Polyploidization Increases the Lipid Content and Improves the Nutritional Quality of Rice

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Research Article

Keywords: LC-MS/MS, nutritional components, tetraploid rice, widely targeted metabolomics

Posted Date: April 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-413313/v1

Abstract

Background: Plant polyploidization is frequently associated with changes in nutrient contents. However, the possible contribution of metabolites to this change has not been investigated by characterizing the metabolite contents of diploid and tetraploid forms of rice (Oryza sativa L.).

Results: We compared the metabolites of a group of diploid–tetraploid japonica brown rice and a group of diploid–tetraploid indica brown rice based on liquid chromatography-tandem mass spectrometry. In total, 401 differential expression metabolites were identified between diploids and tetraploids; of these, between the japonica and indica groups, 180 showed opposite expression trends but 221 showed the same trends (141 up-regulated vs 69 down-regulated). Hierarchical cluster analysis of differential metabolites between diploid and tetraploid species showed a clear grouping pattern, in which the relative contents of lipids, amino acids and derivatives, and phenolic acids increased in tetraploids. Further analysis revealed that the lipids in tetraploid rice increased significantly, especially unsaturated fatty acids and phospholipids.

Conclusions: This study provides more basis for the changes in rice nutritional quality following polyploidization, and may serve as a new theoretical reference for breeding eutrophic or functional rice varieties via polyploidization.

Background

Rice (*Oryza sativa* L.) is one of the most important food crops in the world. While exploring increases in rice yield, the improvement of rice nutritional quality is also pursued (Zhang et al., 2008). Rice grains are mainly composed of starch (85%–90%), protein (7%–12%), lipid (0.3%–3%), mineral elements, and vitamins(Yoshida et al., 2010, Kim et al., 2015). Although the lipid content of rice is low, it has high nutritional value and health care function (Liu et al., 2013). The lipids of rice are divided into non-starch lipids (NSLs) and starch lipids (Juliano, 1983). The NSLs refer to the lipids on the surface of starch granules, which mainly exist in the form of spherical fat bodies (Juliano, 1985). The NSLs mainly include glycerides triglycerides, phospholipids (PLs), and glycolipids] and free fatty acids (Morrison, 1995), and also oryzanol, tocopherol, phenolic acid, phytosterol, squalene, and other physiological active substances (Friedman, 2013, Yu et al., 2016). The main free fatty acids in rice are oleic (C18:1), linoleic (C18:2), palmitic (C16:0), stearic (C18:0), myristic (C14:0), palmitoleic (C16:1), linolenic (C18:3), and arachidonic (C20:4) acids (Zhou et al., 2003, Kitta et al., 2005, Rusydi et al., 2011, Yoon et al., 2012). Most of the free fatty acids in rice are high-quality unsaturated fatty acids, among which arachidonic, linolenic, and linoleic acids have obvious effects in preventing arteriosclerosis and hypercholesterolemia (Sharif, et al., 2014). Low odor threshold volatile compounds, which can be derived from the oxidation of unsaturated fatty acids, are more abundant in the fragrant rice varieties. Thus, the unsaturated fatty acids contribute to rice fragrance, and thereby to overall quality (Concepcion et al., 2018). Compared with starch and protein, increasing the content of unsaturated fatty acids can significantly improve rice eating quality (Yoon et al., 2008). In addition, PLs, another important component of rice NSLs, have important

nutritional value. The PLs are important polar lipids, and widely exist in bacteria, animals, and plants (Liu et al., 2013). Due to their important biochemical functions, many studies have found that dietary PLs are an important way to prevent diabetes, coronary heart disease, inflammation, cancer, and other chronic diseases (Ryan et al., 2007, Bohdanowicz and Grinstein, 2013). Therefore, increasing the lipid content in rice is a way to breed good quality and nutrient-rich varieties.

Polyploids are widely distributed in nature (Wolfe, 2001, Soltis, 2005, Jiao et al., 2011, Peer et al., 2017). Almost all eukaryotes and most angiosperms, as well as crops, such as rice, have experienced polyploidization in the evolutionary process (Paterson et al., 2004, Yu et al., 2005, Rice et al., 2019). Polyploidization often causes gigantism leading to enhanced biomass production, and also changes nutritional quality, for example, contents of carbohydrates, proteins, vitamins, and alkaloids generally increase (Dhawan and Lavania, 1996, Xie et al., 2002). After polyploidization, the nutritional quality of rice also changes (Luo and He, 2001). Compared with diploid rice, the protein content of autotetraploid rice increased by about 30%, amino acid content increased by 20%–30%, but the amylose content decreased by about 12% (Song and Zhang, 1992). These changes improved the nutrition and palatability of rice (Luo and He, 2001). In the past, the changes in nutrients after polyploidization have usually been analyzed using traditional chemical methods. However, the life sciences are currently being transformed by developments in molecular analysis, such as instrumental analysis and biocomputing. In light of the central role played by metabolism in nutrition, metabolomics is rapidly being established as a key analytical method in nutritional studies (Ulaszewska et al., 2018). However, to our knowledge, there is no metabonomics report of the effect of polyploidization on rice nutrition. Here, we compared the metabolites of diploid and tetraploid rice based on liquid chromatography-tandem mass spectrometry (LC-MS/MS). The metabolite expressions among two rice lines with two ploidy levels were analyzed and the significantly differing metabolites identified. Our study provides a basis to use polyploidization to change rice nutritional quality, and is a new theoretical reference for breeding nutrient-rich rice varieties via polyploidization.

Materials And Methods

Rice samples

Diploid rice Balilla (*O. sativa* ssp.*japonica*, 2n = 2*x* = 24) and Yangdao 6 (*O. sativa* ssp.*indica*, 2n = 2*x* = 24) were provided by Academician Longping Yuan (Institute of Agricultural Sciences for Lixiahe Region in Jiangsu, Yangzhou, China). They are abbreviated B-2*x* and Y-2*x* hereafter, respectively. Tetraploid rice Balilla (2n = 4*x* = 48) and Yangdao 6 (2n = 4*x* = 48) were obtained through chromosome doubling by our research group, and abbreviated B-4*x* and Y-4*x*, respectively. Chromosome identification mainly followed the method of Zhang (1996) with some modifications. Rice lines were cultivated using the same planting base and growth conditions. Seeds were harvested when fully ripe and placed in a freezer at −80 °C.

Reagents and instruments

Methanol, acetonitrile, and acetic acid (HPLC/SPECTRO grade) were purchased from Merck, Germany. Deionized water was obtained using the Millpore purification system (Bedford, USA), and lidocaine was purchased from BioBioPha company (Kunming, China). An Ultra Performance Liquid Chromatograph (Shim-pack UFLC CBM20A) was purchased from Shimadzu (Kyoto, Japan). A tandem mass spectrometer (4500 Q TRAP) was purchased from Applied Biosystems (Framingham, MA, USA). The HSS T3 C18 column (100 mm × 2.1 mm × 1.8 μm) was purchased from Waters Corporation (Milford, MA, USA). The Himac CT6E High-Speed Centrifuge was purchased from Hitachi (Tokyo, Japan).

