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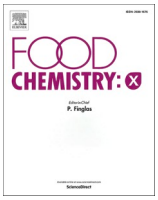

Mechanistic insights into nitrogen-induced changes in pasting characteristics of rice during storage based on proteomics analysis

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ABSTRACT

Nitrogen application delays rice quality deterioration due to changes in its pasting characteristics; however, the underlying mechanisms remain unclear. Using a label-free quantitative proteomics approach, we identified differentially expressed proteins (DEPs) during storage in paddy rice treated with different nitrogen levels. On combining the changes in physiological indicators, high-nitrogen treatment was found to downregulate β-1,3-glucanase, reduce the decomposition of cell wall components, downregulate three proteins involved in starch metabolism, decrease the range of the amylose content and increase the range of the amylopectin, upregulate three proteins related to the lysosomal pathway, and enhance glutelin degradation. In addition, it upregulated three proteins related to flavonoid synthesis, which enhanced the stress response ability of rice, thereby contributing to the stability of biological macromolecules. The discovery of these key DEPs provides potential targets for further control over the deterioration of crop seed storage quality.

1. Introduction

Nitrogen application is a common crop cultivation technique to improve grain yield, milling quality, and nutritional quality, owing to its effects on starch and protein; however, nitrogen may also negatively impact the appearance and eating qualities of rice (Zhang et al., 2020). Surprisingly, a recent study demonstrated that nitrogen application delays the change in the pasting viscosity (one of the key indices of eating quality) of rice during storage (Liang et al., 2021). This suggested that nitrogen application in the field has potential as a valuable preservation measure for paddy rice after harvest. However, the mechanism of action underlying this effect on pasting viscosity is unclear, thereby limiting its widespread application.

Pasting characteristics are the most sensitive indicators of deterioration of the eating quality of rice during storage (Zhou et al., 2002). Reduced pasting viscosity and increased breakdown, setback, pasting temperature, and peak time (Saikrishna et al., 2018) have been identified as markers of rice aging. In the past few decades, extensive research has focused on identifying the mechanism underlying these changes in pasting characteristics during storage. One theory suggests that the changes in the endogenous enzymes and phenolic acids in rice during storage cause lignification of the cell wall, which prevents the entry of water molecules, resulting in reduced pasting viscosity (Zhou et al., 2002). Another theory is that changes in chemical components, including changes in starch components caused by debranching enzymes, the formation of disulfide bonds from sulfhydryl group oxidation in protein molecules (Shi et al., 2017), and the formation of free fatty acids from lipids in the presence of lipase and lipoxygenase (Kim et al.,

Abbreviations: AGC, automatic gain control; AGPS1, glucose-1-phosphate adenylyl-transferase small subunit 1; CHI, chalcone-flavonone isomerase; DEP, differentially expressed protein; E4P, ferythritol-4-phosphate; FV, final viscosity; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDA, malondialdehyde; PEP, phosphoenolpyruvate; PPT, phosphoenolpyruvate/phosphate translocator 1; PRM, parallel reaction monitoring; PV, peak viscosity; ROS, reactive oxygen species; SBE1, 1,4-α-glucan starch branching enzyme; TV, trough viscosity.

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2014), lead to enhanced interactions among starch, lipids, and proteins, thereby decreasing the solubility of starch and protein, ultimately reducing the pasting viscosity. Among the proposed mechanisms, the accumulation of reactive oxygen species (ROS) from fat oxidation is considered to be the main cause of quality deterioration (Li et al., 2017), whereas the changes in fat content have been reported to be independent of pasting characteristics during storage (Zhou et al., 2003). Thus, the causes of the change in pasting characteristics of rice during storage appear to be diverse and complex, involving a variety of endogenous enzyme and chemical composition changes, especially for protein and starch. Recently, researchers have further declared that adding protein into food reduced its pasting viscosity through enhancing the interaction between starch and protein (Wu et al., 2023). In the hot and humid storage environment, the increased interaction between glutelin and starch was thought an important factor for the reduced pasting viscosity of starch (Shi et al., 2020). And the presence of amylopectin, protein, α-amylase has been closed to pasting viscosity of waxy rice (Wang et al., 2022). It can be seen that the change in starch and protein components might affect their interaction in food, thereby impacting its pasting viscosity. However, most studies explored this issue by adding different concentration components into food, and little in view of endogenous components. So far, the deterioration mechanism of pasting viscosity is not yet clear due to the one-sided research perspectives stems from the limited research technology.

