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Lipidomic analysis of grain quality variation in high quality aromatic *japonica* rice

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ABSTRACT

To maintain the purity of the seeds and rice quality of the high-quality rice varieties, five lines with similar field and yield traits were selected from the Nanjing46 population in Liyang and used as study materials, and the original progeny were used as the control material for comparing rice quality and lipid metabolites in this study. The rice quality of the five lines still differed compared to CKN1. The *Badh2-E2* gene was detected in all five lines, but its 2-AP content differed. The C11:0 content in CKN1 and VN1 was significantly greater than that in the other four lines. Most of the differentially abundant metabolites were phospholipids, including PA(16:0/18:2), PC (15:0/16:0) and PG(16:0/16:0). These metabolites can be used as potential metabolic markers for identifying quality variation. This study presents a novel methodology and theoretical framework for investigating varietal degradation and ensuring seed purity authentication.

1. Introduction

With the progress of economic and social life, people's requirements for rice are increasing, and rice quality is becoming the subject of daily scientific research. Rice quality includes processing, appearance, eating and cooking quality and nutritional quality (P. Li et al., 2022). In the "Cooking rice variety quality" (NY/T593–2021) study, the evaluation of rice quality changed from relying solely on human sensory evaluation to using advanced instruments for qualitative and quantitative determination of physical and chemical traits combined with sensory evaluation, allowing for more comprehensive and scientific assessment. Among many indicators, the amylose content, gel consistency, protein content, pasting temperature, taste value and RVA are the main indicators of the eating and cooking quality of rice.

Lipids, as one of the indispensable components of rice, have important impacts on the eating, cooking, nutritional, and storage quality of rice. In recent years, several researchers have shown that a high fat content is a common characteristic shared by most high-quality rice varieties (Shi, Zhang, Wang, & Wu, 2021). Researchers successfully enhanced the oil content of Nanjing 46, the main cultivar in the southern rice region, by synthetic biology technology, and the relative content of oil in its brown rice increased from 2.33% to 11.72% (Liu et al., 2023).

However, it is difficult to determine the difference between high quality rice and ordinary rice planes through only the conventional index evaluation system, which suggests that the fat content of rice is more important to quality than the existing rice evaluation system (L. Liu, Waters, Rose, Bao, & King, 2013; Yu, Zhou, Duan, Min, & Zhu, 2007). The results of quality tests showed that the crude fat content (CFC) of high-quality rice varieties was generally greater than that of nonexcellent varieties and increased significantly with improved rice quality (X. P. Li et al., 2017). Increasing the CFC in rice can significantly improve cooking quality (Gu et al., 2011).

Several researchers have shown significant differences in the fat content of different rice genotypes: glutinous rice > *japonica* rice > *indica* rice > dry rice (Yu et al., 2007; X. M. Zhang & Zhou, 1997) Although the fat content in rice is not high, rice is mostly composed of high-quality unsaturated fatty acids such as oleic acid, linoleic acid and other beneficial components (Concepcion, Calingacion, Garson, & Fitzgerald, 2020), but also contains gluten, sterols, vitamin E, squalene and other physiologically active substances (X. Q. Zhang, Wu, Yuan, & Shu,

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2019), which have a high nutritional value (Kim et al., 2015; X. Q. Zhang et al., 2019). Previous studies have shown that starch supplemented with stearic acid and linoleic acid has a significantly lower peak viscosity and breakdown and a significantly longer peak time (Zhou, Blanchard, Helliwell, & Robards, 2003). It has also been suggested that fat content has a greater effect on the eating and cooking quality of rice than starch and protein content (Jiang et al., 2016). In addition, the fat content of rice is largely independent of other indicators. The fat content was only correlated with the amylose content (X. Q. Zhang et al., 2019), and the amylose content (AC) and fat content could be improved simultaneously. When the fat content increased to a relatively high level, the amylose content (AC) decreased (T. M. Chen et al., 2020), which was highly favourable for improving eating quality. Previous studies have gradually recognized the importance of rice lipids (Xiong et al., 2022). However, the existing quality evaluation systems do not include fat content. Therefore, there is an urgent need to supplement the theoretical research on rice lipids.

Lipidomics is a high-throughput analysis method used to systematically analyse the interactions and functions of all lipid molecules in tissues, organs and cells in living organisms, and subsequently elucidate the related biological activity processes and mechanisms (R. X. Wang, Li, Lam, & Shui, 2020). In recent years, there has been continuous progress in lipidomic technology with the rapid development and support of mass spectrometry and other related technology platforms (X. M. Wang, 2004). Previous studies have shown that the levels of lecithin (PC), ceruloplasmin (PE) and phosphatidylglycerol (PG) decrease significantly, while the level of phosphatidic acid (PA) tends to increase during the ageing of rice, thus revealing the onset of lipid degradation during the ageing process (F. W. Wang, Wang, Jing, & Zhang, 2011).

In this study, we selected Nanjing 46 from Liyang as the material and systematically compared the differences in quality between the different lines. Targeted and nontargeted lipidomic methods were used to analyse the lipid differences metabolites between the different lines and the control, which further revealed the causes of quality degradation among different lines from the same source. This study provides a basis for understanding the causes of germplasm degradation in high-quality rice and provides new technical support for improving the quality and efficiency of high-quality rice.

2. Materials and methods

2.1. Plant materials and growth conditions

Five rice lines (VN1, VN2, VN3, VN4 and VN5) were selected for testing from Nanjing 46 plants grown in a large field in Liyang District, Changzhou city, China, and the control variety (CKN1) was the original prototype provided by the original breeding unit. The experiment was conducted in 2021 and 2022 at the Innovative Experimental Base of President Zhang Hongcheng in Yangzhou City, Jiangsu Province, China. The seeds were sown on May 20th and transplanted on June 18th in 2021. The seeds were sown on May 18th and transplanted on June 17th in 2022. All the culture methods involved the use of blanket plants. The plants in each line were planted in rows with a spacing of 30×12 cm^2, with the plants planted individually. Cultivation measures related to fertilizer and water management as well as pest and weed control were implemented in accordance with local high-yield cultivation requirements.

2.2. Sample collection

Three biological replicates of each sample were collected after maturation of the five lines and the control. Brown rice was produced from the paddy plants using a Korean Ssangyong SY88-TH huller, which produced 18 brown rice samples. Brown rice was produced as milled rice using a Hiba LTJM-2099 milled rice machine.

2.3. Determination of agronomic traits and yield traits

The heading stage refers to the date on which 80% of the rice panicles revealed the flag leaf sheath. The maturation stage is the stage when >95% of the solid kernels are yellow and ripe. Plant height is the height from the ground to the top of the spike (excluding the awn). Ten plants were measured consecutively in the field before harvest. At the time of rice maturity, 10 holes were surveyed in each line to determine the number of spikes per hole, the number of spikelets per panicle, and the percentage of filled grains. After rice harvest, when the moisture content was 14.5%, the 1000-grain weight was determined (1000 grains were weighed, 5 replications).