Sample preparation and extraction

Prior to the experiment, samples were numbered after rice hulling, crushed with a grinding machine, placed in glass sample vials, and stored in a refrigerator at −80 °C. The cryopreserved brown rice samples were crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Then, 100 mg of each powdered sample was dissolved in 1.0 mL of extracting solution (70% methanol). The resulting mixtures were stored overnight at 4 °C and vortexed three times to ensure complete extraction. Following extraction, the pellets were centrifuged at 10,000 × g for 10 min. The extracts were filtered through a microporous membrane (0.22-μm pore size) and stored in a sample vial. Quality control (QC) samples were prepared by mixing sample extracts. During analysis, a QC sample was included in the measurement queue every three test samples to monitor measurement repeatability.

HPLC conditions and ESI-Q TRAP-MS/MS

Chromatographic conditions for metabolites were optimized based on the literature of Chen (2013) and Wang (2018). HPLC conditions: The sample extracts were analyzed using an LC-ESI-MS/MS system, which mainly includes UFLC (Shim-pack UFLC Shimadzu CBM20A system, http://www.shimadzu.com.cn/) and MS (Applied Biosystems 6500 Q TRAP, http://www.appliedbiosystems.com.cn/). Analytical conditions were as follows. The HSS T3 C18 (100 mm × 2.1 mm × 1.8 μm) chromatographic column was used. Samples were rapidly eluted using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Separation was achieved with the following gradients: starting with 5% solvent B and raised to 95% B in 11 min, kept at 95% B for 1 min, dropped quickly to 5% within 0.1 min and kept at 5% B for 3 min. The constant flow rate was 0.4 mL/min, column temperature was 40 °C, and injection volume was 5 μL.

ESI-Q TRAP-MS/MS: Linear ion trap (LIT) and triple quadrupole (QqQ) scans were acquired on a QqQ-LIT mass spectrometer, API 6500 Q TRAP LC/MS/MS system, equipped with an ESI Turbo Ion-Spray interface, operating in positive ion mode and controlled by Analyst 1.6 software (AB Sciex). The ESI source operation parameters follow: ion source, turbo spray, source temperature, 550 °C, ion-spray voltage, 5500 V, ion source gas I, gas II, and curtain gas set at 55, 60, and 25 psi, respectively, and collision gas, high. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QqQ and LIT modes, respectively. The QqQ scans were carried out as

multiple reaction monitoring (MRM) experiments with collision gas (nitrogen) set to 5 psi. Declustering potential (DP) and collision energy (CE) for individual MRM transitions were used for further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Qualitative and quantitative analysis of metabolites

Qualitative analysis: Based on the Metware database (MVDB) and the metabolite information public database, qualitative analysis of primary and secondary mass spectrometry data were obtained by referencing existing MS databases such as MassBank (http://www.massbank.jp), KNAPSAcK (http://kanaya.naist.jp/KNApSAcK), Human Metabolome Database (http://www.hmdb.ca), MoTo DB (http://www.ab.wur.nl/moto), and METLIN (http://metlin.scripps.edu/index.php). The structural analysis of metabolites was determined.

Quantitative analysis: Metabolites were quantified via the MRM mode using QqQ MS. In the MRM mode with detection window of 80 s and a target scan time of 1.5 s, the quadrupole filters the precursor ions (parent ions) of the target substance and excludes the ions corresponding to other molecular weights to prevent interference. After obtaining metabolite data from the different samples, the peak area of the mass spectra of all substances was integrated, and the mass spectra of the same metabolites in different samples were corrected.

Metabolite data analysis

Principal component analysis (PCA), system clustering and data standardization, pattern recognition, and metabolic network analysis of the diploid and tetraploid *japonica* rice metabolites were performed on the MetaboAnalyst 4.0 platform. Partial least squares-discriminant analysis (PLS-DA) was used to maximize the metabolome differences between the diploid and tetraploid *japonica* rice samples. The relative importance of each metabolite to the PLS-DA model was checked using the parameter called variable importance in projection (VIP). Metabolites with VIP ≥ 1 and $p \leq 0.05$ were considered as differential metabolites for group discrimination. A heatmap based on the hierarchical cluster analysis (HCA) method was produced using R software (www.r-project.org). Analysis of metabolic pathways was achieved using the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway database, metabolite set enrichment analysis or pathway analysis, and pathway topology analysis. The content of free fatty acids in brown rice was determined by spectrophotometry.

Results

Phenotype comparison and chromosome identification of diploid and tetraploid rice

Chromosome numbers in root tips of diploid and tetraploid plants were counted. Chromosomes of Balilla-2*x* and Yangdao 6-2*x* were 2n = 2*x* = 24, and of Balilla-4*x* and Yangdao 6-4*x* were 2n = 4*x* = 48 (**Fig. 1C, D**). The phenotypes of diploid and tetraploid brown rice significantly differed. Tetraploid brown rice was larger (**Fig. 1A, B**), with longer and wider grains (**Fig. 1E**). The 1000-grain weight of tetraploid brown rice was 25.56%–28.94% higher than that of diploid brown rice (**Fig. 1F**).

Widely targeted metabolic profiling of diploid and tetraploid brown rice based on LC-MS/MS

Two groups of diploid–tetraploid brown rice (**Fig. 1A, B**), with three biological replicates, making a total of 12 samples were used to portray the metabolic profiles employing the widely targeted metabolomics approach. A typical total ion current plot of one QC sample is shown in **Fig. 2A,** which is the spectrum obtained by continuously summing the intensity of all ions in the mass spectrum at different time points. The multi-substance extracted ion chromatogram is usually used to determine the ion flux spectrum of each extracted substance in MRM mode. The multi-peak detection plot of metabolites in MRM mode is shown in **Fig. 2B**. Based on the MVDB and KEGG databases, and MRM, the qualitative and quantitative mass spectrometry analyses were performed on the metabolites in the samples. In total, 401 metabolites were identified, comprising 70 lipids, 68 amino acids and derivatives, 50 phenolic acids, 2 anthocyanins, 30 flavonoids, 9 flavonols, 22 flavonoid carbonosides, 2 isoflavones, 7 phenolamines, 22 alkaloids, 6 plumeranes, 33 nucleotides and derivatives, 29 organic acids, 27 saccharides and alcohols, 7 vitamins, and 17 others **(Supplementary Table S1)**.

PCA for diploid vs tetraploid groups

The PCA score scatter plots for all samples are shown in **Fig. 2C**, where the abscissa and the ordinate represent the PC1 and PC2 scores, respectively. The distinction between the diploid–tetraploid Balilla (*japonica*) group, the diploid–tetraploid Yangdao 6 (*indica*) group, and the mix group was significant based on the top-ranking PCs, all the samples were within 95% confidence intervals (Hotelling's T-squared ellipse). The PCA results suggested significant differences in metabolic phenotypes between each sample. The values for the diploid and tetraploid brown rice were separated in the PCA score plot of metabolites, and were clearly divided into two categories. The results indicated that after polyploidization of different rice lines, the change trend of their metabolites was similar, which might lead to the same changes in rice nutrients.

Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) for diploid vs tetraploid groups

Compared with PCA, OPLS-DA can maximize the distinction between groups, and is more conducive to finding differential metabolites. The scatter score plots inferred from the inter-group comparison of the diploid–tetraploid Balilla group and the diploid–tetraploid Yangdao 6 group in OPLS-DA are shown in **Fig. 3A and B**, respectively. The R^2Y and Q^2Y scores were all greater than 0.99 in the B-2*x* vs B-4*x***(Fig. 3A**) and Y-2*x* vs Y-4*x* (**Fig. 3B)**, demonstrating that the ploidy difference led to the differential metabolism. The OPLS-DA model was established using many (n = 200) alignment experiments (Trygg & Wold, 2002). The horizontal line corresponds to the R^2 and Q^2 of the original model, and the black and gray points represent the R^2 and Q^2 of the model after Y replacement, respectively (**Fig. 3C, D**). The stable and reproducible model provided a satisfactory explanation of the difference between the two groups of samples. The OPLS-DA results showed that the differential metabolites could be screened according to VIP value in the subsequent analysis.

HCA and volcano plot of differential metabolites for diploid vs tetraploid groups

The HCA can classify metabolites with the same characteristics and identify the differences between groups. So, it can be used to evaluate the characteristic difference of metabolite accumulation caused by ploidy differences. The HCA plot of the differential metabolites identified in comparing the diploid with the tetraploid groups is shown in **Fig. 2D**. The HCA results showed a clear grouping pattern of different species.

Differential metabolites were also analyzed using volcano plots (**Fig. 4F, G**). The points in the volcano plot represent the metabolites, the abscissa indicates the fold change (FC) ($\log_2$) of each substance in the group, and the ordinate indicates the *p*-values ($\log_{10}$) of the Student's t-test. Metabolites with FC of ≥ 2 or FC ≤ 0.5, and $p < 0.05$ were selected. In the *japonica* group, there were 182 up-regulated and 56 down-regulated metabolites in the tetraploid compared with diploid, there were 86 up-regulated and 120 down-regulated metabolites in the *indica* group.

The FC can describe the changes from initial to final values. In the current study, $\log_2$ FC was used to analyze the relative expression changes of metabolites between diploid and tetraploid brown rice. If $\log_2$ FC > 0, this indicates that the relative content of the metabolite was up-regulated, if $\log_2$ FC < 0, the relative content was down-regulated. Of the 401 differential expression metabolites of diploid–tetraploid, among *japonica* and *indica* groups, 180 metabolites showed opposite expression trends but 221 showed the same trends (141 up-regulated vs 69 down-regulated) (**Fig. 4A, B**). Moreover, the numbers of up-regulated metabolites of lipids, amino acids and derivatives, and phenolic acids in tetraploid rice were significantly increased in both the *japonica* and *indica* groups (**Fig. 4A, C–E**). In particular, among the 70 lipid differential metabolites, 53 showed the same trends for the *japonica* and *indica* rice groups, of which, levels of 77.36% (41 out of 53) were up-regulated (**Fig. 4E**).

Clustering, pathway, and enrichment analyses of lipid metabolites for diploid vs tetraploid groups

The previous analysis showed that most (levels of 77.36%) lipid metabolites were up-regulated. The heatmap of lipid metabolite changes for the diploid vs tetraploid groups is shown in **Fig. 5A**. The results showed a clear grouping pattern of different species. The most differential lipid metabolites identified in the study showed similar positively or negatively regulated trends between *japonica* and *indica* groups, consistent with the previous analysis. The results of these lipid metabolite annotations were classified according to the type of pathway in the KEGG database (http://www.genome.jp/kegg/). A total of 13 metabolic pathways were involved (**Fig. 5B**). Among these pathways, “Biosynthesis of unsaturated fatty acids” and “Metabolic pathways” were mainly involved. This indicates that the increase in ploidy mainly changed the content of unsaturated fatty acids of brown rice.

Studies have shown that the main free fatty acids in rice are unsaturated fatty acids (Zhou et al., 2003). In order to verify this previous conclusion, we determined the content of free fatty acids in brown rice (**Fig. 5C**). The content of free fatty acids in Balilla-4*x* was 28.36 nmol/g, which was 45.66% higher than in Balilla-2*x* (19.47 nmol/g), and the content in Yangdao 6-4*x* was 11.82 nmol/g, which was 52.91% higher than that in Yangdao 6-2*x* (7.73 nmol/g). Thus, content of free fatty acids mainly composed of unsaturated fatty acids increased significantly in tetraploid rice, consistent with previous analysis.

Statistical analysis of significant differential lipid metabolites for diploid vs tetraploid groups

In the current study, the VIP and *p*-value were used to analyze the significant differential lipid metabolites. If VIP > 1 and $p < 0.01$, a significant difference in the metabolite exists between the diploid and tetraploid groups. There were 11 metabolites with significant differences (**Table 1)** : two free fatty acids (γ-linolenic and punicic acids), five lysophosphatidylcholines (LysoPC 15:0, LysoPC 16:1, LysoPC 18:1, LysoPC 18:3, and LysoPC 18:3 2n isomer), two glycerol esters (MAG 18:2 and MAG 18:3 isomer1), one sphingolipid (4-hydroxysphinganine), and one phosphatidylcholine (choline alfoscerate). Most of these metabolites were significantly up-regulated in tetraploid rice (**Table 1**). Among them, all the lysophosphatidylcholines increased significantly, and may be important contributors to the increase of PL content in tetraploid rice. Among the annotated free fatty acid metabolites, the level of γ-linolenic increased significantly and indicated that up-regulation of γ-linolenic acid may play an important role in the increase of unsaturated fatty acids in tetraploid rice.

Table 1. Identification of significantly different lipid metabolites in diploid and tetraploid groups based on the criteria of VIP > 1 and $p < 0.01$.

Lipid compounds	B-2*x* vs B-4*x*			Y-2*x* vs Y-4*x*		
	VIP	*p*	Trend	VIP	*p*	Trend
γ-Linolenic acid	2.13	1.85×10^{-3}	↑	1.11	8.38×10^{-4}	↑
Punicic acid	2.22	4.29×10^{-3}	↓	1.55	4.28×10^{-4}	↓
LysoPC (15:0)	1.28	3.73×10^{-4}	↑	1.25	3.94×10^{-4}	↑
LysoPC (16:1)	1.67	8.71×10^{-4}	↑	1.76	3.73×10^{-4}	↑
LysoPC (18:1)	2.50	9.17×10^{-4}	↑	2.31	1.15×10^{-3}	↑
LysoPC (18:3)	5.62	4.16×10^{-5}	↑	2.82	1.97×10^{-3}	↑
LysoPC (18:3) (2n isomer)	5.48	2.69×10^{-4}	↑	3.02	2.54×10^{-3}	↑
4-Hydroxysphinganine	1.07	6.91×10^{-4}	↓	1.82	1.74×10^{-3}	↑
Choline alfoscerate	1.34	06.37×10^{-3}	↑	1.39	7.18×10^{-4}	↑
MAG (18:2)	5.34	2.33×10^{-4}	↑	4.83	9.12×10^{-5}	↑
MAG (18:3) isomer1	2.26	1.89×10^{-4}	↑	1.08	1.13×10^{-4}	↑

Note: the ↑ and ↓ represent increased and reduced levels of lipid metabolites between the two groups, respectively.