In addition, nitrogen application was reported to have a significant influence on the starch and protein components, resulting in significant changes in rice pasting characteristics (Liang et al., 2022). We previously reported that nitrogen application delayed the change in the pasting viscosity of rice during storage, with a 2-month delay detected using application of excessive nitrogen concentrations (Liang et al., 2021). Wen et al. (2017) reported that high-nitrogen treatment increased the protein content, thereby inhibiting the germination of wheat seeds post-harvest. Accordingly, we hypothesized that nitrogen application will lead to sustained influences in starch and protein composition during storage, which will affect the interactions between these two components and ultimately delay changes in pasting characteristics.

To test this hypothesis, we investigated changes in physiological indicators and differentially expressed proteins (DEPs) before and after the storage of paddy rice treated with different nitrogen levels, using a comparative proteomics approach to identify the key proteins involved in the deterioration of rice eating quality. Since proteomics is increasingly being used to investigate all the proteins in cells on a large scale, proteomics was a suitable method for exploring the endogenous enzymatic changes occurring in rice during storage and the effect of nitrogen on this process. Many studies have applied this method to explore the reason for the deterioration of rice seed quality during storage. However, most of the studies revealed the regulatory mechanism for reduced vigor and germination rate (Gao et al., 2016; Lei et al., 2020), and only a few studies have focused on the deterioration of eating quality, especially the pasting characteristics of rice (Jorrin Novo, 2021). Thus, this study may help clarify the mechanism underlying these changes in pasting characteristics during storage from a more comprehensive perspective and further provide molecular-level insights for the development of new strategies and optimization of nitrogen application in the field to delay rice quality deterioration.

2. Materials and methods

2.1. Materials

The rice variety Yanfeng 47, the main local rice variety, was planted in Panjin, Liaoning Province, China (122°14′17″N, 41°9′31″E). The physical and chemical properties of the 0–20 cm soil was as follows: pH 8.2, bulk density 1.39 g/cm^3, organic matter 22.57 g/kg, available potassium 164.22 mg/kg, available phosphorus 21.61 mg/kg, alkali nitrogen 105.24 mg/kg, and total nitrogen 1.42 g/kg. According to our previous study (Liang et al., 2021), three nitrogen application rates i.e., 0, 260, and 420 kg/ha, were selected as the experimental groups to compare the effects of nitrogen application and concentration on rice pasting characteristics, which were labeled as treatments N0, N260, and N420, respectively. Other cultivation and management methods were consistent with local traditional methods. Rice seeds were transplanted in May and harvested in October 2018.

According to standard post-harvest treatment in the rice industry, all harvested paddies were air-dried for 1 month to reduce the moisture content to approximately 14 %. Each dried paddy sample (500 g) was then packed in a nylon net bag, placed in a carton, and stored under laboratory conditions for 12 months. The temperature and humidity were recorded every 3 days. At 0, 2, 4, 6, 8, 10, and 12 months of storage, 500 g samples were milled into brown rice by a ridge mill (FC2K, Yamamoto, Japan) and into milled rice by a milling machine (VP-32 T, Yamamoto, Japan) successively. Brown rice and milled rice samples (80 g each) were respectively ground into flour and then passed through a 100-mesh screen for subsequent determination. In addition, paddy rice samples (20 g) were manually shelled, snap-frozen in liquid nitrogen, and stored at –80 °C until analysis.

2.2. Determination of rice pasting viscosity during storage

Three grams of milled rice flour was added to simple cups containing 25 mL of distilled water each, and the pasting characteristics (peak viscosity, PV; trough viscosity, TV; final viscosity, FV; setback; breakdown; peak time; and pasting temperature) were measured with a rapid viscosity analyzer (RVA-4, Perten, Sweden) according to the method of Mao et al. (2021).

2.3. Measurement of starch and protein components, and moisture in rice during storage

The amylose and amylopectin contents of the rice were determined according to Ma et al. (2021) with a minor modification: milled rice flour defatted with petroleum ether was used. In detail, 10 mL of 0.5 M KOH was added to 0.1000 g defatted milled rice flour and maintained at 75 °C for 20 min until fully dissolved, which was then diluted to 50 mL with distilled water. After filtration, 5 mL of the filtrate was added to 25 mL of distilled water. The pH was adjusted to 3.0 using 0.1 M HCl and 0.1 M NaOH, 0.5 mL of iodine was added, and then the mixture was diluted to 50 mL with distilled water. With distilled water used as a control, the absorbance values Aλ1, Aλ2, Aλ3, and Aλ4 were determined at 630 nm, 480 nm, 550 nm, and 735 nm, respectively. The values of ΔA1 (Aλ1 – Aλ2) and ΔA2 (Aλ3 – Aλ4) were calculated. A standard curve was constructed using the amylose and amylopectin standard solutions, which were used to calculate the amylose and amylopectin contents in the rice, and the total starch content was calculated as the sum of the amylose and amylopectin contents.

