Unveiling the Impact of Different Nitrogen Fertilizer Levels on Rice’s Eating Quality through Metabolite Evaluation

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Abstract: We investigated the variations in metabolites associated with the quality of rice consumption when exposed to varying nitrogen fertilizer levels, as well as the regulatory role of pivotal metabolites within metabolic pathways. This research employed Hongyang 5 as the subject of experimentation, examining the metabolites of Hongyang 5 at three different nitrogen levels using non-targeted metabonomic analysis. The findings indicated that the overall assessment of the eating quality/palatability (CEQ) and amylose contents (AC) of Low nitrogen (D1: 180 kg·ha⁻¹) was notably greater than that of Medium nitrogen (D2: 270 kg·ha⁻¹) and High nitrogen (D3: 315 kg·ha⁻¹). Conversely, the amylopectin (APC), total starch (SC), and protein contents (AP) of D1 were remarkably lower than those observed in D2 and D3. The starch debranching enzyme (DBE) and granule-bound starch synthetase (GBSS) of D1 were remarkably higher than those of D2 and D3. The soluble starch synthase (SSS) of D1 was the lowest. The ADP-glucose pyro-phosphorylase (AGP) and starch branching enzyme (SBE) of D3 were remarkably higher than that of D1 and D2. We identified 76 differential metabolites (DMs) between D1 and D2 (20 up-regulated and 56 down-regulated). A total of 88 DMs were identified between D3 and D1 (42 up-regulated and 46 down-regulated). A total of 57 DMs were identified between D3 and D2. Most of the DMs related to rice-eating quality were involved in the lipid metabolic pathway and amino acid metabolic pathway. The essential metabolites within the metabolic pathway are classified as lipid metabolites and are (13(S)-hydroperoxy-linolenic acid, PGB2, 3-phosphocholine, 7-epijasmonic acid, 20-carboxyleukotriene B4 and 11-dehydro-thromboxane B2), amino acid metabolites (4-guanidinobutanoic acid, (3R, 5S)-1-pyrroline-3-hydroxy-5-carboxylic acid, citric acid, (S)-2-Acetolactate, L-glutamine, L-2, 4-aminobutyric acid and putrescine). These key metabolites may be affected by nitrogen fertilizer conditions and play critical regulatory roles in the metabolic pathway, resulting in differences in rice eating quality.

Keywords: rice eating quality; nitrogen fertilizer; metabolomics; metabolic pathway

1. Introduction

Rice (Oryza sativa L.) is a prominent global food crop and serves as a fundamental dietary staple for roughly half of the world’s population [1,2]. In recent years, with the improvement of rice yield and people’s living standards, the market demand for high-quality rice with good eating quality is growing rapidly [3,4]. Starch, comprising over 80% of the total composition, is the main constituent of rice and has a vital role in determining its quality [5,6]. Protein constitutes the second largest component of rice, comprising 8–10% of the dry weight of brown rice. The presence of protein in rice not only serves as a crucial indicator of its nutritional quality but also significantly influences its palatability. In addition, there are trace storage substances such as fatty acids, vitamins, and mineral
elements in rice [7]. The quality or palatability of rice is influenced by both genetic traits and environmental factors and cultivation practices [8,9]. Extensive research worldwide has been carried out to examine the effects of nitrogen fertilizer application on both the yield and nutritional quality of rice [10,11]. Nevertheless, limited research investigating the enhancement of rice-eating quality has been conducted when nitrogen fertilizer is integrated with metabolomics techniques.

Metabolomics is a prominent field within the realm of “omics” sciences. It focuses on the analysis of biologically synthesized or degraded compounds in organisms, aiming to unveil the biological functions of organisms from a metabolic standpoint and provide insights into various life phenomena [12,13]. As omics technology continues to advance and evolve, metabolomics is assuming an overwhelmingly significant contribution in the analysis and detection of the nutritional quality of crops. It facilitates comprehensive investigations into the metabolites present in crops, thereby facilitating a deeper understanding of their nutritional profiles [14]. Metabolomics, at a biological level, can identify variations in small molecular metabolites within crop growth organs and quantitatively measure their content, thus providing valuable insights into the metabolic composition of crops [15]. Nitrogen is an important nutrient element of rice, which plays a considerable role in the regulation of the quality of rice, so many studies have been carried out on the effects of nitrogen fertilizer on rice quality. Starch synthesis, accumulation, and metabolism are mainly related to the activities of starch synthase (SS, EC 2.4.1.21), sucrose synthase (SuSy, EC 2.4.1.13), ADPG-pyrophosphatase (AGPase, EC 2.7.7.27), starch debranching enzyme (DBE, EC 3.2.1.68) and starch branching enzyme (SBE, EC 2.4.1.18) [16]. In the early filling stage, the application of high nitrogen treatment substantially enhanced the rate at which amylose accumulated. Moreover, a remarkable positive correlation was observed between amylose accumulation and the activity of Granule-Bound Starch Synthase (GBSS, EC 2.4.1.21) [17]. A higher nitrogen application rate significantly decreased the activities of AGPase and SBE in rice leaf sheath at the heading stage [18]. There was a similar trend in rice grains. Xiong et al. conducted a study investigating the variations in metabolites associated with rice quality formation under various nitrogen fertilizers and densities of plantations [19]. The analysis revealed that the identified differential metabolites were primarily enhanced in pathways linked to lipid metabolism and amino acid metabolism [19].