The γ-linolenic acid is formed by the elongation and desaturation of palmitic acid carbon chain. The pathways for synthesis of certain unsaturated fatty acids as affected by rice polyploidization are shown in **Fig. 6.** Heatmap and $\log_2$ FC value of lipid metabolite changes between diploid and tetraploid brown rice indicated that the level of palmitoleic (16:1, Δ^{9}), stearic (18:0), linoleic (18:2, $\Delta^{9,12}$), α-linolenic (18:3, $\Delta^{9,12,15}$), and γ-linolenic (18:3, $\Delta^{6,9,12}$) acids were increased. These unsaturated fatty acids are the main components of free fatty acids in rice. It indicated that the expression level of palmitoleic acid, stearic acid, linoleic acid, α-linolenic acid, and especially γ-linolenic acid were up-regulated after polyploidization. Finally, the content of free fatty acids in tetraploid rice was increased significantly.

Discussion

Differential metabolites between diploid and tetraploid rice

Metabolomics is a method of qualitatively and quantitatively analyzing all metabolites in an organism, which can be used to explore dynamic changes in metabolites and the accumulation patterns and genetic origins of plant metabolites (Weckwerth, 2003, Dettmer et al., 2007). Widely targeted metabolomic

analysis is a novel approach that can simultaneously quantify hundreds of known metabolites and nearly 1000 known and unknown metabolites (Sawada et al., 2007, Chen et al., 2013). Due to the important role of metabolism in nutrition, metabonomics has been increasingly used to analyze rice nutrients. Hu et al. (2014) compared 121 metabolites in mature seeds of a wide panel of *japonica* and *indica* cultivars, which laid a foundation for improving rice quality through metabolic engineering. Later, Hu et al. (2016) investigated the dynamic metabolic changes during rice grain development of two *japonica* and two *indica* cultivars using a non-targeted metabolomics approach. Analyses revealed both cultivar- and developmental stage-dependent metabolic changes in rice grains. Analysis of rice by gas chromatography-MS has revealed several odor-active volatile compounds that may provide the characteristic aroma of different rice varieties (Calingacion et al., 2012, Mumm et al., 2016, Concepcion et al., 2018). The secondary metabolites of the Chinese and North American wild rice were analyzed using an UHPLC-QqQ-MS-based metabolomics approach, which provided reference values for isolation and identification of functional compounds from wild rice (Yan et al., 2019).

Researchers have compared nutrients between diploid and autotetraploid rice, such as amino acids, proteins, and starches (Song & Zhang, 1992, Gu et al., 2015). However, there is no report on comparative analyses of secondary metabolomics in diploid and tetraploid rice. In this study, 401 differential expression metabolites of diploid–tetraploid brown rice were identified based on LC-MS/MS. The analysis revealed very different relative contents of metabolites between diploid and tetraploid groups. Especially lipids, amino acids and derivatives, and phenolic acids increased obviously in tetraploids of both *indica* and *japonica* rice. The result once again confirmed that the amino acid content increased significantly after polyploidization. Additionally, significant increases in lipid substances (e.g., unsaturated fatty acids and PLs) were also found for the first time. Among the lipid metabolites with significant differences (VIP > 1, $p < 0.01$), the γ-linolenic acid and LysoPC 15:0, LysoPC 16:1, LysoPC 18:1, LysoPC 18:3, and LysoPC 18:3 2n isomers were significantly up-regulated in tetraploid rice. Among unsaturated fatty acids, the level of γ-linolenic acid increased significantly, meanwhile, the relative contents of most other unsaturated fatty acids in the pathway of γ-linolenic acid synthesis have also increased. Therefore, the up-regulation of most unsaturated fatty acid metabolites (especially γ-linolenic acid) and PL metabolites (especially the five LysoPCs) were important contributors to the increase in lipids after rice polyploidization.

Polyploidization changes rice nutrition

Polyploidization usually leads to changes in plant nutrition (Xie et al., 2002). For example, the soluble protein amount doubled in polyploid *Panicum virgatum* (Warmer et al., 1987) and amino acid content increased in polyploid sorghum (Luo et al., 1992). In autotetraploid barley, protein and glutenin contents increased (Tiwari et al., 1980). Autotetraploid *Setaria tomentosa* had superior starch, total soluble sugar, and lipid contents (Shashi and Sachdeva, 1990). Polyploidization significantly changes the rice phenotype. Tetraploid rice seeds often become longer, wider, and heavier. At the same time, the chalkiness degree, chalkiness rate, gel consistency, alkali spreading value, and gelatinization temperature are also

changed (Gu et al., 2015). Nutrient substances, such as protein and amino acid contents, clearly increase but the amylose content often decreases in tetraploid rice (Song and Zhang, 1992, Liu et al., 2007, Xie et al., 2007, Gu et al., 2015). In addition, our study showed that phenolic acids and lipids (especially unsaturated fatty acids and PLs) also increased significantly. Rice grains contain smaller proportions of lipids than starch and protein, however, these lipids may make a significant contribution to processing and nutritional properties (Yoshida et al., 2010, Kim et al., 2015). For instance, rice oil is a popular cooking oil in several countries, which has a direct impact on human nutrition and health (Ghosh, 2007). Therefore, it will be valuable to cultivate rice varieties with high lipid contents developed through polyploidization. Autotetraploid rice was first reported in 1933 (Nakamori, 1933), making a history of nearly 90 years. However, the low-fertility autotetraploid rice has always been difficult to use in agriculture. In recent years, the polyploid meiosis stability tetraploid rice lines and neo-tetraploid rice lines with high seed and pollen fertilities have opened the door to the use of autotetraploid varieties in rice breeding and cultivation (Cai et al., 2007, Guo et al., 2017, Koide et al., 2020). These high seed-fertility tetraploid rice lines make it possible to breed nutrient-rich varieties, including of proteins, amino acids, and lipids.

Conclusions

The present study used widely targeted metabolites based on the LC-MS/MS detection platform to analyze the metabolic differences in diploid and tetraploid rice. This is the first analysis of the effects of rice polyploidization on nutritional quality from the perspective of metabonomics. Among the 401 differential expression metabolites identified, the relative contents of lipids, amino acids and derivatives, and phenolic acids clearly increased in tetraploid rice. In particular, the lipid contents, especially of unsaturated fatty acids and PLs, increased significantly after polyploidization in both *indica* and *japonica* rice, which has not been reported before. Overall, the present work provides more knowledge concerning the changes in rice nutritional quality after polyploidization, and more reference for breeding eutrophic or functional rice, such as lipid-rich polyploid rice varieties.