The contents of total protein and the component proteins were determined by the methods of Zhang, with minor modifications (Zhang et al., 2020): 0.1000 g milled rice flour was successively extracted with distilled water, 5 % NaCl, 70 % ethanol, and 0.05 M NaOH in a material to liquid ratio of 1:10 to collect the albumin, globulin, prolamin, and glutelin solutions, respectively; each extraction process was repeated three times. The contents of the four protein components were determined using a BCA protein assay kit (Banxia, Beijing, China). The total protein concentration was calculated as the sum of the four proteins. Finally, the constant-weight method was used to determine rice moisture at 105 °C in an oven.

2.4. Determination of fat and malondialdehyde (MDA) contents and the acidity of rice during storage

The fat content in 1 g brown rice flour was determined using the

Soxhlet extraction method with petroleum ether. One gram of brown rice flour was extracted with ethanol (1:2), phenolphthalein solution was then added, and the solution was titrated with 0.1 M KOH to determine the total free fat acid contents. The MDA content of brown rice was determined using an MDA assay kit (A003-3–1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.5. Rice protein extraction and quantification

For proteomic analysis, paddy rice were collected and stored for 0 and 12 months. After manual shelling, brown rice grains were ground with liquid nitrogen, and proteins were extracted with lysis buffer (4 % sodium dodecyl sulfate, 1 mM dithiothreitol, and 100 mM Tris-HCl; pH 7.6). The trypsin hydrolysis protein sample was analyzed using the filter-aided proteome preparation method (Wiśniewski et al., 2009). The enzymatically hydrolyzed peptides were desalted using a C18-reversed phase column, redissolved in 40 μL of dissolution buffer after freeze-drying, and quantified according to the absorbance at 280 nm. Brown rice grains in the N0, N260, and N420 treatment groups stored for 0 months were labeled A1, A2, and A3, respectively, and those stored for 12 months were labeled B1, B2, and B3, respectively. Each experimental group consisted of three replicates.

2.6. Label-free quantitative proteomics and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

For label-free quantitative proteomics, the proteins were separated using a nano high-performance liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) with buffer A (0.1 % formic acid) and buffer B (0.1 % formic acid, 84 % acetonitrile). The protein fractions were eluted onto an analytical column (Thermo Scientific Easy column, 75 μm × 10 cm × 3 μm, C18) with a gradient of buffer B at a flow rate of 250 nL/min, using the following elution program: 0 % to 35 % buffer B for 0 to 50 min, 35 % to 100 % buffer B for 50 to 58 min, and 100 % buffer B for 58 to 60 min.

After chromatographic separation, the samples were analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). The scanning range of the parent ion was 300–1800 m/z, the resolution of primary mass spectrometry was 70,000, the m/z was 200, and the automatic gain control (AGC) target was 3e6. The m/z of the polypeptide fragments was determined according to the following method: 10 fragment maps (MS2 scan) were collected after each full scan. The MS2 activation type was HCD, the isolation window was 2 m/z, the resolution of secondary mass spectrometry was 17,500, the m/z was 200, the normalized collection energy was 30 eV, and the underfill ratio was 0.1 %.

2.7. Protein identification, annotation, and functional analysis

Raw data from LC-MS/MS were searched against the Uniprot Oryza Sativa 242,148 20,201,103 protein database using the MaxQuant software (version 1.5.5.1) to identify and quantify the proteins. The false discovery rate was adjusted to < 0.01. DEPs were identified on the basis of the ratio value between two groups (A1 vs. B1, A2 vs. B2, and A3 vs. B3), and P-values were calculated through a t-test of each protein. In this study, proteins with a fold change ≥ 1.2 (or ≤ 0.83) and $P < 0.05$ were considered DEPs.

OmicsBean software (https://www.omicsbean.cn/) was used to compare the distribution of each Gene Ontology (GO) classification or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for the DEPs and to perform the GO enrichment and KEGG pathway analyses; $P < 0.05$ was used as the threshold to determine the significant enrichment of DEPs.

