a), all sample points were located in different areas of the elliptical confidence interval (95%). Diploid and tetraploid samples were completely separated, but the sample replicates were clustered together, indicative of the reproducibility and reliability of the experiment. The first and second principal components respectively explained 96.2% and 2.8% of the variance (i.e., 99% combined). Different classes of metabolites were distributed in different quadrants, with flavonoids and polyphenols contributing greatly to the metabolite content of the tetraploid tartary buckwheat. Accordingly, there were significant differences in the metabolites between the diploid and tetraploid samples.

3.5 Analysis of Differentially Abundant Metabolites

PLS-DA and OPLS-DA were performed. The visualization of the PLS-DA and OPLS-DA data indicated that all samples were within the 95% confidence interval (Figs. 4b, 4c). Additionally, the diploid and tetraploid samples were differentiated and all samples of each seed type were clustered together. In the displacement test chart, all of the blue Q2 points were lower than the original far right blue Q2 points for the PLS-DA and OPLS-DA (Figs. 4d, 4e). For both models, the Q2 values for the different groups exceeded 0.9, indicating that the models were appropriate and the subsequent analysis of the differentially abundant metabolites could proceed.

The untargeted metabolomics analysis detected 83 differentially abundant metabolites between the diploid and tetraploid samples, of which 59 and 24 were respectively more and less abundant in the tetraploid sample (Fig. 5a). By clustering the differentially abundant metabolites, we determined that the contents of more than half of the metabolites were higher in the tetraploid than in the diploid. These metabolites were mainly related to secondary metabolism (e.g., flavonoids, terpenoids, alkaloids, polyphenols, amino acids, and their derivatives) (Figs. 5b, 5c). Different classes of metabolites were distributed in different groups, with flavonoids and polyphenols contributing greatly to the metabolite content of the tetraploid tartary buckwheat. Accordingly, there were significant differences in the metabolites between the diploid and tetraploid samples.

According to the analysis of MetaboAnalyst software, the corresponding enrichment metabolic pathways of most differential metabolites were obtained in the pathway map, including pentose and glucuronate interconversions, aminoacyl-tRNA biosynthesis, glycerolipid metabolism, alanine, aspartate and glutamate metabolisms, biosynthesis of unsaturated fatty acids, glycolysis/gluconeogenesis, fatty acid biosynthesis, and purine metabolism. These metabolic pathways are mainly associated with biosynthesis of fatty acid and amino acid metabolism. Furthermore, there were higher levels of metabolics related to flavonoid biosynthesis, phenylpropanoid biosynthesis and stilbenoid, diarylheptanoid and gingerol biosynthesis.