2.4. Determination of rice quality

The rice plants were harvested, threshed, dried and stored indoors. High-quality rice plants (GB/T 17891–2017) were used to determine the brown rice percentage (BR), milled rice percentage (MR), and head milled rice percentage (HMR). The chalkiness rate (CR) and chalkiness degree (CD) were determined by a Wanshen SC-E rice appearance quality detection and rice quality determination instrument. The "Determination of amylose content in rice-Spectrophotometry method" (NY/T 2639–2014) was applied for the determination of amylose content (AC). The crude protein content (CPC) was calculated by determining the nitrogen content via the Kjeldahl method and multiplying the result by a conversion factor of 6.25 (J. Q. Wang et al., 2018). The sucrose content (SuC) and starch content (StC) were determined using kits from Suzhou Michy Biomedical Technology Co., Ltd. (Suzhou, China). The crude fat content (CFC) was determined by the Soxhlet extraction method (GB 5009.6–2016). The taste value (TV) was determined using a rice flavour metre (SATAKE RICE TASTE ANALYSER). The hardness, springiness, stickiness, and balance of cold rice were determined using the SMS Texture Analyser Physical Property Analyser P/36R probe.

2.5. Determination of the solubility and swelling power of starch

Starch extraction was performed according to Wei's research methods (Wei et al., 2011) with slight modifications. The solubility (SL) and swelling power (SP) of the starch were determined according to the methods of Konik-Rose (Konik-Rose et al., 2001). Approximately 35.00 mg of pure starch sample (m0) was weighed into a weighed 2 ml Eppendorf centrifuge tube (ml), and 1 ml of ultrapure water was added. The sample was shaken in a water bath at 90 °C for 1 h and centrifuged at 4000 rpm for 10 min, after which the total weight of the supernatant was determined by pipetting the liquid with a liquid pipette (m2). The sample was dried and weighed at 60 °C (m3). The amount of water-absorbed colloid adhering to the wall of the tube was taken as the swelling weight.

Solubility (SL) = 100*(m0 + m1-m3)/m0*100%; swelling power (SP) = (m2-m1)/(m3-m1).

2.6. Detection of the fragrance gene

The primer *fgr-InDel-E2* developed by Jun Wang (J. Wang, Yang, Chen, & Zhong, 2008) was used for the detection of the fragrance gene *fgr*. Rice genomic DNA was extracted via the CTAB method from fresh leaves collected at the peak tillering stage. PCR was performed using a 20 μl system of 10.00 μl Vazyme 2 × Taq Master Mix, 1 μl each of forwards and reverse primers, 2 μl of DNA template (approximately 100 ng), and ultrapure water, which was adjusted to 20.00 μl. The PCR program was as follows: predenaturation at 95 °C for 10 min; denaturation at 95 °C for 30 s; annealing at 55 °C for 30 s; extension at 72 °C for 30 s; 35 cycles; of extension at 72 °C for 10 min; cooling at 12 °C for 10 min; and separation of the target fragments via 4% agarose gel electrophoresis.

2.7. Determination of 2-AP content

Combined with the findings of Wu and Hinge (Hinge, Patil, & Nadaf, 2016; Wu, Chou, Wu, Chen, & Huang, 2009), the extraction of 2-AP preprocessed samples was improved.

Gas chromatography–mass spectrometry (GC–MS): The relative composition of 2-AP was determined by a Shimadzu GC–MS QP 2010 plus, and the standard internal technique was applied with 2,4,6-trimethylpyrimidine (TMP) as the internal standard (Shan, Zhang, Chen, Zhang, & Gao, 2015). The chromatographic column used was an RTX-5MS (30 m × 0.32 mm × 0.25 μm). The RTX-5MS column temperature was adjusted to 40 °C after injection. After 1 min, the temperature was increased to 65 °C at a rate of 2 °C min^{-1} for a period of 1 min. Subsequently, the temperature was increased to 220 °C at 10 °C min^{-1}. The carrier gas was high-purity helium (≥99.999%). The volume of the injection administered was 2 μl. The mass spectrometer was operated in electron impact (EI) ionization mode with an EI energy of 70 eV. The ion source temperature was set to 200 °C. The temperature of the interface was recorded as 250 °C, while the quadrupole temperature was 150 °C. The process of acquiring mass spectra involved a scan ranging from *m/z* 30 to 350.

2.8. Targeted lipidomics

For preparation of the mixed standard, one millilitre of hexane was used to dissolve 100 mg of a 51 fatty acid methyl ester mixed standard to obtain 51 fatty acid methyl ester mixed standard reserve solution A. A total of 100 μl of solution A was combined with 900 μl of hexane to obtain 51 fatty acid methyl ester mixed standard intermediate solution B. Intermediate solution B was diluted into the working solution and loaded into an injection vial for GC–MS analysis.

Briefly, 50 mg of the sample was weighed in a 2 ml grinding tube, a small steel ball was added, 1 ml of dichloromethane:methanol (*v*/v = 1:1) was added, and the mixture was ground in a cryomill (50 Hz) for 3 min. The mixture was sonicated at low temperature for 15 min and allowed to stand at −20 °C for 15 min. The mixture was centrifuged at 4 °C and 13,000 rcf for 10 min, after which 500 μl of the supernatant was aspirated into a 1.5 ml EP tube, after which the mixture was blown dry under nitrogen. After blow-drying, 0.5 ml of methylation reagent (0.5 mol l^{-1} sodium hydroxide methanol solution) was added, and the mixture was vortexed for 30 s, sonicated at 4 °C for 10 min, and placed in a water bath at 60 °C for 0.5 h. After cooling, 0.5 ml of hexane was added, and then the mixture was vortexed for 30 s, and centrifuged at 4 °C and 13,000 rcf for 10 min. A total of 100 μl of the upper layer (hexane layer) was transferred to an injection vial for subsequent GC–MS analysis.

An Agilent Technologies Inc. CA (UAS) 8890B—7000D GC/MSD GC–MS instrument was used. The chromatographic conditions were as follows: Agilent CP7489 CP-Sil 88 for FAME capillary column (100 m × 250 μm × 0.2 μm; Agilent J&W Scientific, Folsom, CA, USA); high-purity helium (purity not <99.999%) as the carrier gas; a flow rate of 1.0 ml·min^{-1}; and an injection temperature of 260 °C. The injection volume was 1 μl, and the sample was injected in split mode, with a split ratio of 50:1 and a solvent delay of 9.9 min. The initial temperature of the column temperature chamber was 80 °C for 0 min, and the temperature was increased to 180 °C and then 220 °C at 4 °C min^{-1} and then 230 °C at 2 °C min^{-1}, which was held for 13.5 min. Afterwards, the temperature was increased to 235 °C and maintained for 2 min. The mass spectrometric conditions were as follows: source, EI; ion source temperature, 230 °C; quadrupole temperature, 150 °C; transmission line temperature, 240 °C; EI ionization energy, 70 eV; and scanning mode, selected ion monitoring (SIM).

2.9. LC–MS/MS analysis

The methods of Xie and Zhu were used (Zhu et al., 2022), and 18 samples of brown rice were collected over time and subjected to sample preparation and LC–MS analysis. The metabolite data were analysed using the R software package ropls (version 1.6.2).

2.10. Statistical analysis

At least three replicate measurements were taken for the determination of the rice parameters, unless otherwise stated. Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) was used to organize and calculate the average value of the rice quality data. Each test was conducted twice. Statistical analysis, one-way analysis of variance (ANOVA), Duncan's test and a post hoc test were conducted using SPSS statistical software (version 22.0; IBM, Armonk, New York, NY, USA). The graphing was also completed using Origin 2021 graphing software.