2.8. Parallel reaction monitoring (PRM) validations

To verify the protein expression levels obtained by the label-free proteomics analysis, 20 DEPs were randomly selected based on the label-free results and further quantified by a PRM assay. Briefly, the peptides were prepared as described above for the label-free assays. The obtained peptide mixtures were subjected to PRM analysis using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). The scanning range of the parent ion was 300–1800 m/z, the resolution of the primary mass spectrometer was 60,000 (200 m/z), and the AGC target was 3e6. Twenty PRM scans (MS2 scans) were obtained after each full scan. The MS2 activation type was HCD, and the normalized collection energy was 27 eV. Three biological replicates were analyzed for each sample. The resulting MS data were processed using the Skyline (v 0.3.6) program.

2.9. Statistical analysis

All data were processed using Microsoft Excel 2019 and analyzed for significance using SPSS 22 software with Duncan's test ($P < 0.05$). The mean values of three replicates are reported. All tables were generated using Microsoft Word 2019. Graphs were plotted using Origin 8.5.

3. Results and discussion

3.1. Changes in physicochemical properties of rice during storage with different nitrogen application rates

3.1.1. Effects of nitrogen application rate on the pasting characteristics of rice

As shown in Fig. 1, nitrogen application delayed the changes in the pasting viscosity indices (PV, TV, and FV) during storage, indicating that nitrogen application has the potential to delay the deterioration of rice eating quality. Compared to the no-nitrogen (N0) treatment, medium- (N260) and high-nitrogen (N420) treatments delayed the peak times of PV, TV, and FV in the first rising stage by 2 months (Fig. 1A). The N420 treatment delayed the peak time of TV and FV by 2 and 4 months, respectively, and N260 treatment delayed the FV peak time by 2 months (Fig. 1B, C). Moreover, the N420 treatment resulted in a smaller change in the TV at both the ascending and descending stages. The TV of the rice under N0, N260, and N420 treatments increased by 16 %, 6 %, and 6 % in the ascending stage, and decreased by 27 %, 24 %, and 22 % in the descending stage, respectively (Fig. 1B). And there were no significant differences in other pasting characteristics (setback, breakdown, peak time, and pasting temperature) between different nitrogen treatments (Fig. S1).

The viscosity indices (PV, TV, and FV) of the pasting characteristics reflect the binding ability between rice flour (mainly starch) and water at the different pasting stages (Islam et al., 2020). These indices were previously shown to be correlated with the expansion rate of rice and to be negatively correlated with hardness (Champagne et al., 1999). The reduction of these pasting viscosity indices thus indicates the deterioration of eating quality in rice during storage. As previous study displayed (Saikrishna et al., 2018), PV, TV and FV showed a similar trend of increasing after the short to intermediate term of storage (0–8 months), and then TV and FV decreased with longer storage time in our results. Among which the initial increase may be attributed to the reduced α-amylase activity (Park et al., 2012), and the subsequent decrease has been attributed to the interaction between starch and non-starch components (Zhou et al., 2003). Further, we detected that nitrogen application could delay and inhibit the change in three viscosity indices of rice during storage, with the highest nitrogen application concentration having the best effect on delaying this deterioration.