Previously, numerous works on the impact of nitrogen fertilizer on the eating quality of rice have been conducted; however, there has been a dearth of research exploring the key metabolites and metabolic pathways influenced by nitrogen fertilizer that affect rice eating quality. To investigate the disparity in metabolites of Hongyang 5 rice variety under varying nitrogen application rates and its association with rice eating quality, three distinct treatment schemes were established: high nitrogen, medium nitrogen, and low nitrogen. By comparing the differences in metabolites among different treatments, the key differential metabolic pathways were screened to further clarify the formation processes involved in rice eating quality. In this research, untargeted metabolomics technology has been employed to identify and characterize metabolites present in milled rice, aiming to identify potential markers that can be utilized for the subsequent enhancement of the quality of rice taste.

2. Materials and Methods

2.1. Growth Conditions Employed and Plant Materials Used

Oryza sativa L. ssp. japonica (Hongyang 5) variety cultivation was carried out at the Yangzhou University farm in Yangzhou City, Jiangsu Province (32°39′ N, 119°42′ E) during the growing season of rice in 2022. The type of soil was identified as sandy loam, with nitrogen availability of 1.5 g kg⁻¹, phosphorus availability of 36.6 mg kg⁻¹, and potassium availability of 90.8 mg kg⁻¹. The rice was sown on 25 May 2022, using blanket seedlings of age 25 days. Transplantation of seedlings of rice was conducted at the one-heart and three-leaf stage, where four seedlings were planted per hole. We implemented three treatments:
low nitrogen (D1: 180 kg·ha$^{-1}$), medium nitrogen (D2: 270 kg·ha$^{-1}$), and high nitrogen (D3: 315 kg·ha$^{-1}$). The hill spacing was set at 28 cm × 12 cm, accommodating four seedlings at each hill. Each treatment was repeated three times, and the area of the plot measured, 15 m$^2$ (5 m × 3 m).

To achieve isolation between sub-districts within the field, ridges were employed and covered with a plastic film ensuring separate irrigation and drainage. Disease, insect pest, and weed control were implemented as per the protocol of the conventional process of cultivating rice with high yields. In three splits, nitrogen was applied: as 35% basal fertilizer, 35% at the tillering initiation, and 30% at the panicle initiation, using urea (46.4% N) as the nitrogen source. Each plot received a basal fertilizer application of calcium superphosphate (P$_2$O$_5$ content: 12%) at a rate of 135 kg P$_2$O$_5$ ha$^{-1}$ and potassium chloride (K$_2$O content: 60%) at a rate of 135 kg K$_2$O ha$^{-1}$, both at the panicle initiation stage as well. The experimental field was flooded post-transplant and remained flooded until 7 days before maturity. To prevent losses in the quality of rice and its yield, intensive control measures were undertaken to manage insects, diseases, and weeds using chemical methods.

2.2. Rice Eating Quality Analysis

The assessment of total starch contents (SC) in rice flour was conducted using kits commercially manufactured by Sigma-Aldrich (St. Louis, MO, USA) on the basis of the method by Zhu et al. [20]. Amylopectin contents (APC) and amylose contents (AC) were evaluated following the protocol outlined by Wang [21]. The content of amylopectin was quantified at 556 nm and 737 nm whereas the quantification of amylose content was conducted at 620 nm and 479 nm. The milled rice N content was determined with the aid of Kjleco™ 8400 equipment (Infratec 1241, Hillerød, Denmark), and the content of protein (AP) was calculated by multiplying 5.95 by the N content [22].

Eating quality was evaluated with a cooked rice taste analyzer (STA1, Satake, Hiroshima, Japan) that converts various physicochemical indexes of the rice samples into “taste” scores, based on known correlations previously established between the near-infrared reflectance measurements of the key constituents and preference sensory scores. The appearance (0–10), hardness (0–10), viscosity (0–10), degree of balance (0–10), and the comprehensive evaluation of eating quality (CEQ) (0–100) of the rice were also obtained [23].

The reflected light of rice was measured using reflected wavelengths of 540 nm (R1) and 970 nm (R2). The transmittance of rice was measured using transmission wavelengths of 540 nm (T1) and 640 nm (T2). The absorbance A of reflected or transmitted light of $\lambda_i$ is obtained from the following equation [24]:

$$A(\lambda_i) = K(\lambda_i) \times \left[1/I(\lambda_i)/I(0)\right];$$

Among these, $A(\lambda_i)$ is the absorbance of the sample; $K(\lambda_i)$ is the correction coefficient; $I(\lambda_i)$ is the sensor output for the sample; $I(0)$ is the sensor output for the reference board.