3. Results

3.1. Analysis of agronomic and yield traits

We analysed the agronomic and yield traits of five lines and CKN1 in 2021 and 2022 (Table S1). The five lines did not significantly differ in critical fertility, plant height or yield traits compared to those of CKN1 ($P < 0.05$). However, there were significant differences in plant height, number of spikelets per panicle, percentage of filled grains and 1000-grain weight between years ($P < 0.05$).

3.2. Analysis of rice quality traits

The rice quality of five different lines from the same source differed somewhat from that of CKN1 (Table 1). The processing quality of VN2 was the worst and was significantly inferior to that of CKN1 ($P < 0.05$). The BR of VN5 was significantly greater than that of CKN1, but the MR and HMR were highly significantly lower than those of CKN1 ($P < 0.05$). The HMRs of VN1, VN3 and VN4 were significantly lower than that of CKN1 ($P < 0.05$). A comparison of the appearance quality of each line revealed that VN2 and VN4 had better appearance quality and that their CR and CD were significantly lower than those of CKN1 ($P < 0.05$). The StCs of VN2, VN3 and VN4 were significantly lower than that of CKN1 by 15.74, 14.15 and 22.94%, respectively ($P < 0.05$). The ACs of VN1 and VN4 were significantly lower than that of CKN1, which were 10.67 and 13.03% lower, respectively ($P < 0.05$). The SL of all five lines was significantly lower than that of CKN1 ($P < 0.05$). The SPs of VN1, VN3 and VN5 were greater than that of CKN1 and were >22.55, 17.18 and 14.11%, respectively. CKN1 had the highest SuC of 14.24 mg g^{-1}, while VN2, VN3 and VN4 all had significantly lower SuC than CKN1 ($P < 0.05$), as shown by CKN1 > VN5 > VN1 > VN4 > VN3 > VN2. The CPCs of VN2 and VN4 were significantly lower than that of CKN1 by 5.36 and 8.81%, respectively ($P < 0.05$). VN4 had the lowest CFC of 0.97%, which was 31.69% lower than that of CKN1. In terms of the taste value, only VN2 had a significantly lower TV than CKN1 by 6.89% ($P < 0.05$). There were no significant differences in hardness, stickiness, springiness or balance between the five lines and CKN1 ($P < 0.05$).

We detected the functional markers *fgr-Indel-E2* in each line and CKN1 and found that the target band of 100 bp was amplified in 6 materials. However, the contents of the main aroma substance 2-AP were different. The 2-AP content in CKN1 was 67.19 ng g^{-1}, which was greater than that in the other lines. The order of incidence was CKN1 > VN4 > VN1 > VN3 > VN2 > VN5, and all the differences were significant ($P < 0.05$).

The results of the correlation analysis showed (Table 2) that there was a significant positive correlation between MR and the HMR ($P < 0.05$). There were significant positive correlations between StC and CR and between CFC and stickiness ($P < 0.01$). A significant positive correlation was found between CFC and SuC ($P < 0.01$). There was a significant negative correlation between TV and hardness and a significant positive correlation between balance and SP ($P < 0.01$).

Table 1
Analysis of the difference in rice quality traits among lines.

	CKN1	VN1	VN2	VN3	VN4	VN5
BR (%)	85.33 ± 0.79b	85.54 ± 0.34ab	82.37 ± 0.20c	85.24 ± 0.47b	85.34 ± 1.08b	86.50 ± 0.10a
MR (%)	77.35 ± 1.32a	76.64 ± 0.20ab	74.18 ± 0.42c	77.58 ± 1.20a	75.44 ± 0.61bc	75.14 ± 0.62bc
HMR (%)	76.31 ± 1.28a	72.65 ± 2.76bc	68.15 ± 0.47d	73.75 ± 1.97ab	71.13 ± 1.14bcd	70.34 ± 1.25 cd
CR	52.09 ± 2.83a	47.43 ± 0.92b	41.58 ± 0.57c	52.20 ± 0.82a	42.59 ± 0.43c	53.61 ± 2.87a
CD	20.29 ± 1.17a	15.25 ± 2.39b	12.27 ± 1.47c	17.61 ± 0.99b	12.43 ± 0.77c	17.51 ± 0.71b
StC (mg g^{-1})	547.70 ± 32.16a	490.81 ± 26.54ab	461.50 ± 20.46bc	470.20 ± 39.40bc	422.04 ± 47.41c	551.05 ± 35.20a
AC (%)	12.96 ± 0.32a	11.58 ± 0.89b	13.21 ± 0.16a	12.54 ± 0.31a	11.27 ± 0.61b	12.51 ± 0.14a
SuC (mg g^{-1})	14.24 ± 0.85a	12.89 ± 0.46ab	10.83 ± 0.46d	11.56 ± 0.84bc	11.98 ± 0.87bc	12.99 ± 0.99ab
CPC (mg g^{-1})	54.87 ± 2.13a	51.93 ± 1.80bc	55.25 ± 0.42a	53.75 ± 0.95ab	54.78 ± 0.76a	50.04 ± 0.69c
CFC (%)	1.42 ± 0.19a	1.37 ± 0.02ab	1.04 ± 0.04bc	1.15 ± 0.38abc	0.97 ± 0.10c	1.34 ± 0.09ab
TV	73.53 ± 0.91ab	73.10 ± 5.62ab	68.47 ± 10.06b	72.70 ± 6.03ab	79.47 ± 1.81a	72.5 ± 2.40ab
Hardness (g)	121.68 ± 25.72a	130.47 ± 30.08a	124.86 ± 35.53a	111.63 ± 31.25a	86.94 ± 26.02a	124.07 ± 49.95a
Sprining (%)	0.498 ± 0.021ab	0.500 ± 0.008a	0.502 ± 0.016a	0.48 ± 0.017ab	0.475 ± 0.016b	0.490 ± 0.016ab
Stickness (g)	1184.68 ± 298.51a	1059.31 ± 82.08a	1149.20 ± 33.10a	1080.11 ± 93.75a	1009.33 ± 162.14a	1212.68 ± 144.94a
Balance	0.256 ± 0.028a	0.272 ± 0.020a	0.272 ± 0.018a	0.272 ± 0.035a	0.260 ± 0.057a	0.270 ± 0.019a
SL	0.254 ± 0.017a	0.122 ± 0.004b	0.108 ± 0.014b	0.118 ± 0.004b	0.117 ± 0.007b	0.126 ± 0.004b
SP	9.12 ± 1.06c	11.17 ± 0.14a	10.04 ± 0.41bc	10.68 ± 0.51ab	9.87 ± 0.39bc	10.41 ± 0.50ab
2-AP Content (ng g^{-1})	67.19 ± 7.26a	42.21 ± 2.08b	33.86 ± 8.13bc	38.03 ± 1.96bc	59.25 ± 1.71a	30.60 ± 1.02c
Genotype	*N*	*N*	*N*	*N*	*N*	*N*

The lowercased letters that succeed the data in the same line denote a statistically significant difference at the $P < 0.05$. BR: brown rice rate, MR: milled rice rate, HMR: head milled rice rate, CR: chalkiness rate, CD: chalkiness degree, StC: starch content, AC: amylose content, SuC: sucrose content, CPC:crude protein content, CFC: crude fat content, TV: taste value, SL: solubility, SP: swelling power. The notation "*N*" represents a negative value, while "*P*" represents a positive value.