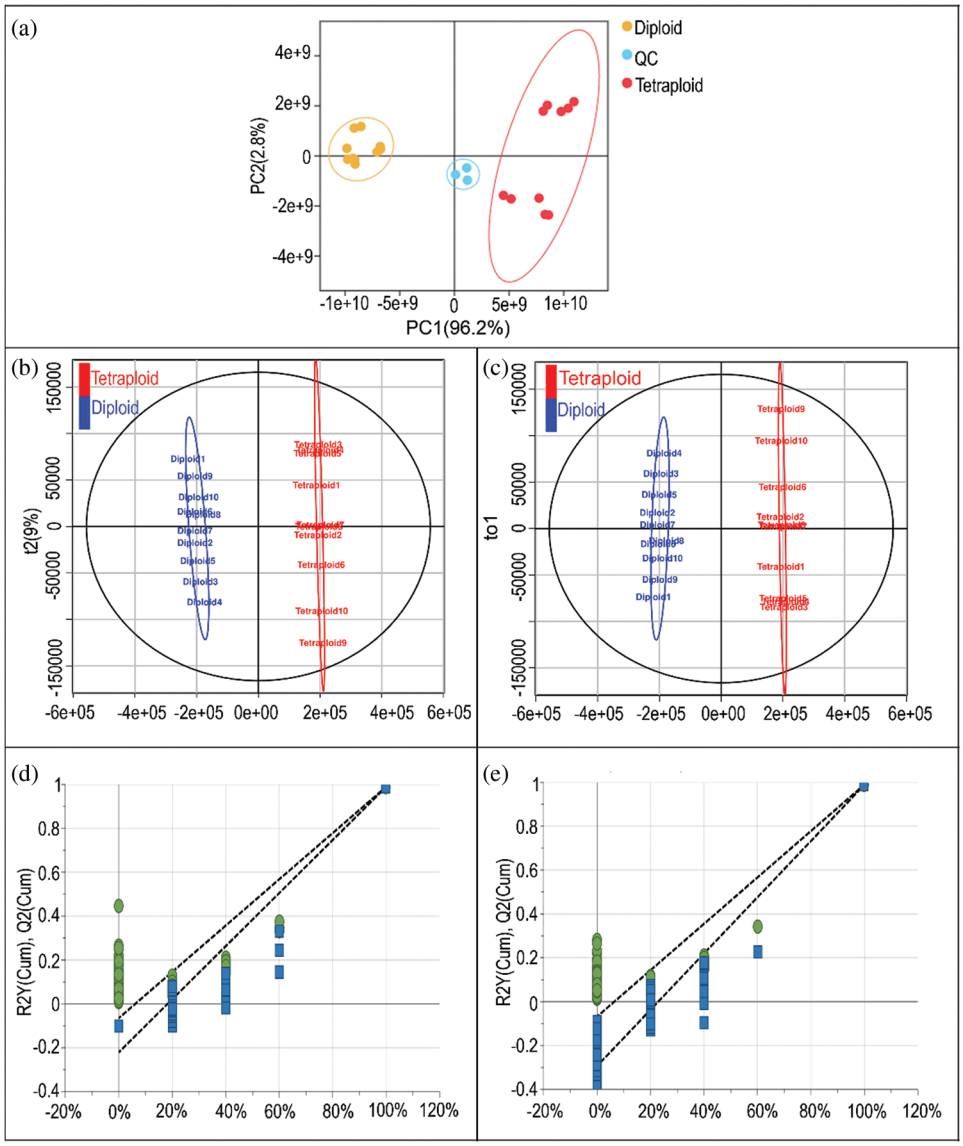

Figure 4: Different metabolites analysis on the basis of principal component analysis (PCA) score plot (a); Partial least squares-discriminant analysis (PLS-DA) (b); Orthogonal signal correction and partial least squares-discriminant analysis (OPLS-DA) (c); Permutation plot of PLS-DA (d) and OPLS-DA (e)