Abbreviations

NSLs: non-starch lipids, PLs: phospholipids, LC-MS/MS: liquid chromatography-tandem mass spectrometry, HPLC: High performance liquid chromatography, UHPLC: Ultra high pressure liquid chromatography, MM: mixer mill, QC: Quality control, LIT: Linear ion trap, QqQ: triple quadrupole, MRM: multiple reaction monitoring, DP: Declustering potential, CE: collision energy, MVDB: Metware database, PCA: Principal component analysis, PLS-DA: Partial least squares-discriminant analysis, VIP: variable importance in projection, HCA: hierarchical cluster analysis, KEGG: Kyoto Encyclopedia of Genes and Genomes, FC: fold change, MAG: Monoacylglycerol ester, PC: Phosphatidylcholine.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

XZ and DC designed and supervised the works. WW, QT, RC, YX, QX performed experiments. PL, ZS, LL analyzed the data and prepared the figures. XZ, WW, YH wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the National Key R & D Plan (2017YFD100400), the Natural Science Foundation of Hubei Province (2018CFB633), the National Natural Science Foundation of China (No.31960068), the Wuhan Science and Technology Major Project "Development and application of polyploid rice", the Open Research Fund of State Key Laboratory of Hybrid Rice (Hunan Hybrid Rice Research Center) (19KF06).

Availability of Data and Materials

The datasets supporting the conclusions of this article are provided within the article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

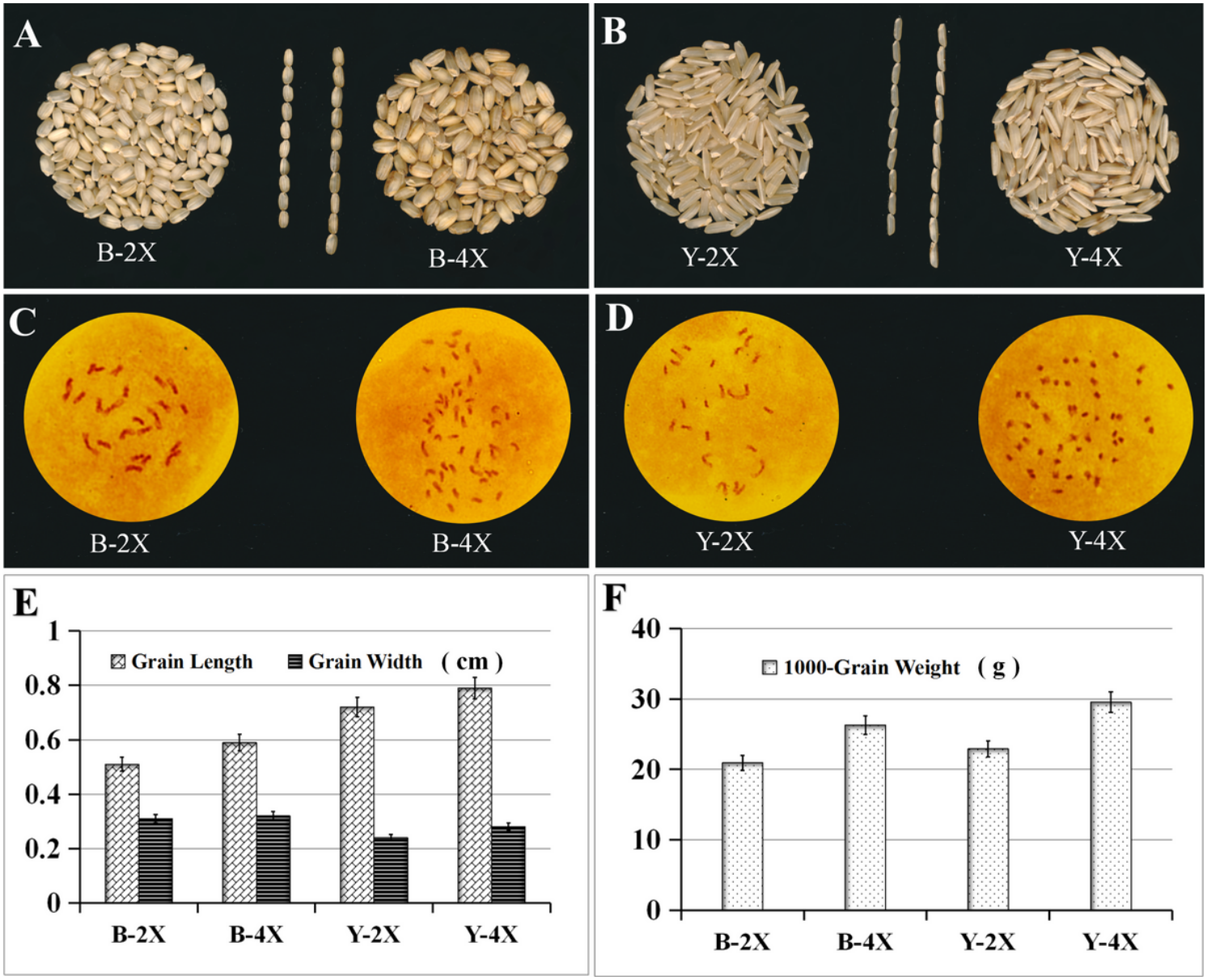

Figure 1

Brown rice comparison and chromosome identification between diploid and tetraploid rice. (A) Diploid and tetraploid brown rice of Balilla (japonica). (B) Diploid and tetraploid brown rice of Yangdao 6 (indica). Root tip chromosomes of diploid and tetraploid rice: (C) Balilla and (D) Yangdao 6. Comparison between diploid and tetraploid brown rice: (E) grain length and width and (F) 1000-grain weight.