3.3. Multivariate statistical analysis

To understand the sampling of the samples to be tested and the reliability of the test results, a quality control sample (QC) was added to monitor the system stability of this test, while the samples were tested by LC–MS/MS. As shown in Fig. 1A, we evaluated the QC samples by relative standard deviation (RSD), and the results showed that the cumulative percentage of peaks reached 0.8994 when the RSD of the QC samples was <30, which indicated that the overall data were qualified (the dashed line indicates the data before preprocessing, and the solid line indicates the data after preprocessing). Therefore, this study initially determined that this analytical technique was sufficiently stable and reliable for the determination of the samples, providing good testing conditions for subsequent in-depth evaluation and analysis of test results.

Before in-depth analysis of the sample data, we first performed principal component analysis (PCA) of the samples based on the expression of the principal components of the samples' nontargeted metabolomics data to evaluate the similarity of the samples within the group and the difference in the samples between the groups. According to the PCA score plot, the first two principal components, PC1 and PC2, explained 34.00% and 9.54% of the data variance, respectively, and the sum of the contributions of the two components was 43.54% (Fig. 1B). This indicates that there is a high degree of similarity between the samples and that further analysis of the data is needed. Partial least squares discriminant analysis (PLS-DA) revealed that Component 1 and Component 2 explained 49.8% and 9.81% of the data variance, respectively (Fig. 1C, Fig. 1D). After 200 substitution tests, the random arrangement produced R2 and Q2 values that were smaller than the original values, and the slope of the straight line was large, indicating that PLS-DA did not cause model overfitting, leading to a more reliable substitution test (Fig. 1E). All the CKN1 replicates were significantly different from the remaining five samples. The greater the separation from the remaining samples is, the more significant the classification effect.

3.4. Differential lipid metabolites

We analysed the whole lipidome of five different lines and CKN1 cells using LC–MS/MS, and 38 differential lipid subclasses were detected, totaling 405 lipid molecules (Table S2). These 38 lipid subclasses were dominated by triglycerides (TGs), diglycerides (DGs), ceramides (Cers) and lecithin (PC). TG was the most abundant group, containing 89 lipid molecules; DG contained 43 lipid molecules; Cer contained 42 lipid molecules; and PC contained 31 lipid molecules. Each line also had its own unique metabolite map (Fig. 1F). There were also specific differentially abundant metabolites (DAMs) between the 5 lines and CKN1 (Fig. 1G).

A total of 91 DAMs were identified in the VN1 and CKN1 comparison groups, of which 72 exhibited a decrease (Fig. 1H, Table S3). A total of 126 DAMs were identified in the VN2 and CKN1 comparison group, 90 of which were upregulated (Fig. 1H, Table S4). A total of 129 DAMs were identified in the VN3 and CKN1 comparison group, 73 of which were downregulated (Fig. 1H, Table S5). A total of 119 DAMs were identified in the VN4 and CKN1 comparison group, 69 of which were downregulated (Fig. 1H, Table S6). A total of 156 DAMs were identified in the VN5 and CKN1 comparison groups, with 97 showing downregulation (Fig. 1H, Table S7). In addition, we highlighted the DAMs in the form of clustering trees and VIP bar charts (Fig. S1) and visualized the DAMs of the different comparison groups in the form of volcano plots, with each point representing a DAM (Fig. 2).

In this study (Table S8), the DAMs in the VN and CKN1 comparison groups were found to be involved mainly in glycerophospholipid metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism, sphingolipid metabolism, ether lipid metabolism, and arachidonic acid metabolism pathways (Table S3). DAMs in the VN2 and CKN1 comparison groups were involved mainly in glycerophospholipid metabolism, arachidonic acid metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism, glycerolipid metabolism, the phosphatidylinositol signalling system, and the sphingolipid metabolism pathway (Table S4). DAMs in the VN3 and CKN1 comparison groups were involved mainly in glycerophospholipid metabolism, glycerolipid metabolism, the phosphatidylinositol signalling system, arachidonic acid metabolism, linoleic acid metabolism, alpha-linolenic acid metabolism, and the ether lipid metabolism pathway (Table S5). DAMs in the VN4 and CKN1 comparison groups were mainly involved in linoleic acid metabolism, alpha-linolenic acid metabolism, arachidonic acid metabolism, and glycerophospholipid metabolism pathways (Table S6). DAMs in the VN5 and CKN1 comparison groups were involved mainly in glycerophospholipid metabolism, linoleic acid metabolism, arachidonic acid metabolism, the phosphatidylinositol signalling system, glycerolipid metabolism, and the alpha-linolenic acid metabolism pathway (Table S7).

Table 2
Correlation analysis of rice quality characters.

	MR	HMR	CR	CD	StC	AC	SuC	CPC	CFC	TV	Hardness	Sprining	Stickness	Balance	SL	SP	2-AP Content
BR	0.473	0.479	0.698	0.554	0.446	−0.481	0.656	−0.676	0.534	0.545	−0.115	−0.407	0.010	−0.217	0.200	0.183	0.162
MR		0.935**	0.614	0.720	0.248	−0.090	0.497	0.025	0.492	0.221	0.030	−0.168	−0.158	−0.240	0.506	0.048	0.422
HMR			0.601	0.794	0.390	−0.016	0.710	0.112	0.580	0.287	0.017	−0.075	0.000	−0.529	0.765	−0.258	0.651
CR				0.924**	0.789	0.246	0.611	−0.558	0.739	−0.090	0.343	−0.049	0.520	−0.022	0.434	0.055	−0.062
CD					0.814*	0.360	0.749	−0.272	0.796	−0.090	0.358	0.100	0.545	−0.283	0.731	−0.220	0.236
StC						0.458	0.756	−0.528	0.892*	−0.340	0.659	0.461	0.842*	−0.118	0.609	−0.176	−0.031
AC							−0.049	0.215	0.175	−0.794	0.524	0.504	0.787	0.098	0.347	−0.396	−0.227
SuC								−0.303	0.843*	0.252	0.253	0.211	0.373	−0.584	0.804	−0.326	0.544
CPC									−0.534	0.074	−0.404	−0.049	−0.295	−0.442	0.249	−0.590	0.541
CFC										−0.214	0.692	0.505	0.541	−0.088	0.600	0.049	0.089
TV											−0.815*	−0.737	−0.608	−0.606	0.071	−0.169	0.627
Hardness												0.876*	0.627	0.462	0.169	0.276	−0.442
Sprining													0.522	0.211	0.270	−0.005	−0.173
Stickness-														−0.006	0.440	−0.348	−0.233
Balance															−0.755	0.839*	−0.937**
SL																−0.716	0.723
SP																	−0.686

* and ** reveal significant difference at $P < 0.05$ and $P < 0.01$, respectively.

3.5. Targeted metabolomics analysis of medium and long chain fatty acids

In this study, 51 medium- and long-chain fatty acids (MLFAs) were detected and analysed, and the TIC plots showed that the separation of each index was high and that the peak shapes were good (Fig. S2). Linear regression standard curves were plotted using the mass spectral peak areas of the target standard solutions as the vertical coordinates and the concentrations as the horizontal coordinates. The linear R2 of all the targets was >0.99, indicating good linearity (Table S9). The results showed that the RSDs of all the targets were <15%, which indicated that the method and the analytical system were stable and reliable and could be used for the quantitative determination of the samples.