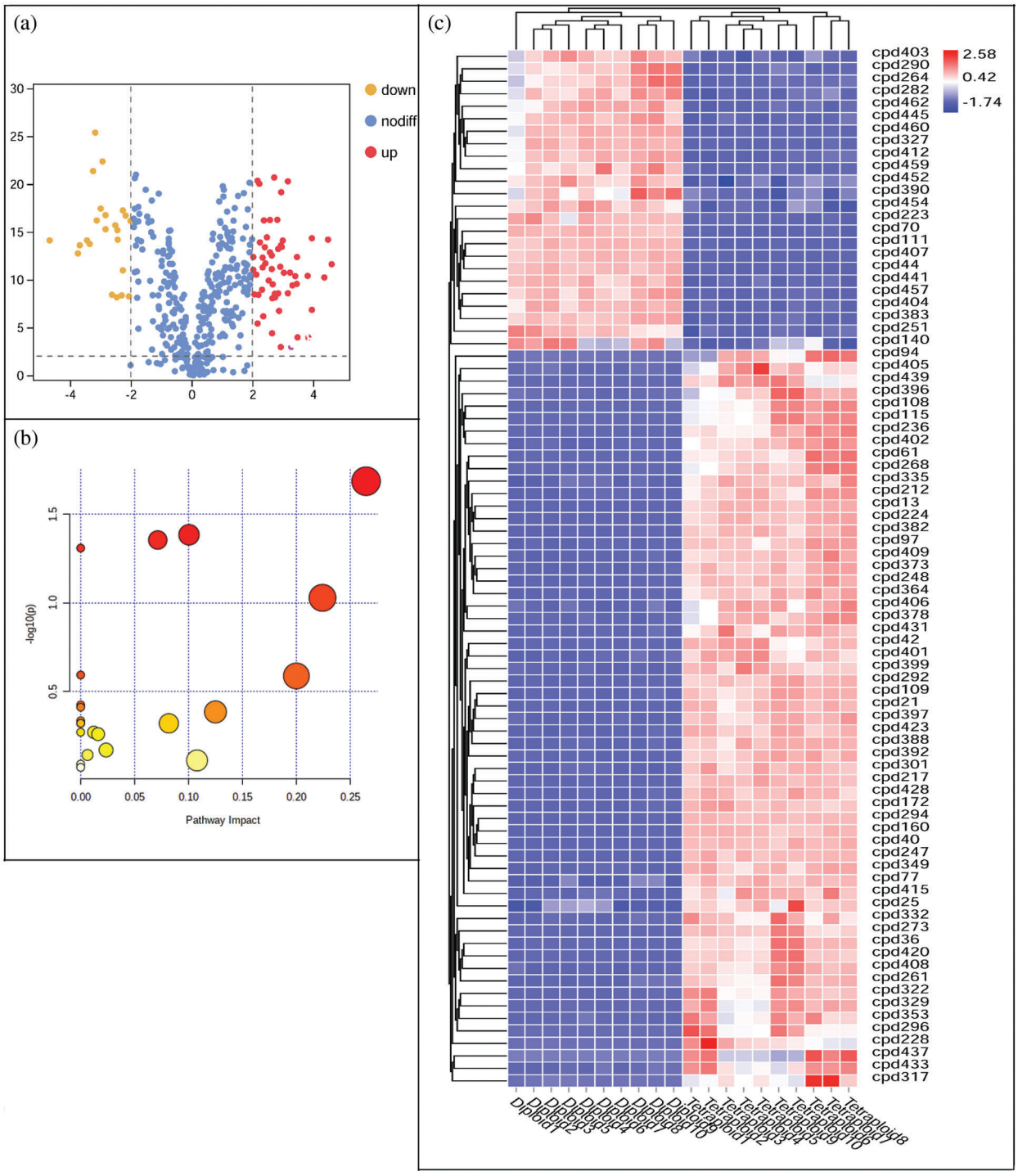

Figure 5: Volcano plot (a), KEGG pathways (b) and clustering heat map (c) of the different contents of metabolites

4 Discussion

An increase in the ploidy level leads to increased species diversity, but it is also accompanied by the loss of repeated genes and the production of new functional genes, which greatly enriches the genetic diversity of the generated polyploid [26]. Naturally formed polyploids are the results of long-term natural selection during evolution, which enables them to adapt to environmental conditions and have a higher mortality ratio than diploid ones [27,28]. A survey of the rich buckwheat germplasm resources revealed natural buckwheat tetraploids, including wild buckwheat (e.g., *Fagopyrum gracilipes* and *Fagopyrum crispatifolium*), which are not cultivated [29,30]. Artificial mutagenesis technology can efficiently induce targeted crop polyploidy. It has been successfully used to expand the genomes of many kinds of plants. Regardless of whether an autopolyploid or an allopolyploid is produced, phenotypic variations are common during the formation of a polyploid species, which explains the enhanced plasticity of polyploids during natural or artificial selection. Polyploidization also results in the production of a lot of materials, which is conducive to the evolution of organisms in nature. Germplasm resources with varying ploidy levels are important for buckwheat breeding and germplasm resource innovation. The artificial polyploidization of the plant is typically achieved through a colchicine treatment [31–33]. Flow cytometry is widely used to rapidly and precisely identify polyploid plants [34,35]. In this study, the materials were examined by flow cytometry to identify the autotetraploid tartary buckwheat with stable inheritance. Additionally, the artificial breeding of tartary buckwheat polyploids can overcome problems, including the long production cycle of the pure parental lines. Furthermore, varieties developed through chromosome doubling rather than genetic modifications (e.g., gene engineering) are more easily accepted as cultivars.