Using the absorbance A of the four wavelengths (R1, R2, T1, T2) of the sample as the independent variable and the sensory comprehensive score as the dependent variable, a multiple regression analysis was conducted to obtain a multiple regression equation for the sensory comprehensive score based on the absorbance of the four wavelengths [25]:

$$C = f_0 + f_1 \times A(R1) + f_2 \times A(R2) + f_3 \times A(T1) + f_4 \times A(T2);$$

Among these, C is the comprehensive evaluation of eating quality; $f_0$ is the parameter of the regression equation; $f_1$–$f_4$ are the regression coefficients of each wavelength in the regression equation; A is the absorbance of each wavelength.

2.3. Extraction and Assay of the Enzyme

Ten grains were manually homogenized by grinding them at 4 °C using 5 mL of extraction buffer (100 mmol/L Tricine-NaOH, 8 mmol/L MgCl$_2$, pH 7.5, 2 mmol/L EDTA, 12.5% glycerol, 50 mmol/L β-mercaptoethanol, and 1% PVP-40). Post centrifugation at
10,000× g and at 4 °C for 10 min, the generated supernatant was utilized for enzyme activity determination. The activities of Granule-Bound Starch Synthase (GBSS, EC 2.4.1.21), soluble starch synthase (SSS, EC 2.4.1.21), and ADPG-pyrophosphatase (AGPase, EC 2.7.7.27) were assessed with slight modifications in accordance with the approach described by Yang et al. [26]. Similarly, the analysis of starch debranching enzyme (DBE, EC 3.2.1.68) and starch branching enzyme (SBE, EC 2.4.1.18) activities was conducted based on the methodology proposed by Yang et al. [26].

2.4. Sample Metabolite Extraction

For the metabolite extraction and data pre-processing, we followed the method described by Xiong et al. (2022). Accurately weighed head-milled rice samples (50 mg) were transferred to centrifuge tubes of 2 mL volume, and a bead of 6 mm diameter was added to each tube. Then, to each centrifuge tube, a 400 µL of extract (methanol: water = 4:1 (v:v)) containing a 0.02 mg mL⁻¹ internal standard (L-2-chlorophenyl alanine) was added. The samples were subjected to grinding in a frozen tissue grinder for 6 min at −10 °C and 50 Hz. Low-temperature ultrasonic extraction was then performed for 30 min at 5 °C and 40 KHz. After being held for 30 min at −20 °C, for 15 min the samples were centrifuged at a rate of 13,000× g and a temperature of 4 °C. For subsequent analysis, the supernatant was transferred to sample vials having inner cannulas. In addition, from each sample 20 µL of the supernatant was pooled to create a sample as a quality control (QC).

2.5. LC-MS/MS Analysis

Chromatographically, the metabolites were separated using a Thermo UHPLC system equipped with an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters, Milford, MA, USA). The chromatographic conditions involved mobile phase A (95% water and 5% acetonitrile with 0.1% formic acid) and mobile phase B (47.5% acetonitrile, 47.5% isopropanol, and 5% water with 0.1% formic acid). The injection volume was 2 µL, and the temperature of the column was set at 40 °C. Using a Thermo UHPLC-Q Exactive HF-X Mass Spectrometer having an electrospray ionization (ESI) source operating in positive or negative ion mode, the mass spectrometric data were collected. The optimum conditions followed were as follows: capillary temperature, 325 °C; heater temperature, 425 °C; sheath gas flow rate, aux gas flow rate, 50 arb; 13 arb; normalized collision energy, 20–40–60 V rolling for MS/MS; ion-spray voltage floating (ISVF), −3500 V in negative mode and 3500 V in positive mode. MS/MS resolution was set to 7500, and Full MS resolution was set to 60,000. Data acquisition was conducted utilizing the data-dependent acquisition (DDA) mode, with a detection range of 70–1050 m/z.

After UPLC-MS analyses, the raw data were imported into the Progenesis QI 2.3 (Nonlinear Dynamics, Waters, Milford, MA, USA) for peak detection and alignment. The preprocessing results generated a data matrix that consisted of the retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. Metabolic features detected at least 80% in any set of samples were retained. After filtering, minimum metabolite values were imputed for specific samples in which the metabolite levels fell below the lower limit of quantitation and all Metabolic features were normalized by sum. The internal standard was used for data QC (reproducibility), and Metabolic features for which the relative standard deviation (RSD) of QC > 30% were discarded. Following normalization procedures and imputation, statistical analysis was performed on log transformed data to identify significant differences in metabolite levels between comparable groups. Mass spectra of these metabolic features were identified by using the accurate mass, MS/MS fragments spectra and isotope ratio difference by searching in reliable biochemical databases, such as the Human metabolome database (HMDB) (http://www.hmdb.ca/ (accessed on 11 December 2022)) and Metlin database (https://metlin.scripps.edu/ (accessed on 13 December 2022)). Concretely, the mass tolerance between the measured m/z values and the exact mass of the components of interest was ±10 ppm. For metabolites having MS/MS
confirmation, only those with MS/MS fragments score above 30 were considered as confidently identified. Otherwise, metabolites had only tentative assignments.