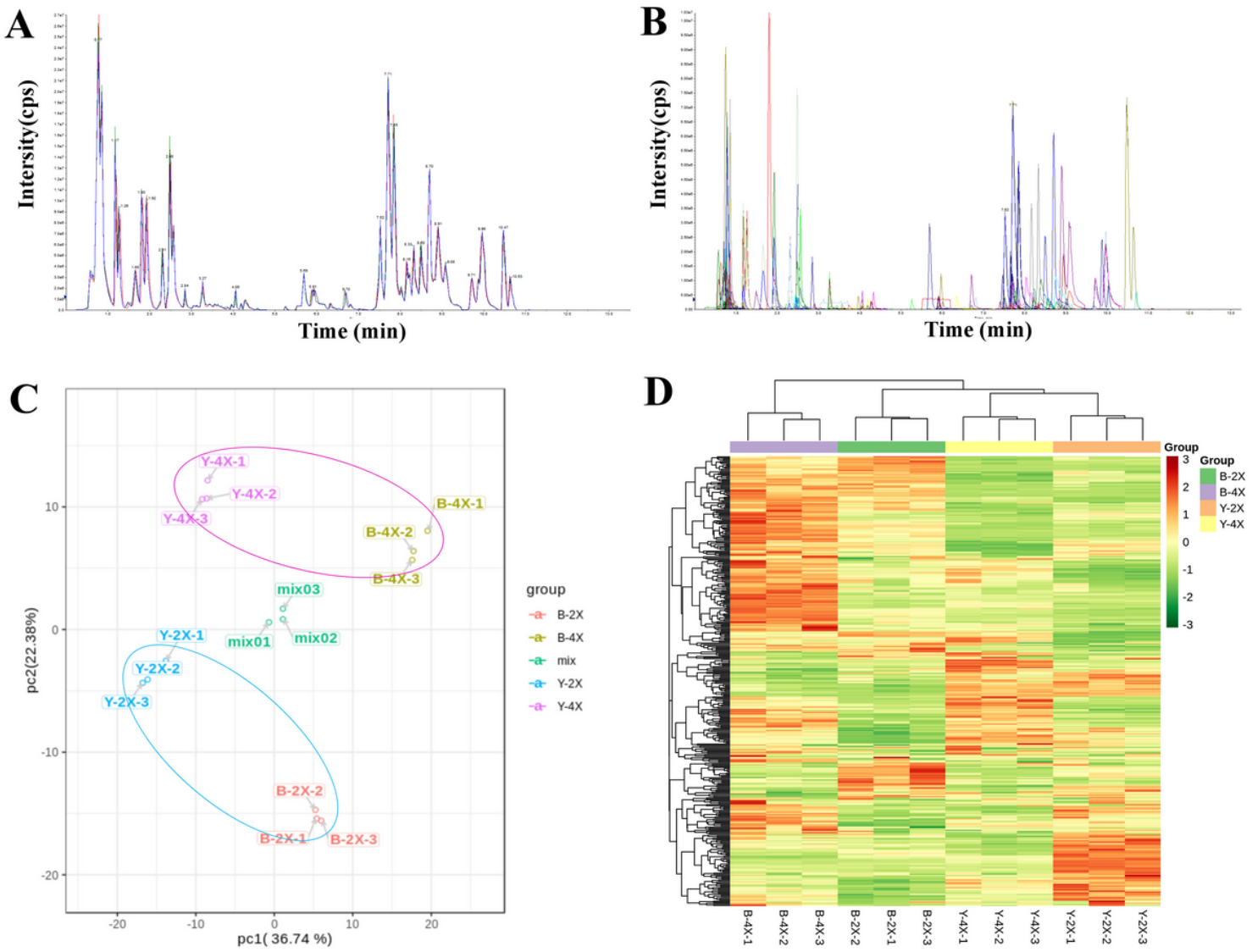

Figure 2

Detection and analysis of metabolites according to LC–MS/MS. Detection of the TIC overlap map by (A) QC sample mass spectrometry and (B) MRM metabolite detection multipeak map. (C) PCA scores plot map and (D) hierarchical cluster analysis of diploid vs tetraploid groups.

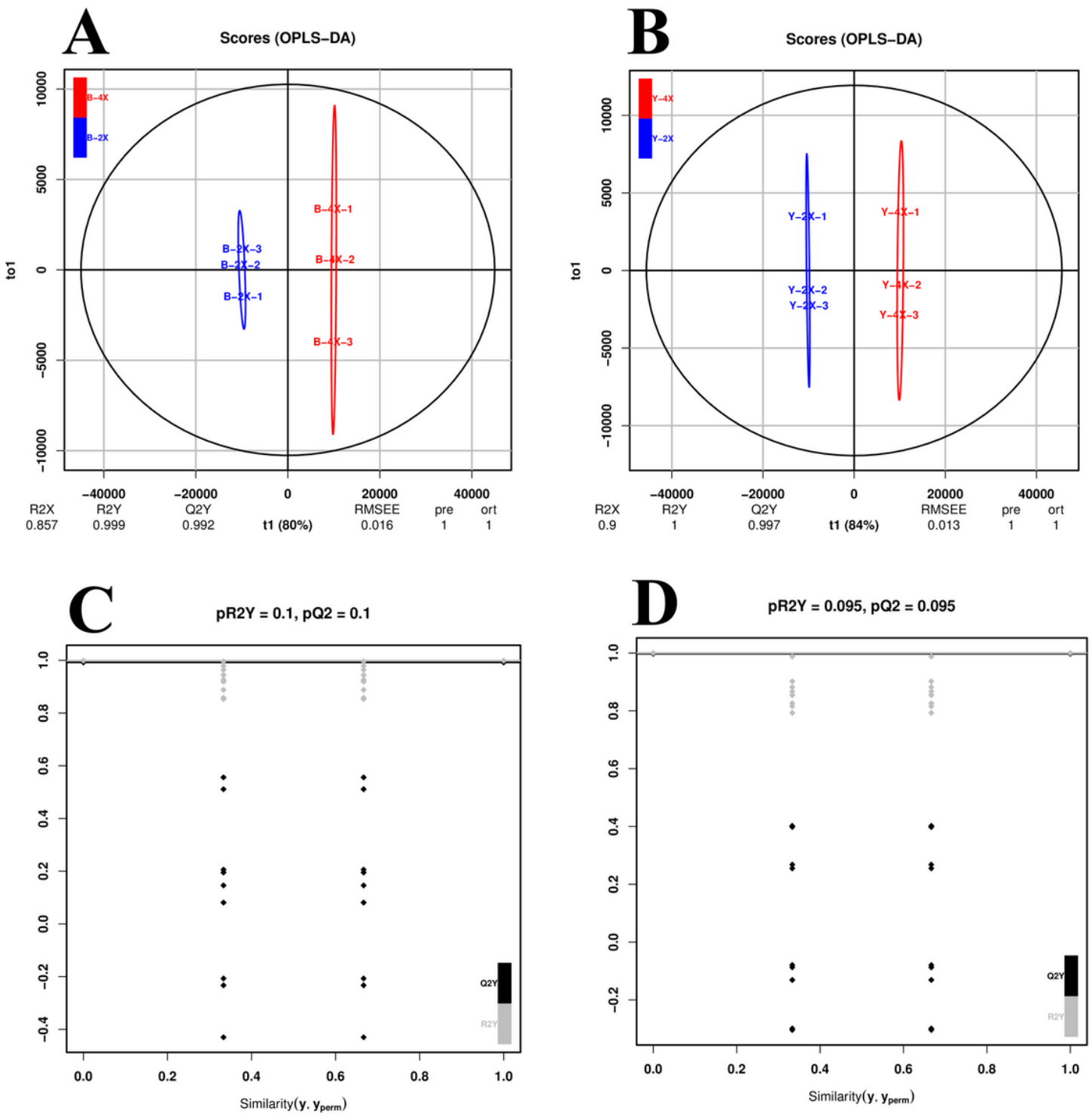

Figure 3

OPLS-DA scores and permutation verification. Scores of the OPLS-DA model with (A) Balilla-2x vs Balilla-4x and (B) Yangdao 6-2x vs Yangdao 6-4x. OPLS-DA permutation analysis model verification chart of (C) Balilla-2x vs Balilla-4x and (D) Yangdao 6-2x vs Yangdao 6-4x. R2Y and Q2 represent the interpretation rate of the model to the Y matrix and the prediction ability of the model, respectively. A value closer to 1 means that the model is more stable and reliable, and when Q2 > 0.9, the model is excellent. The

horizontal line corresponds to the R2 and Q2 of the original model, and the black and gray points represent the R2, and Q2, of the model after Y replacement, respectively.