In this study, 51 MLFAs were analysed (Table S10). The contents of C14:1 T, C14:1, C15:1 T, C15:1, C16:1 T, C17:1 T, C18:1n6t, C18:1n11t, C18:1n6c, C18:2n6t, C19:1n7t, C20:1 T, C22:1 T, C23:0, C20:3n3, C20:4n6, C24:0, C20:5n3, C24:1, C22:4, C22:5n3, C22:5n6, and C22:6n3 were very low in the five lines and control materials. The most abundant fatty acid was C18:2n6c, followed by C18:1n9c and C16:0. These three fatty acids accounted for >90% of the total fatty acid content in the sample. Only C11:0 was detected in CKN1 and VN1, and its content was significantly greater than that of the other four lines ($P < 0.05$).

3.6. Correlation analysis between rice quality and MLFAs

To investigate the relationship between rice quality and MLFAs, we analysed the correlation between MLFAs and rice quality in five lines and CKN1 (Fig. S3). The relationships between 28 identified MLFAs and rice quality (processing quality, appearance quality, cooking and taste quality, and nutritional quality) were analysed.

C11:0 was significantly positively correlated with the MR, HMR, SuC, CFC, SL and 2-AP contents and significantly negatively correlated with stickiness ($P < 0.01$). C14:0 was significantly negatively correlated with StC ($P < 0.01$). Balance was significantly positively correlated with C16:0 and C18:2n6c ($P < 0.01$). C17:1 was significantly positively correlated with the CPC ($P < 0.01$). SL was significantly positively correlated with C16:0, C17:0, C17:1, C18:0, C20:0 and C20:1 ($P < 0.01$). The 2-AP content was significantly positively correlated with C17:0, C17:1, C20:0 and C20:1 ($P < 0.01$).

3.7. Analysis of the correlation between rice quality and DAMS

To investigate the relationship between rice quality and DAMs, we analysed the correlation between DAMs and rice quality in five line materials and in CKN1 (Fig. 3). The 23 identified metabolites, including ceramides (Cers), diglycerides (DGs), Lysophosphatidylcholines (LPCs), lecithin (PCs), Phosphatidylethanolamines (PEs), Phosphatidylglycerols (PGs), triglycerides (TGs) and so on, were analysed for their association with rice quality (processing quality, appearance quality, cooking and taste quality, nutritional quality). AC was significantly negatively correlated with Cer(t18:0/25:0 + O), Cer(t18:0/24:0), Cer(t18:0/26:0) and Cer(t18:0/22:0) and significantly positively correlated with DG (20:0/18:2), DG(20:1/18:2), TG(14:1e/6:0/18:2), PG(28:0/18:1), Cer (t18:0/18:2), and Cer(d18:1/18:1 + O) ($P < 0.01$). TV was significantly negatively correlated with DG(16:1/18:2) and DG(34:0/18:3) and was significantly positively correlated with TG(18:2/14:2/18:2), PC(16:1/18:2), PC(18:1/18:1), PE(17:1/16:0) and Cer(t18:0/22:0) ($P < 0.01$). The 2-AP content was significantly positively correlated with TG(16:0/14:1/18:2), Cer(t18:0/24:0), Cer(t18:0/22:0) and TG(26:0/18:1/18:2). Additionally, it was significantly negatively correlated with DG(16:1/18:2), PG(28:0/18:1) and TG(17:0/18:2/18:2) ($P < 0.01$). StC and CFC were significantly positively correlated with TG(16:0/18:2/18:3) and TG(18:3/18:2/18:3) ($P < 0.01$). The CFC concentration was significantly negatively correlated with TG(12:1e/6:0/14:1), DG(14:0/18:2), DG(18:1/14:0) and TG(12:1e/6:0/14:0) ($P < 0.01$). StC was

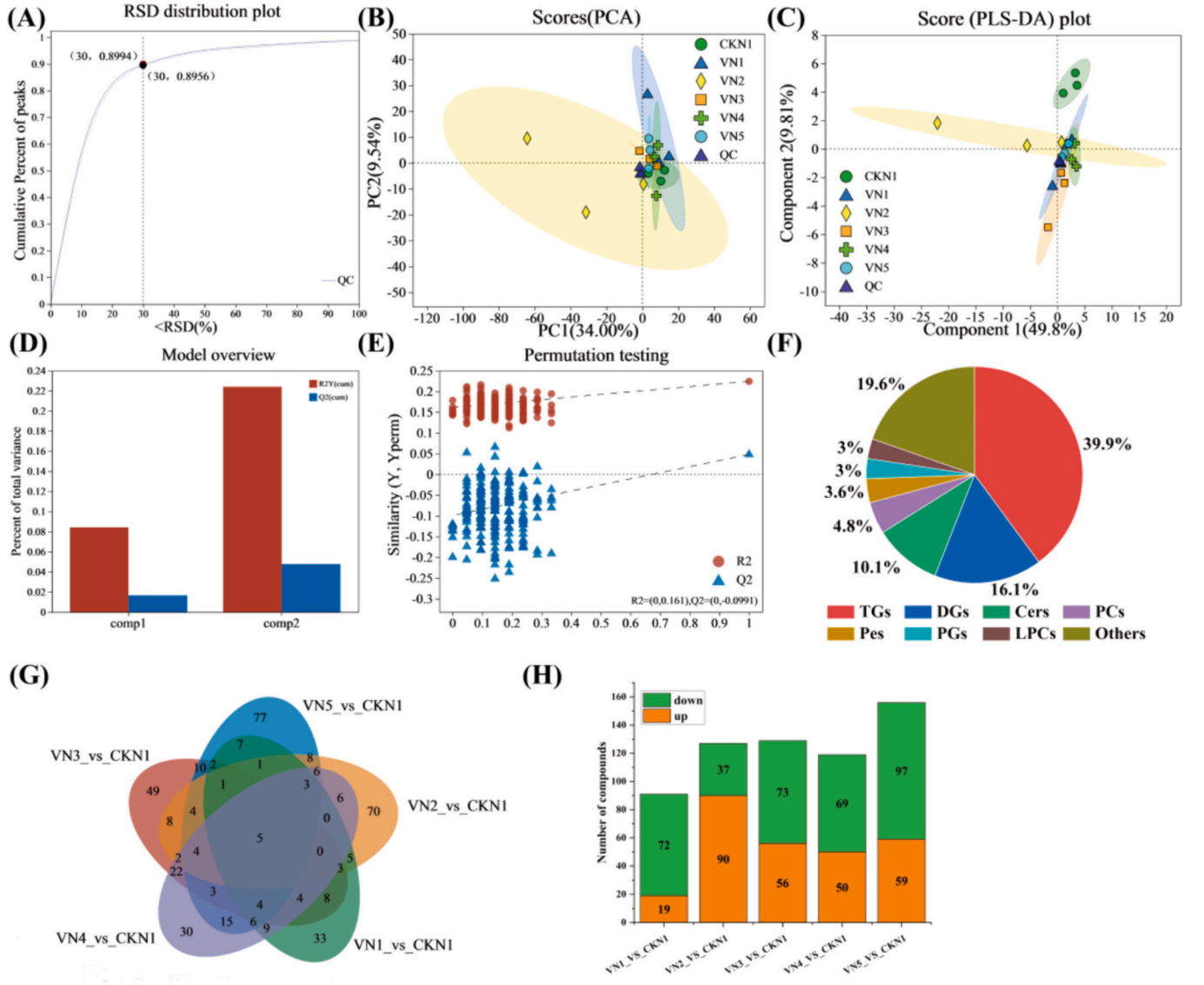

Fig. 1. Testing for permutations and multivariate statistical scroes among the strains and Metabolite analysis. (A) RSD distribution plot, (B) PCA, (C) PLS-DA, (D) Overview of the PLS-DA, (E) PLS-DA permulatation testing, (F) Statistical map of compounds, (G)Venn distribution of metabolites, (H)Comparison of different metabolites between strains and CKN1. RSD: relative standard deviation; PCA: principal component analysis; PC: principal component; PLS-DA: partial least squares discriminant analysis.