2.6. Differential Metabolites and Statistical Analysis

The identification of DMs were done based on the criteria of variable importance for projection (VIP) that were greater than 1 and less than 0.05 $p$ values. Utilizing the ropls software (Version 1.6.2) within the R package, multivariate statistical analysis was conducted. The DMs between the two groups were mapped and summarized onto biochemical pathways via metabolic enrichment and pathway analysis using a database search, KEGG. These metabolites were categorized based on their involvement in specific pathways or their functional roles. Enrichment analysis focused on examining whether a metabolite group appeared in a particular function node. The annotation analysis initially focused on individual metabolites and then extended to a group of metabolites. Scipy.stats (Python packages, Version1.0.0) (https://docs.scipy.org/doc/scipy/ (accessed on 15 December 2022)) was utilized to identify statistically significantly enriched pathways by means of Fisher’s exact test.

2.7. Data Analysis

Data analysis involved sorting and calculating the average values using WPS 2021 software. SPSS 18.0 statistical software was utilized for the variance analysis of data on rice quality, and to generate consolidated figures, Adobe Illustrator CS6 software was employed.

3. Results

3.1. Effects of Nitrogen Fertilizer on Rice Eating Quality

The eating quality of Hongyang 5 was significantly different under different nitrogen fertilizer treatments (Table 1). The comprehensive evaluation of eating quality (CEQ) under D1 was the highest, which was 6.14% and 12.92% higher than that under D2 and D3 respectively. This may be related to the lower hardness and higher viscosity degree of balance of rice under D1 treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Appearance</th>
<th>Hardness</th>
<th>Viscosity</th>
<th>Degree of Balance</th>
<th>CEQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>8.4 a</td>
<td>5.4 b</td>
<td>8.3 a</td>
<td>8.5 b</td>
<td>81.3 a</td>
</tr>
<tr>
<td>D2</td>
<td>7.6 b</td>
<td>6.3 a</td>
<td>7.6 b</td>
<td>7.5 b</td>
<td>76.6 b</td>
</tr>
<tr>
<td>D3</td>
<td>7.5 b</td>
<td>6.2 a</td>
<td>7.0 c</td>
<td>6.8 c</td>
<td>72.0 c</td>
</tr>
</tbody>
</table>

1 Note: Within a column, values followed by different lowercase letters are significantly different at the $p = 0.05$ level. The same applies to the subsequent tables; D1: low nitrogen (180 kg·ha$^{-1}$); D2: medium nitrogen (270 kg·ha$^{-1}$); D3: high nitrogen (315 kg·ha$^{-1}$); CEQ: comprehensive evaluation of eating quality.

3.2. Rice Components Content and Key Enzyme Activity in Starch Synthesis

In this study, the differences in starch components in rice under three nitrogen fertilizer levels were analyzed (Table 2). The amylose content (AC) in D1 exhibited significant increases of 2.81% and 7.17% compared to D2 and D3, respectively. Similarly, the amylopectin content (APC) in D1 showed significant decreases of 2.01% and 3.24% compared to D2 and D3, respectively. Additionally, the total starch content (SC) in D1 demonstrated significant decreases of 0.68% and 2.08% compared to D2 and D3, respectively. Notably, as the nitrogen application rate increased, the trend of rice protein content mirrored that of the total starch content, displaying a consistent upward trend. The data of rice eating quality, rice component content and enzymatic activities under three nitrogen treatments were evaluated using principal component analysis (PCA). In the PCA of enzyme activities under nitrogen treatments, the first two principal components (PC1 and PC2)
collectively explained 90.229% and 7.60% of the variance in the data, respectively (Figure 1A). In the PCA of rice eating quality under nitrogen treatments, the first two principal components (PC1 and PC2) collectively explained 95.089% and 4.012% of the variance in the data, respectively (Figure 1B). In the PCA of rice component content under nitrogen treatments, the first two principal components (PC1 and PC2) collectively explained 95.711% and 3.566% of the variance in the data, respectively (Figure 1C).