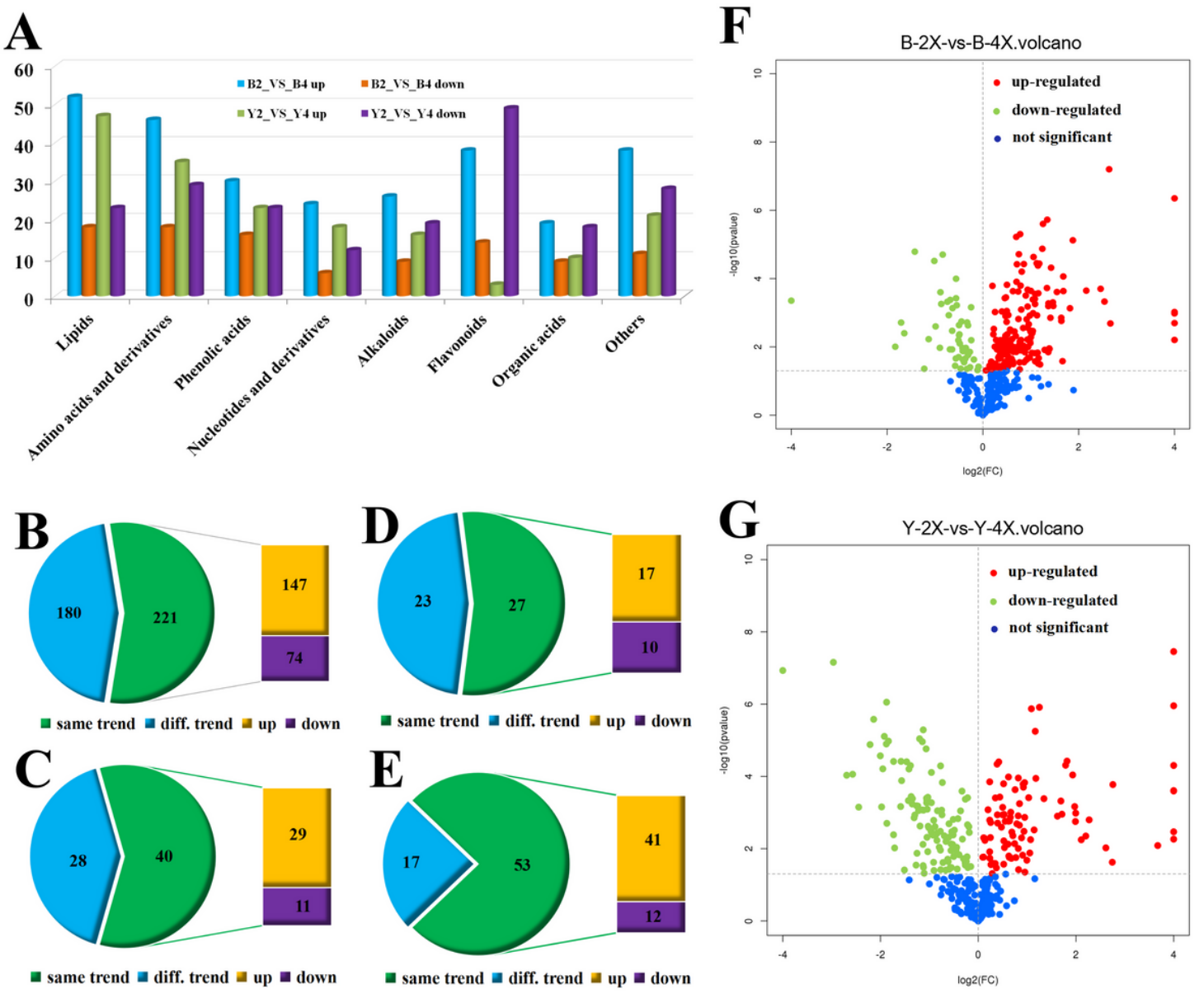

Figure 4

Number of different types of differential metabolites for diploid vs tetraploid groups and volcano plot of differential metabolites. (A) Number of different types of differential metabolites for diploid vs tetraploid. Blue color indicates the number of up-regulated metabolites of Balilla-2x vs Balilla-4x, and orange color indicates the number of down-regulated. Green color indicates the number of up-regulated metabolites of Yangdao 6-2x vs Yangdao 6-4x, and purple indicates the number of down-regulated. The expression trend of differential metabolites for diploid vs tetraploid between japonica and indica groups. Green color indicates the number of same regulated trend metabolites, and blue indicates the number of differentially regulated trend. Yellow color indicates the number of up-regulated metabolites, and purple indicates the number of down-regulated: (B) total metabolites, (C) amino acids and derivatives, (D) phenolic acids, and (E) lipids. (Volcano plot of (F) Balilla-2x vs Balilla-4x and (G) Yangdao 6-2x vs Yangdao 6-4x. The colors

of the scatter points indicate the final screening results: red indicates metabolites that were significantly up-regulated, green indicates metabolites that were significantly down-regulated, and blue indicates metabolites with no significant difference.