significantly negatively correlated with TG(26:0/18:1/18:2), TG(18:1/18:2/24:0), TG(26:0/16:0/18:1) and TG(26:0/18:1/18:1) ($P < 0.01$). DG(11:0/18:1) was significantly negatively correlated with processing quality and appearance quality ($P < 0.01$). Processing qualities showed significant negative correlations with DG(16:1/18:1), DG(16:1/18:2), DG(14:0/18:2), DG(18:2/18:2), DG(18:1/14:0) and DG(20:1/18:2) ($P < 0.01$). PC(16:0/16:0) was significantly positively correlated with SuC ($P < 0.01$). PC(16:0/14:0) was significantly positively correlated with BR and negatively correlated with springiness ($P < 0.01$). PC(20:1/18:2) was significantly positively correlated with SuC and BR ($P < 0.01$).

3.8. Candidate metabolic biomarkers

We analysed the variable importance in the projection (VIP) values of the identified metabolites and compared these metabolites with rice quality (Fig. 3, Fig. S1). PA(16:0/18:2), PC(15:0/16:0) and PG(16:0/16:0) can be used as potential metabolic biomarkers, and LPC(24:0), PA(18:0/18:2) and Cer(d18:1/18:1) can be used as secondary metabolites. We also analysed the detailed metabolic biomarkers, including the Metab ID, Formula, KEGG Compound ID, KEGG Pathway, Retention Time, HMDB Superclass, HMDB Class, HMDB Subclass, VIP_OPLS-DA, and VIP_PLS-DA. In addition, we mapped the metabolic pathways that regulate many key metabolites (Fig. 4).

3.9. ROC analysis and abundance value analysis

We analysed the screened potential metabolic biomarkers and secondary metabolites by receiver operating characteristic (ROC) curve analysis. The results showed that the potential biometabolites PA (16:0/18:2), PC (15:0/16:0) and PG (16:0/16:0) had an area under the curve (AUC) of 1.0000 in the five comparisons (Fig. S4), which indicated a sufficient diagnostic effect with high accuracy. However, the secondary metabolites LPC(24:0), PA(18:0/18:2) and Cer(d18:1/18:1) exhibited different effects (Fig. S5). In the VN1 and CKN1 comparison groups, the AUC of Cer(d18:1/18:1) was 0.6667, the specificity of LPC(24:0) was 0.33, and the AUC was 0.8889. The AUC values for LPC(24:0), PA(18:0/18:2) and Cer(d18:1/18:1) in the comparison between VN4 and CKN1

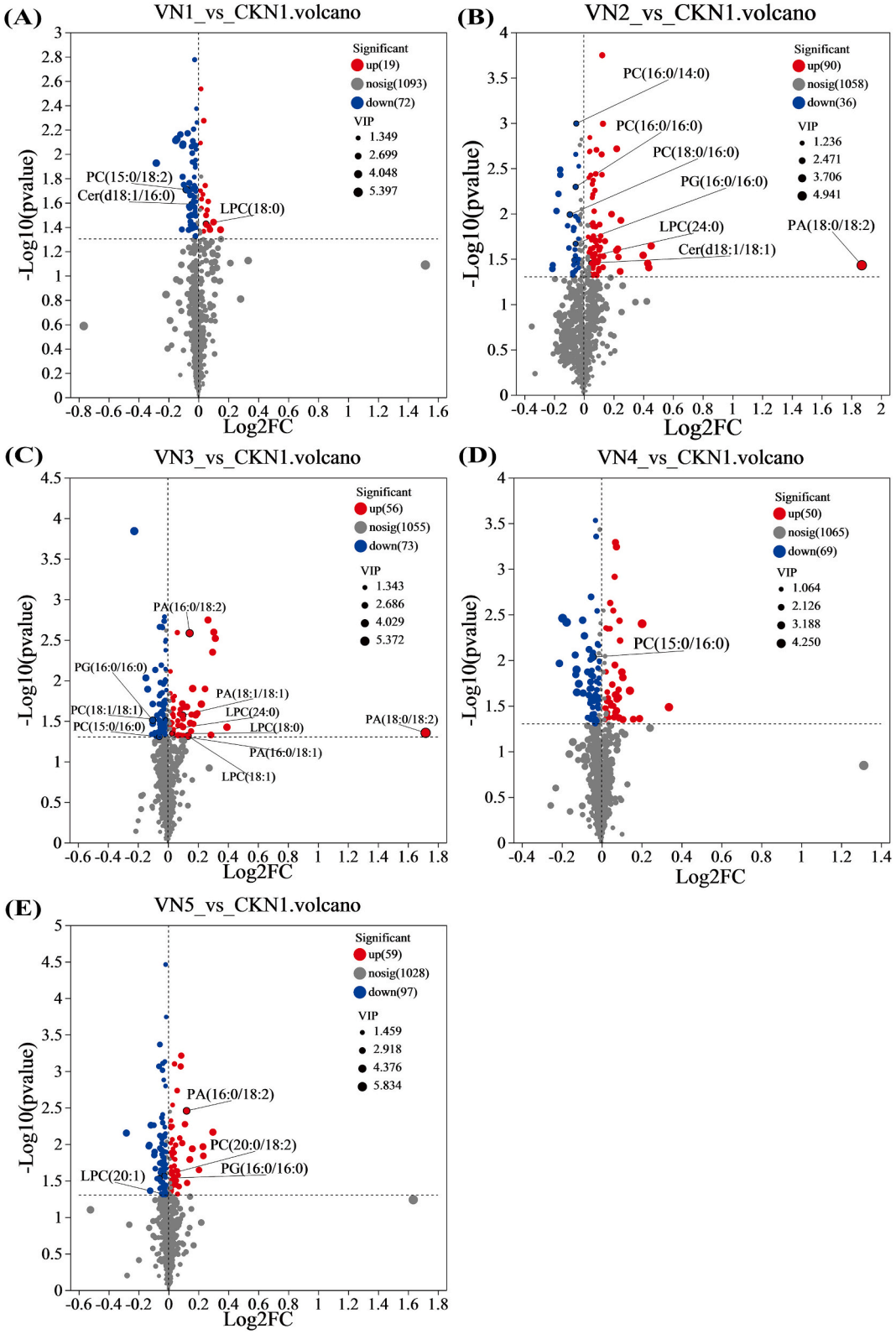

Fig. 2. DMs volcano map. All dots demonstrate specific metabolites, and the dot's size demonstrates the VIP value. On the left are the metabolites with different down-regulation, and on the right are the metabolites with different up-regulation; the greater the left and right are, the greater the significance of the above point. (A) VN1 and CKN1. (B) VN2 and CKN1. (C) VN3 and CKN1. (D) VN4 and CKN1. (E) VN5 and CKN1.

were lower than 0.9000. The AUC of LPC(24:0) was 0.6667 in the comparison between VN5 and CKN1. These findings suggested that these secondary metabolites are less accurate than the other metabolites and can be detected only by secondary assays.