Table 2. Differences of rice component content and key enzyme activities in rice under three kinds of nitrogen fertilizer levels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AC (%)</th>
<th>APC (%)</th>
<th>SC (%)</th>
<th>AP (%)</th>
<th>AGP (nmol min$^{-1}$ mg protein$^{-1}$)</th>
<th>GBSS (nmol min$^{-1}$ mg protein$^{-1}$)</th>
<th>SSS (nmol min$^{-1}$ mg protein$^{-1}$)</th>
<th>SBE (U mg protein$^{-1}$)</th>
<th>DBE (mg min$^{-1}$ mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>13.16 a</td>
<td>70.08 c</td>
<td>83.83 c</td>
<td>7.31 c</td>
<td>25.94 c</td>
<td>14.09 a</td>
<td>7.63 b</td>
<td>3.76 b</td>
<td>0.53 a</td>
</tr>
<tr>
<td>D2</td>
<td>12.80 b</td>
<td>71.52 b</td>
<td>84.40 b</td>
<td>7.81 b</td>
<td>29.60 b</td>
<td>12.59 b</td>
<td>7.86 ab</td>
<td>3.78 b</td>
<td>0.48 b</td>
</tr>
<tr>
<td>D3</td>
<td>12.28 c</td>
<td>72.43 a</td>
<td>85.61 a</td>
<td>8.11 a</td>
<td>31.50 a</td>
<td>10.07 c</td>
<td>8.95 a</td>
<td>4.34 a</td>
<td>0.41 c</td>
</tr>
</tbody>
</table>

1 Values followed by different lowercase letters within a column are significantly different at the p = 0.05 level. AC: amylose content; APC: amylopectin content; SC: total starch content; AP: protein content; AGP: ADP-glucose pyro-phosphorylase; GBSS: granule-bound starch synthetase; SSS: soluble starch synthase; SBE: starch branching enzyme; DBE: starch debranching enzyme.

Figure 1. The PCA of rice quality and enzyme activities. (A) PCA of enzyme activities under nitrogen treatments; (B) PCA of rice eating quality under nitrogen treatments; (C) PCA of rice component content under nitrogen treatments. The ellipses of different colors indicate that the “real” samples of this group are distributed in this region with 95% confidence; Exceeding this area indicates that the sample may be abnormal.

The levels of granule-bound starch synthetase (GBSS) and starch debranching enzyme (DBE) in D1 were significantly higher compared to D2 and D3. No substantial difference was observed between D2 and D3 in terms of soluble starch synthase (SSS), but its activity was significantly higher than that of D1. Starch branching enzyme (SBE) and ADP-glucose pyro-phosphorylase (AGP) exhibited the highest levels in D3, which were significantly higher than those in D1 and D2. The correlation analysis of the quality index (Table 3) revealed significant positive correlations between the comprehensive evaluation of eating quality (CEQ) and AC, GBSS, and DBE. Additionally, significant negative correlations were observed between CEQ and APC, SC, and AP. AC exhibited significant positive correlations with GBSS and DBE. APC and SC exhibited significant positive correlations with AGP, SSS, and SBE. Conversely, AC showed significant negative correlations with AGP, SSS, and SBE. APC and SC were significantly negatively correlated with GBSS and DBE.

Table 3. Correlation analysis of eating quality indexes of rice.

<table>
<thead>
<tr>
<th>Character</th>
<th>AC</th>
<th>APC</th>
<th>SC</th>
<th>AP</th>
<th>AGP</th>
<th>GBSS</th>
<th>SSS</th>
<th>SBE</th>
<th>DBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>-0.928 **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>-0.974 **</td>
<td>0.916 **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
AP $-0.904 \times 0.960 \times 0.914 \times$
AGP $-0.848 \times 0.933 \times 0.867 \times 0.876 \times$
GBSS $0.919 \times -0.915 \times -0.927 \times -0.927 \times -0.830 \times$
SSS $-0.773 \times 0.693 \times 0.860 \times 0.784 \times -0.763 \times$
SBE $-0.860 \times 0.753 \times 0.894 \times 0.683 \times 0.755 \times -0.823 \times 0.752 \times$
DBE $0.848 \times -0.920 \times -0.901 \times -0.926 \times -0.889 \times 0.860 \times -0.817 \times -0.778 \times$
CEQ $0.942 \times -0.888 \times -0.914 \times -0.886 \times -0.897 \times 0.887 \times -0.743 \times -0.787 \times 0.807 \times$