Plant phenotypic traits are intuitive manifestations of plant genetic changes and are often used to evaluate germplasm. The polyploidization of plant species is often accompanied by phenotypic and adaptive changes, some of which may result in significantly enlarged tissues, organs, and cells as well as enhanced seed traits. Previous study has proved that tetraploid tartary buckwheat seeds are significantly larger than diploid tartary buckwheat seeds [36]. Consistent with this earlier finding, we observed that changing the ploidy level of tartary buckwheat affects the seed size, with significant increases in length, width, and a thousand-grain weight. Because of the dosage effect of chromosome replications, the genome content affects the size of cell nuclei, which in turn leads to changes in seed structure and size. Both seed size and the thousand-grain weight are critical indicators of crop yield. Doubling the number of chromosomes can increase tartary buckwheat seed size and weight, with positive implications for crop yield. Although an increase in the ploidy level can enhance seed size to a certain extent, it often leads to a decrease in seed plumpness [23]. Similarly, our results revealed the low seed filling rate for the tetraploid tartary buckwheat generated in this study, which may adversely affect its utility for the commercial production of buckwheat. Scanning electron microscopy images indicated that the starch granules in tetraploid tartary buckwheat seeds were loosely arranged, which may have contributed to the decrease in the seed filling rate. An examination of the seed structure detected a relatively large gap between the husk and the kernel of the tetraploid tartary buckwheat seed. Unlike common buckwheat, the shell and the kernel of tartary buckwheat seeds are very close, which is difficult to remove [37]. The shell needs to be powdered with tartary buckwheat flour or subjected to complex processing (e.g., steam shelling), but both methods adversely affect the quality of tartary buckwheat flour. Whether tetraploid tartary buckwheat can be used to solve tartary buckwheat's dehulling issue remains to be determined.

Seed germination involves the absorption of water and the activation of the metabolic system, which leads to the radicle breaking through the seed coat. This growth stage influences the subsequent plant growth and development. Willenborg et al. proved that the germination rate of diploid wheat seeds is significantly higher than that of tetraploid and hexaploid wheat seeds [38]. In other words, the germination rate and emergence rate of small-grained seeds are higher than the corresponding rates of

large-grained seeds. In the current study, the germination rate was significantly affected by a change to the ploidy level of tartary buckwheat. The germination rate of autotetraploid tartary buckwheat seeds was lower than that of diploid seeds, especially during the initial seed germination stage. Seed germination is affected by many factors, including seed characteristics, external environmental conditions, maturity, and endogenous hormone contents [39–41]. The difference in the germination rates of diploid and autotetraploid seeds will need to be thoroughly characterized to develop technical methods for improving tetraploid seed germination. A moderate germination delay is beneficial because it can prevent the occurrence of tartary buckwheat spikes and it can solve other problems associated with the tartary buckwheat harvesting and rainy seasons. However, compared with diploid tartary buckwheat seeds, embryo abortion is relatively common in tetraploid tartary buckwheat seeds, which is also an important problem affecting the utility of polyploid seeds for agricultural production. Tartary buckwheat proteins and flavonoids are biologically active substances with beneficial effects on health. Plant polyploidization leads to altered gene expression levels, which can modulate plant nutrient and secondary metabolite contents [42,43]. The phenolic acid and flavonoid contents and the antioxidant capacity are significantly higher in tetraploid *Lonicera japonica* than in diploid plants during most growth stages [44]. The results of this study indicate that polyploidization affects flavonoid synthesis in plants. Tetraploid tartary buckwheat is an ideal material for future research on the effects of genome duplications on the formation of specific substances and for the development of new high-quality tartary buckwheat varieties.

5 Conclusions

The differences in seed morphology and metabolites between tetraploid and corresponding diploid accessions were studied. The length, width, area, and a thousand-grain weight were greater for the tetraploid than for the diploid seeds. Additionally, the seed flavonoid contents of tetraploid tartary buckwheat were higher than those of the diploid tartary buckwheat. Based on the UPLC-Q Exactive orbit trap high-resolution mass spectrometry method, 83 differentially abundant compounds were identified, which were mainly associated with biosynthesis of fatty acid, amino acid metabolism and flavonoid metabolites. The results of this study could not only enrich the buckwheat germplasm resources, but also provide material for breeding high-quality buckwheat varieties.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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