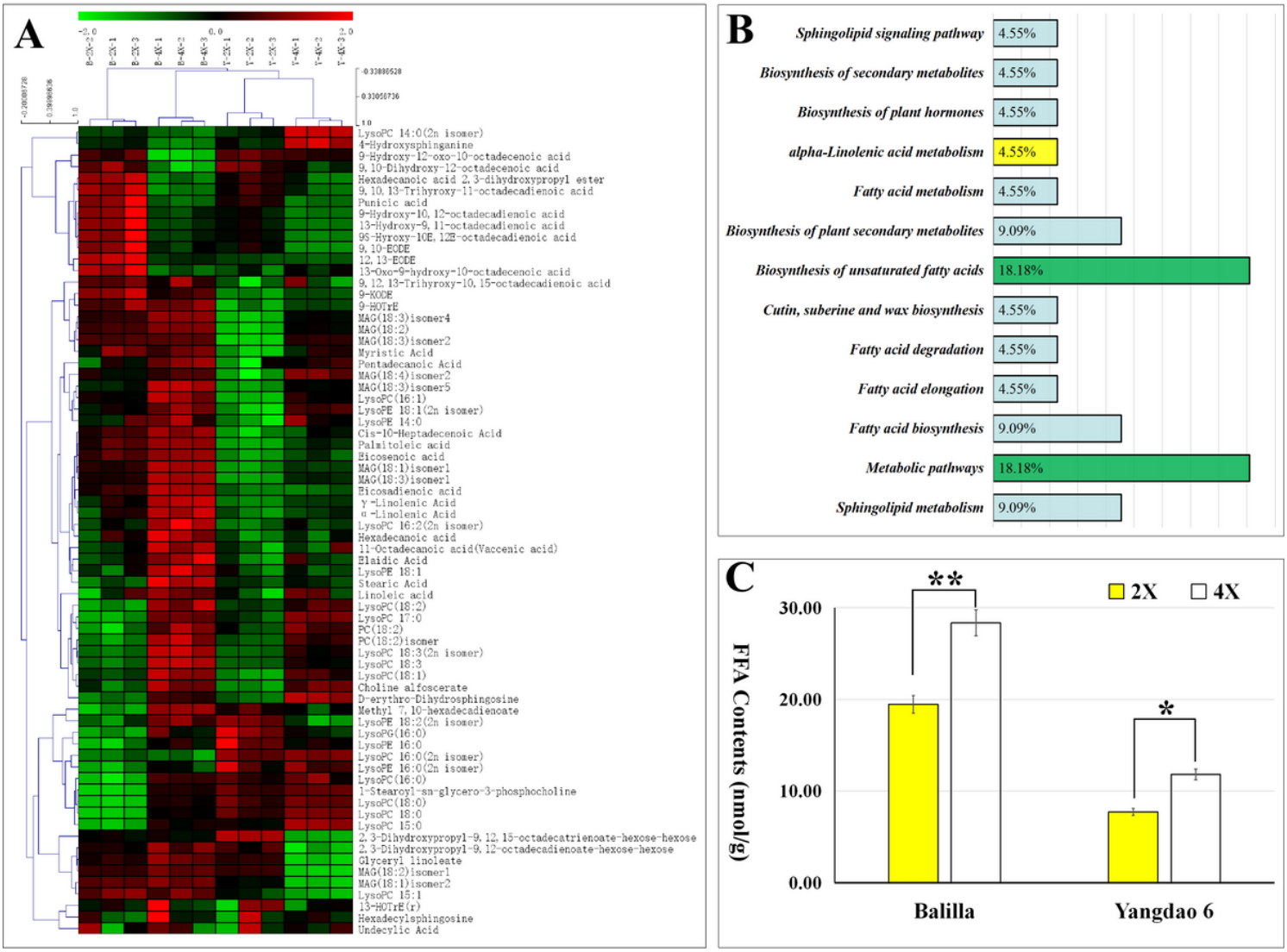

Figure 5

Clustering analysis and pathway analysis of lipid metabolites for diploid vs tetraploid groups, and determination of free fatty acids in brown rice. (A) Heat map of lipid metabolite changes in brown rice grains. Red segments indicate a relatively high content of metabolites, and green segments indicate a relatively low content of metabolites. (B) Functional annotation and KEGG classification of lipid metabolites. The ordinate is the name of the KEGG metabolic pathway, the abscissa is the number of metabolites annotated to the pathway and its proportion in the total number of lipid metabolites annotated. (C) Determination of free fatty acids in diploid (yellow) and tetraploid (white) brown rice.

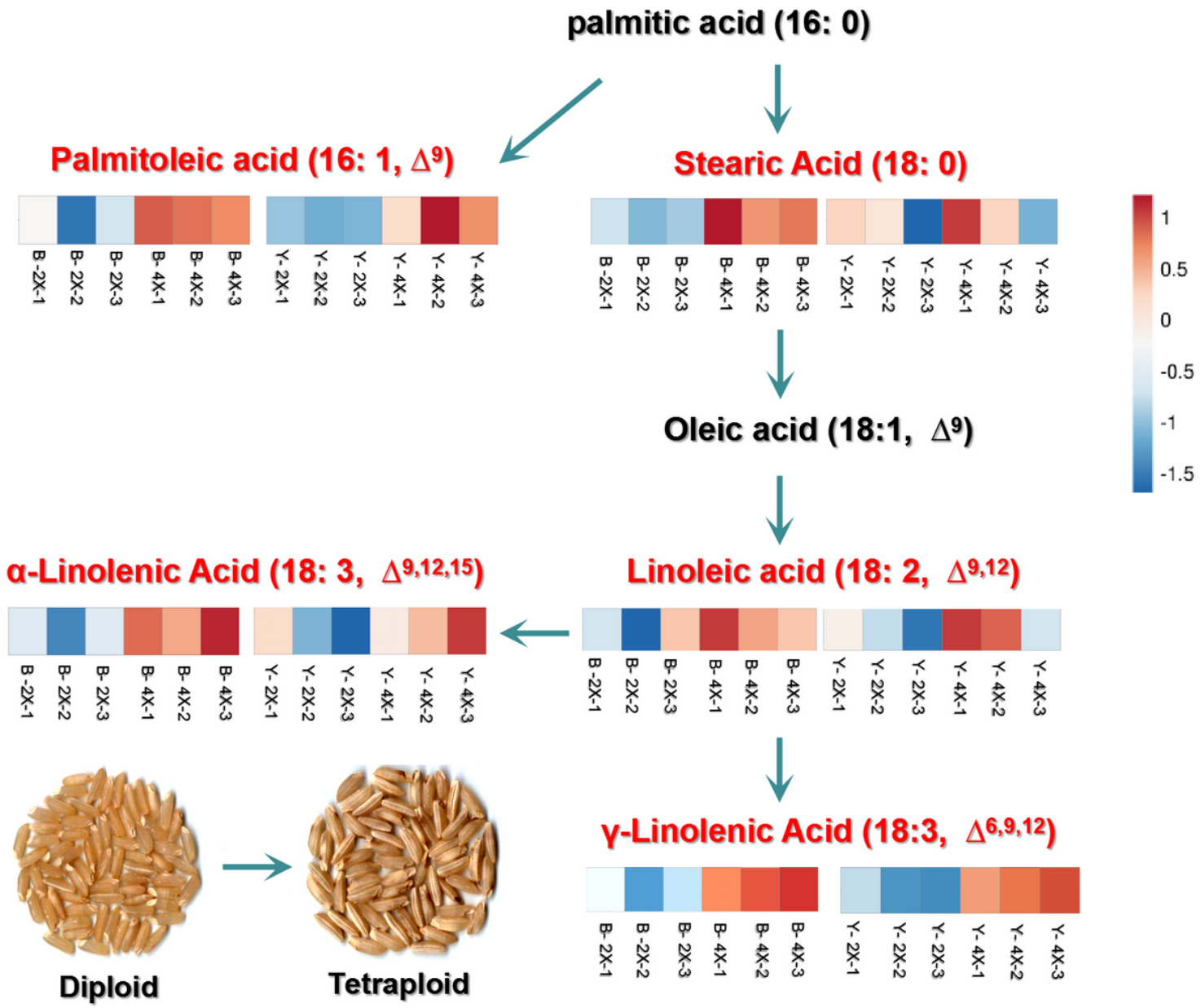

Figure 6

Schematic presentation of the pathway for certain unsaturated fatty acids metabolites as affected by polyploidization in rice. Red represents increased. The checkerboard is the heatmap of metabolites for the diploid vs tetraploid groups. Ratios of fold changes are given by shades of red or blue color according to the scale bar.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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