1 The * indicate statistical significance at the $p = 0.05$ level, whereas ** Significant at the $p = 0.01$ level.

3.3. Multivariate Statistical and Metabolic Profiling

The data of metabolites under three nitrogen treatments were evaluated using principal component analysis (PCA). In the PCA score chart, the first two principal components (PC1 and PC2) collectively explained 65.70% and 9.08% of the variance in the data, respectively (Figure 2A). The results of the partial least squares discriminant analysis (PLS-DA) indicated that Component 1 and Component 2 explained 76.9% and 4.09% of the variance, respectively (Figure 2B). We identified 76 DMs between D1 and D2 (20 up-regulated and 56 down-regulated) (Figure 2C; Table S1). We show the names of selected HMDB levels (Superclass) and the percentage of metabolites in order of the number of metabolites, from highest to lowest. A different color in each pie chart represents a different HMDB classification, and its area represents the relative proportion of metabolites in that classification (Figure 3). Lipids and lipid-like accounted for 22.54% of the analyzed metabolites, while organic acids and derivatives accounted for 18.31%. Organoheterocyclic compounds accounted for 15.49%. Phenylpropanoids and polyketides accounted for 12.68%. Benzenoids accounted for 11.27%. Organic oxygen compounds accounted for 8.46%. Organic nitrogen compounds accounted for 5.63%. Nucleotides, nucleosides, and analogs accounted for 4.23% (Figure 3A). 88 DMs were identified between D3 and D1 (42 up-regulated and 46 down-regulated) (Figure 2C; Table S2). Lipids and lipid-like molecules accounted for 32.14% of the analyzed metabolites. Organo-heterocyclic compounds accounted for 14.29%. Organic acids and derivatives accounted for 13.10%. Phenylpropanoids and polyketides accounted for 13.10%. Benzenoids accounted for 9.52%. Organic oxygen compounds accounted for 4.76% of the analyzed metabolites, while organic nitrogen compounds accounted for 2.38% of the total metabolites. Nucleotides, nucleosides, and analogs accounted for 1.19%. Alkaloids and derivatives accounted for 2.38% (Figure 3B). 57 DMs were identified between D3 and D2 (38 up-regulated and 19 down-regulated) (Figure 2C; Table S3). Lipids and lipid-like molecules accounted for 42.31% of the analyzed metabolites. Organo-heterocyclic compounds accounted for 7.69%. Organic acids and derivatives accounted for 7.69% of the analyzed metabolites. Phenylpropanoids and polyketides accounted for 11.54%. Benzenoids accounted for 13.46%. Organic oxygen compounds accounted for 9.62% of the analyzed metabolites. Organic nitrogen compounds accounted for 1.92%. Nucleotides, nucleosides, and analogs, as well as alkaloids and derivatives, each accounted for 1.92% of the metabolites. Hydrocarbon derivatives also accounted for 1.92% (Figure 3C). The comparison among the three treatments revealed a total of 548 common metabolites, indicating similarities in their metabolic profiles (Figure 2D). Each treatment also exhibited its own unique set of metabolites, highlighting the distinct metabolic characteristics of each treatment. Furthermore, each comparison metabolite group was visualized using a volcano plot, providing a comprehensive view of the differential metabolites and their statistical significance (Figure 4A–C).
Figure 2. Exploration of multivariate statistical analysis and differential metabolites under three nitrogen fertilizer treatments. (A) PCA score plot illustrating the distribution of samples as per their metabolic profiles. The ellipses of different colors indicate that the "real" samples of this group are distributed in this region with 95% confidence; Exceeding this area indicates that the sample may be abnormal. (B) PLS-DA score plot demonstrating the discrimination between different treatments. In the figure, the greater the degree of separation between the two groups of samples, the smaller the overlap of ellipses of different colors, indicating that the classification effect is more significant. (C) DMs up-regulated and down-regulated in different comparison groups. (D) Venn distribution map depicting the overlapping and unique metabolites among the treatments. Different colors represent different groups, overlapping numbers represent the number of metabolites common to multiple groups, and non-overlapping numbers represent the number of metabolites unique to the corresponding group.

3.4. KEGG Pathway

The metabolic processes in plants give rise to intricate pathways and interconnected networks involving various molecules, ultimately resulting in comprehensive alterations within the metabolome. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, we discovered that the DMs in the comparison groups D1_vs_D2 were associated with various metabolic pathways. These pathways include arginine biosynthesis, leucine, and isoleucine biosynthesis, valine, linoleic acid metabolism, glycerophospholipid metabolism, glycerolipids metabolism, serine, glycine, and threonine metabolism, tyrosine metabolism, alpha-linolenic acid metabolism, arachidonic acid metabolism, aspartate, alanine, and glutamate metabolism, as well as proline and arginine metabolism (Table S4). Regarding the D3_vs_D1 comparison groups, the DMs are involved in various metabolic pathways. These pathways include the biosynthesis of unsaturated fatty acids, linoleic acid, glycerophospholipid metabolism, alpha-linolenic acid metabolism, tryptophan metabolism, and arachidonic acid metabolism (Table S4). Regarding the D3_vs_D2 comparison groups, the DMs are involved in several metabolic pathways. These pathways include valine, leucine, and isoleucine biosynthesis, glycerophospholipid metabolism, glycerolipids metabolism, arachidonic acid metabolism, alpha-linolenic acid metabolism, arginine, and proline metabolism, as well as the biosynthesis of unsaturated fatty acids (Table S4).
Figure 3. Statistical map of compounds based on HMDB hierarchy (Class). (A) Comparison between D1 and D2 treatments showing the metabolites organized by class, listed in descending sequence of abundance. (B) Comparison between D3 and D1 treatments displaying the metabolites categorized by class, listed in descending sequence of abundance. (C) Comparison between D3 and D2 treatments indicating the metabolites grouped by class, listed in descending sequence of abundance.
Figure 4. Volcano plot of DMs. Each dot depicts a specific metabolite, with the dot size corresponding to the VIP value. p values were estimated with paired Student’s t-test on Single dimensional statistical analysis. VIP > 1, fold-change = 1, p < 0.05 is a significant difference metabolite. The down-regulated metabolites are on the left side, while the up-regulated metabolites are on the right side. The further to the right or left a point is, the higher significant the differential expression. (A) D1 and D2 treatment comparison (B) D3 and D1 treatments comparison. (C) D3 and D2 treatments comparison.