Moreover, we analysed the abundance values of these metabolites (Fig. S6). The results revealed that the PA(16:0/18:2), PG(16:0/16:0), LPC(24:0), PA(18:0/18:2), and Cer(d18:1/18:1) of VN2 exhibited significant differences from those of CKN1. PC(15:0/16:0) was significantly

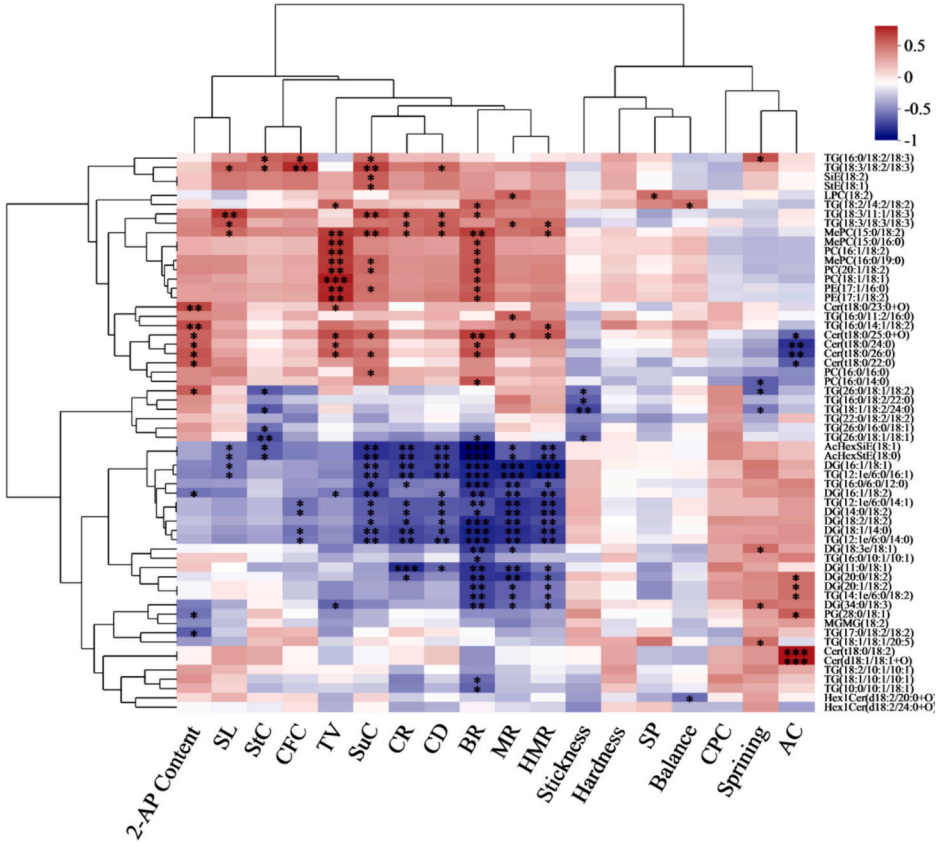

Fig. 3. Correlation analysis of rice quality with DMs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

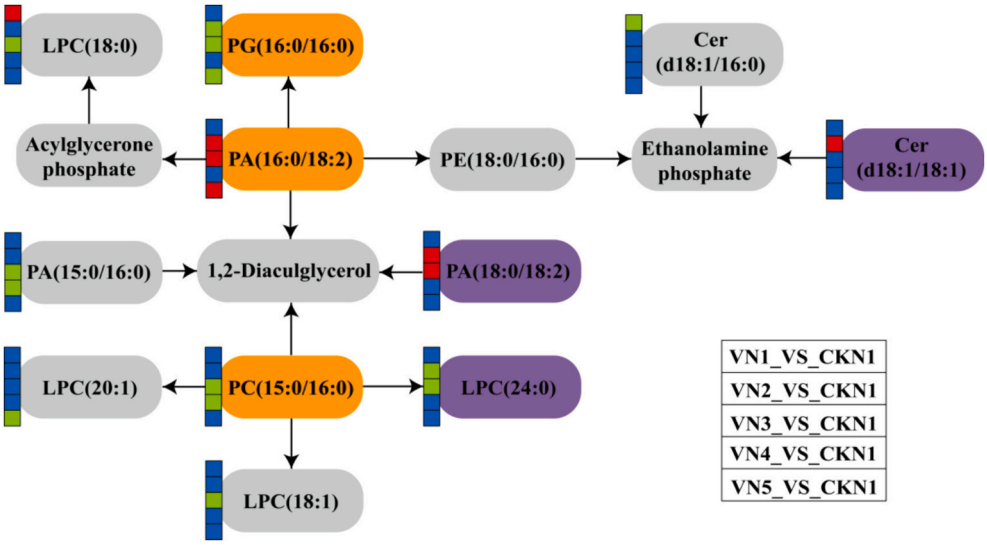

Fig. 4. An overview of a few key metabolites that play a critical role in various metabolic pathways. The orange rectangles represent crucial metabolites. The purple rectangles represent secondary metabolites. Red represents overexpression, green indicates underexpression, and blue indicates no significant variation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower than CKN1 in VN4. In VN5, PA(16:0/18:2) was significantly greater than that in CKN1, while PG(16:0/16:0) was significantly lower than that in CKN1. These findings indicate that the screened potential metabolites and secondary metabolites can better differentiate between the lines and CKN1.

4. Discussions

4.1. Differences in rice quality

With the improvement of living standards, people are pursuing higher rice quality. In this study, we analysed the differences in rice quality between five lines and CKN1 (Table 1) and found that the processing quality of all five lines was lower than that of CKN1; moreover,