3.5. Analysis of the Correlation between Rice Quality Indexes and Metabolite Levels

To gain a deeper understanding of the relationship between rice quality indexes and metabolite levels, we conducted a correlation analysis between the two, which allowed us to examine the associations and potential links between specific rice quality indexes and the levels of different metabolites (Figure 5). N-oleoyl arginine, tirofiban, lubiprostone, 1-oleoyl lysophosphatidic acid (sodium salt), drostanolone, PGB2, 13(S)-hydroperoxylinolenic acid, (9Z,11S,16S)-1-acetoxy-9,17-octadecadiene-12,14-diyne-11,16-diol and Pro-Pro-Phe levels were positively correlated with AC, GBSS CEQ, viscosity, Degree of balance and DBE. N-oleoyl arginine, palmitoyl glucuronide, PGB2, lysPA (0:0/18:2 (9Z,12Z)), 13(S)-hydroperoxylinolenic acid, (9Z,11S,16S)-1-acetoxy-9,17-octadecadiene-12,14-diyne-11,16-diol and Pro-Pro-Phe levels were significantly negatively correlated with APC, SC, AGP, SSS, hardness and SBE. Scoparin 2″-glucoside, meloside, and isovitexin 2″-o-glucoside levels were significantly positively correlated with APC, AGP, and SBE. Meloside, isovitexin 2″-o-glucoside, isoschaftoside, 4-guanidinobutanoic acid, 6-thioinosine-5′-monophosphate, DG (18:0/18:1 (12Z)-O (9S,10R)/0:0) and delta-tocotrienol levels were significantly negatively correlated with GBSS, DBE and appearance. Furthermore, we elucidated the regulatory role of key metabolites by mapping their involvement in metabolic pathways (Figure 6). 4-guanidinobutanoic acid, (3R,5S)-1-pyrroline-3-hydroxy-5-carboxylic acid, citric acid, (S)-2-acetolactate, L-glutamine, L-2,4-diaminobutyric acid and putrescine are amino acid metabolism, 13(S)-hydroperoxylinolenic acid, PGB2, 3-phosphocholine, 7-epijasmonic acid, 20-carboxyleukotriene B4 and 11-dehydro-thromboxane B2 are lipid metabolites (Table S5).
Figure 5. Correlation analysis between rice enzyme activities and DMs, unraveling potential links between enzymatic processes and metabolic regulation in rice. Each cell represents the correlation between two attributes (metabolites and associated features), with different colors representing the magnitude of the correlation coefficient between attributes. * indicates statistical significance at the $p = 0.05$ level, whereas ** significance at the $p = 0.01$ level.

Figure 6. A snapshot of the potential regulation of essential metabolites in metabolic pathways during pairwise comparisons of three nitrogen levels. Key metabolites are highlighted within orange rectangles. Small red rectangles denote significant metabolite content upregulation, while small blue rectangles denote significant downregulation. Small grey rectangles denote no significant difference.
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in metabolite content. This visualization provides insights into the regulatory patterns of essential metabolites under different nitrogen levels.

4. Discussion

The eating quality of rice refers to the sensory properties of rice when it is eaten by people, such as softness, viscoelasticity, and so on. Sensory evaluation has strong subjectivity. The analysis and determination of eating quality by instruments and equipment has the characteristics of high efficiency and small error. The comprehensive evaluation of eating quality measured by the taste meter is significantly positively correlated with the sensory score, indicating that the rice taste meter can be used to evaluate the taste quality of rice [27]. Most previous studies showed that nitrogen fertilizer is an important agronomic measure in regulating the rice’s eating quality [28]. In the present study, the eating quality evaluation (CEQ) of Hongyang 5 exhibited a decline as the nitrogen application rate increased. Conversely, the protein content exhibited an opposite trend. Furthermore, correlation analysis revealed a significant negative correlation between CEQ and protein content (AP). This observation is basically consistent with the outputs of previous studies [29]. Nitrogen fertilizer not only affects the protein content of rice but also affects the starch content by regulating enzyme activity. Ultimately, it changed the rice eating quality [28,30]. The amylose content of the southern semi-glutinous japonica rice was observed to be lower compared to that of northern japonica rice. This lower amylose content significantly contributed to a reduction in rice hardness, while simultaneously increasing rice viscosity. Ultimately, these characteristics led to the development of soft and sticky rice with a more desirable texture [27,31]. To some extent, reducing the content of AC is beneficial to improve the rice eating quality [32]. In contrast to previous findings, our study revealed that the amylose content (AC) of D1 was remarkably greater than that of D2 by 2.81% and D3 by 7.17%. Furthermore, the activities of the starch debranching enzyme (DBE) and granule-bound starch synthetase (GBSS) were significantly higher in D1 compared to D2 and D3. Conversely, D3 exhibited the highest levels of soluble starch synthase (SSS), ADP-glucose pyro-phosphorylase (AGP), and starch branching enzyme (SBE) (Table 2). Moreover, these findings demonstrated significant positive correlations between the comprehensive evaluation of eating quality (CEQ) and variables such as AC, GBSS, and DBE. Hongyang 5 had significantly lower amylose content under the condition of high nitrogen fertilizer but did not form a higher CEQ. We guessed that the decrease of AC in Hongyang 5 was less than the increase in AP, which may be the cause of the low CEQ of Hongyang 5.

Nitrogen is among the three major nutrient elements that regulate the growth and development of rice [33]. Different levels of nitrogen application can change the accumulation of plant metabolites and affect yield and rice quality [19]. In this present study, we observed that different comparison groups of Hongyang 5 exhibited their own specific metabolites, as depicted in Figure 2D. Each comparison group exhibited a distinct set of metabolites, highlighting the unique metabolic profiles associated with different experimental conditions. Furthermore, Figure 3 illustrates the variations in the metabolites’ number and proportion across the different comparison groups, providing significant insights into the compositional differences and metabolic diversity within Hongyang 5 under different conditions. Prior research has indicated that elevating the nitrogen application rate can lead to significant increases in the levels of several amino acids, including threonine, valine, methionine, lysine, leucine, phenylalanine, and isoleucine [34]. In the present study, the metabolic pathways of amino acids such as proline and arginine were enriched (Table S5). (S)-2-acetolactate, citric acid, L-glutamine, L-2,4-diaminobutyric acid, 4-guanidinobutanoic acid, putrescine, and (3R,5S)-1-pyrroline-3-hydroxy-5-carboxylic acid are the critical amino acid metabolites (Figure 6; Table S6) and play vital roles in amino acid metabolism pathways. Lipids are another important storage material in rice, mainly including phospholipids and fats, which are most abundant in the embryo and aleurone layer, but mainly in the form of lipid-amylose complex in the endosperm [35,36].
Lipids are not only a crucial determinant of rice nutritional quality but also a significant factor influencing the eating quality of rice [19]. There were significant differences in lipid metabolites of Hongyang 5 under different nitrogen fertilizer treatments, among which 7-epijasmonic acid, 20-hydroxy-leukotriene E4, stearidonic acid, 3-phosphocholine, PGB2, 13(S)-hydroperoxylinolenic acid, 11-dehydro-thromboxane B2, 20-carboxyleukotriene B4 were all identified as lipid metabolites. Previous studies have found that the higher plant onion can synthesize prostaglandins [37]. The prostaglandins are also present in the buds and cambial zone of poplar and the Siberian larch [38]. There are few reports on prostaglandin synthesis in higher plants, which may be related to the low level of prostaglandin synthesis in higher plants and the low resolution of previous detection instruments and the weak ability to distinguish peaks [39]. In this study, the PGB2, 13(S)-hydroperoxylinolenic acid positively correlated with CEQ, viscosity, and degree of balance. Therefore, we hypothesized that rice eating quality could be improved by changing key metabolites of lipid metabolism by regulating the nitrogen application rate.

5. Conclusions

The amount of nitrogen applied had a remarkable influence on the eating quality of Hongyang 5 rice. As the nitrogen application rate increased, the amylose content decreased while the protein content increased. The taste value was highest when a low-nitrogen fertilizer was used. Metabonomic investigations revealed that, under varying nitrogen fertilizer conditions, specific metabolites in the grains were associated with amino acid metabolism and lipid metabolism pathways. These key metabolites may be regulated by nitrogen fertilizer and play an important role in modulating the eating quality characteristics of the Hongyang 5 rice variety.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13082123/s1, Table S1: DMs between D1 and D2; Table S2: DMs between D3 and D1; Table S3: DMs between D3 and D2; Table S4: KEGG pathways associated with DMs; Table S5: KEGG pathways associated with DMs; Table S6: Metabolite information related to rice quality traits and metabolic pathways.

Author Contributions: Writing—original draft preparation, N.Z. and Y.Z.; review and editing, T.S. and J.H.; formal analysis, J.Z.; funding acquisition, Q.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Jiangsu (Haian) Modern Agriculture (Rice and Wheat) Science and Technology Comprehensive Demonstration Base (JATS [2020]492), Jiangsu Province Seed Industry Revitalization Project [JBCS(2021)036], the project was also funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, China.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interests.

References


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