the processing quality of VN2 was significantly inferior to that of CKN1, which indicated that the processing quality significantly deteriorated despite the similarity of the line structure in the field. These findings suggest that processing quality can be used as an important indicator of rice germplasm degradation. Although the lipid content in rice is very low, lipids play a very important role in the appearance and cooking quality of rice (Zhou, Robards, Helliwell, & Blanchard, 2002). It is worthwhile to study whether this variation exists in rice populations and is not easily observed in the field, therefore, new quality identification indices should be added for the breeding of high-quality rice in the future. Within a certain range, a higher fat content is associated with better grain integrity, oilier rice, higher gloss, and better rice palatability and aroma (Ahmed, Butt, Sharif, & Iqbal, 2016; Concepcion et al., 2020). Lipids also have an effect on the textural properties of rice (Bi, Zhang, Zhou, & Yang, 2018). A previous study revealed that crude fat content was negatively correlated with hardness and positively correlated with viscosity (Cameron & Wang, 2005). In this study, VN2 and VN4, which had significantly lower CFCs than did CKN1, had significantly better appearance quality than did CKN1. StC, SuC and SL were significantly lower than those in CKN1. However, no significant correlation was found between the CFC and textural characteristics (Table 2). This may be because although degradation occurred, the differences were not significant due to the close lineage between the lines of the same variety. Aroma, a key quality attribute in determining the market value of rice, has been emphasized by crop scientists in recent years for its application in breeding (Imran et al., 2023), and 2-AP has been reported to be the major molecule for aroma in aromatic rice varieties (Buttery, Ling, Juliano, & Turnbaugh, 2002; Starkenmann, Niclass, Vuichoud, Schweizer, & He, 2019). In the present study, the functional marker *fgr*-InDel-E2 did not differ between the lines and CKN1. However, the 2-AP content in CKN1 was 67.19 ng g^{-1}, which was significantly greater than that in the other 5 lines. Taken together, these findings indicated that during the years of planting and breeding in Nanjing 46, the amount of aroma substances also decreased with decreasing variety. Therefore, the 2-AP content can be used as an important index for studying the degradation of aroma rice germplasm.

4.2. Differences in medium and long chain fatty acids(MLFAs) between lines

MCFAs are an important class of functional chemicals that are characterized by rapid digestion and absorption and do not accumulate in fat; thus, they have great potential in the development and utilization of healthy foods (Kleerebezem & van Loosdrecht, 2007). In this study, through targeted metabolomics analysis of 51 MCFAs, we found that the major fatty acids present in the five lines and CK were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), which accounted for 90% of the total fatty acid content. Among these fatty acids, palmitic (C16:0) and linoleic acid (C18:2) were the most abundant. Similar results were illustrated in previously published studies based on high-performance liquid chromatography mass spectrometry (L. Liu et al., 2013). Moreover, only C11:0 showed variability between VN1 and CKN1, and VN2, VN3, VN4, and VN5 had very low levels of C11:0 (Table S10). C11:0 was significantly positively correlated with the 2-AP content and significantly negatively correlated with stickiness. This finding suggested that C11:0 can be used as a minor identification tool for germplasm degradation studies.

4.3. Differences in lipid metabolites between 5 lines and CKN1

Although the content of fat in rice is low, its structure and components, such as fatty acids and phospholipids, have important influences on taste quality, appearance quality and quality changes during storage (Z. K. Chen et al., 2021; Zhou et al., 2003). Previous studies have shown that the fat content of rice plants is approximately 0.3–3%, and these plants are mainly distributed in the germ, followed by the seed coat and the dextrinous layer, while less fat is present in the endosperm (Zhou et al., 2003). Researchers determined the fat content of 92 rice varieties and found that there was variation among the different varieties of rice and that there was a highly significant negative correlation between fat content and AC (Yu et al., 2007).When exploring the interaction between lipids and flavour substances, Li et al. (R. Li & Gao, 2017) suggested that lipids play an important role in the perception, stability, and formation of flavour substances. Lipid molecules can form polymers in various forms to provide stable storage space and class release of flavour substances, illustrating the close relationship between flavour substances and lipids. According to our preliminary study and the literature, PC, PE, FA, DG and Cer are the major non-starch lipids exhibiting biological effects in rice, while LPC, LPE and LPG are the major starch lipids in rice(D. Zhang, Zhao, Wang, Wang, Liu, Wang, et al., 2022). Among them, PA, LPG, various lysophospholipids, sphingolipids and fatty acids are important signalling lipids involved in various abiotic stress responses in plants. (Hou, Ufer, & Bartels, 2016). Sphingolipids exert various biological functions by regulating signalling and metabolic pathways, and Cer, a sphingolipid, is degraded to a low degree during storage, and its biological function is not impaired(D. Zhang et al., 2022). PG is an important phospholipid component of higher plant cystoid membranes, and is involved in PSII light and electron transport, and the formation of light-trapping complexes, etc., is essential for cystoid membrane development(Babiychuk et al., 2003; Sakurai, Mizusawa, Ohashi, Kobayashi, & Wada, 2007).The major lipids included TGs, DGs, Cers, and PCs, totalling 38 differential lipid subclasses and 405 lipid molecules (Table S2). Correlation analysis of differential lipid metabolites with rice quality by comparing the lines with CKN1 (Fig. 3) revealed that Cer(d18:1/18:1) was significantly positively correlated with AC and significantly negatively correlated with TV. PC(18:1/18:1) was significantly positively correlated with BR. Therefore, the metabolites PA(16:0/18:2), PC(15:0/16:0) and PG(16:0/16:0) can be used as potential metabolic biomarkers. The metabolites LPC(24:0), PA(18:0/18:2) and Cer(d18:1/18:1) can be secondary metabolites; in this way, we constructed metabolic pathways for key lipid metabolites (Fig. 4). We also performed ROC analysis and abundance value analysis of these metabolites (Fig. S4; Fig. S5; Fig. S6). The results also showed that the AUC of the screened potential metabolic biomarkers was 1.0000, indicating that the diagnostic effects were highly accurate. This approach can provide a new method and theoretical basis for the study of plant variety degradation and identification of seed purity.

5. Conclusion

In this study, although all five lines were from Liyang, their quality changed to different degrees. VN2 had the worst processing quality, with BR, MR, and HMR 3.46, 4.10, and 10.69% lower than that of CKN1, respectively. The ACs of VN1 and VN4 were significantly lower than that of CKN1 by 10.67 and 13.03%, respectively. The CPCs of VN2 and VN4 were significantly lower than that of CKN1 by 5.36 and 8.81%, respectively. The CFC of VN4 was the lowest at 0.97%, which was 31.69% lower than that of CKN1. The TV of VN2 was significantly lower than that of CKN1. Although all 5 lines exhibited an amplified target band of 100 bp, there were differences in the main fragrance substance 2-AP, which was lower than that of CKN1 in all the other lines. C11:0 was detected only in CKN1 and VN1, and its content was significantly greater than that of the remaining four lines. Most of the DAMs were enriched in glycerophospholipid metabolism, sphingolipid metabolism and glycerolipid metabolism, whereas the key metabolites in the metabolic pathway were predominantly phospholipids, which included PA(16:0/18:2), PC(15:0/16:0), PG(16:0/16:0), LPC(24:0), PA(18:0/18:2), and Cer(d18:1/18:1). These metabolites play important regulatory roles in metabolic pathways. This study provides new insights into the degradation of high-quality rice germplasm. The identification of these key metabolites can aid in the development of potential biomarkers to screen

for varietal degradation that occurs during long-term cultivation and provide a basis for seed purity characterization. However, the combined contribution of specific secondary metabolites to the study of rice germplasm degradation needs to be further investigated.

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CRediT authorship contribution statement

Qiang Shi: Writing – original draft, Investigation, Data curation. **Wenjie Lu:** Investigation, Formal analysis, Data curation. **Runnan Wang:** Visualization, Investigation. **Jinlong Hu:** Writing – review & editing, Methodology, Conceptualization. **Jinyan Zhu:** Writing – review & editing, Methodology, Conceptualization. **Hongcheng Zhang:** Writing – review & editing, Methodology, Conceptualization. **Nianbin Zhou:** Writing – review & editing. **Qiangqiang Xiong:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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