3.1.2. Effects of nitrogen application rate on the contents of starch, protein components, and moisture content of rice

Except for the moisture content, starch and protein are the main

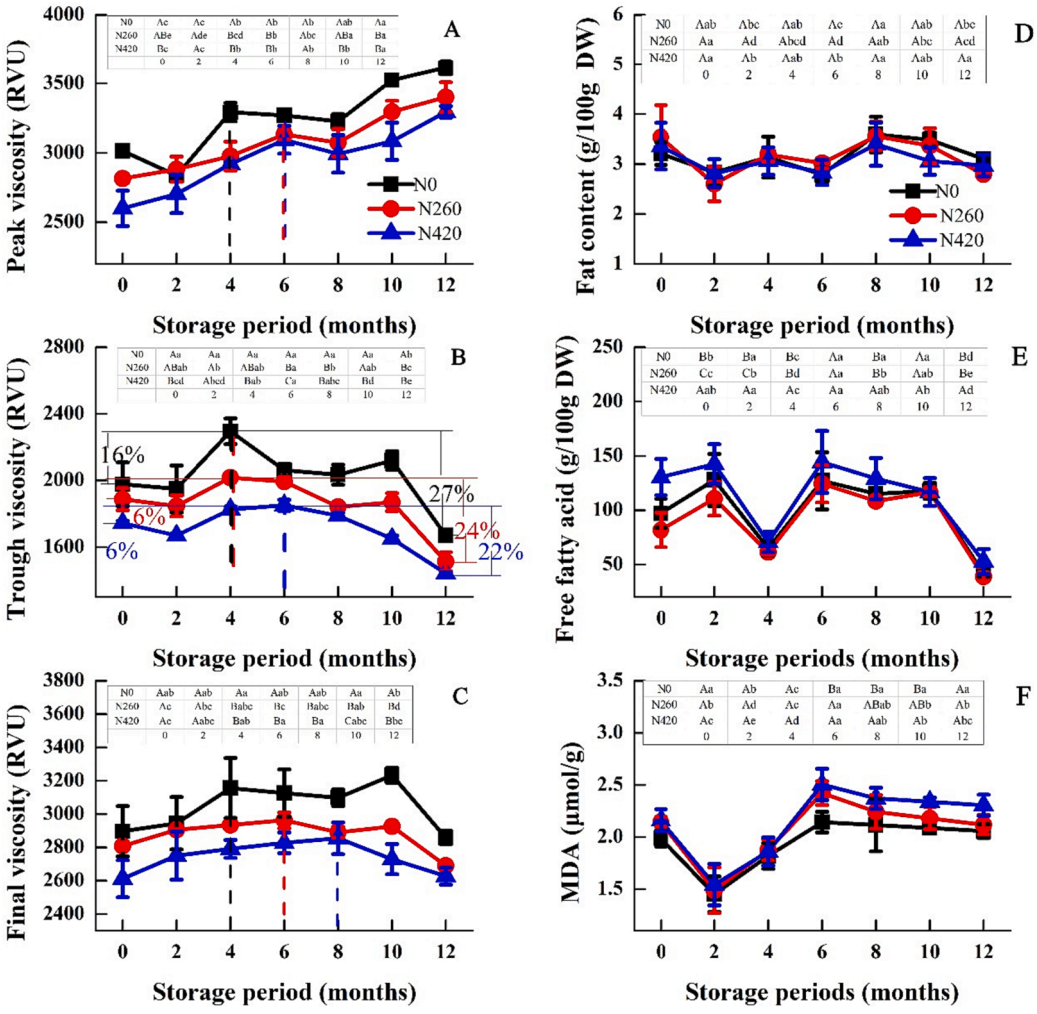

Fig. 1. Changes of pasting characteristics, the pH of rice soup, fat content, fatty acid value and malondialdehyde content of rice during storage under different nitrogen application rates. Note: The content of all compositions was calculated by dry weight (DW) of rice.

constituents of rice, and the proportions of their components significantly influence the pasting characteristics of rice.

In previous research, some researchers believe there was a degradation of amylopectin, resulting a decreased amylopectin in rice during storage (Wu et al., 2019). Other experts argued that the degraded components in rice after storage was amylose rather than amylopectin (Gu et al., 2019). Our results showed that amylose decreased, whereas both amylopectin and total starch increased in rice after storage. This indicated that changes in starch components in rice during storage do not involve simply a one-way transformation from one component to another but that more complex changes are involved, which will be further explained in conjunction with proteomics data related to starch metabolism. Furthermore, we found that compared with the control rice without storage, rice with N420 treatment after storage exhibited a decline in the range of the amylose content and an increase in the range of the amylopectin and total starch contents, which were all lower than those detected with the other treatments (Fig. 2A–C). This could be the main reason for the relatively lower change range of the pasting viscosity indices in rice with the N420 treatment. The pasting process of rice is mainly a combination of starch and water, and the amylose: amylopectin ratio is highly related to the pasting viscosity (Lu et al., 2009). Rice under N420 treatment had a lower range of changes than the other treatments with respect to both the degradation of amylose and the generation of amylopectin. This indicated that there were smaller changes in starch composition under N420 treatment, thus providing a stable material basis for the stability of the pasting viscosity of rice during storage.

Nitrogen application also changed the trend of protein components after storage, which may be related to the differences in the rate of rice quality deterioration. The seed storability was previously reported to be negatively related to glutelin but positively related to globulin (Gao et al., 2016). In this study, high-nitrogen application elevated the decrease in glutelin but reduced the decrease in globulin, which might lead to stronger storage stability in rice, resulting in delayed quality deterioration. In addition, increased prolamin content and decreased contents of the three other protein components in the high-nitrogen treatment group were detected in rice (Fig. 2D–H), which differed from the result for *Calicotome villosa* seeds in Boughalleb's study (2020). This might be attributed to species differences.

Throughout the storage period, the moisture content of rice between different nitrogen application treatments showed no significant differences and only changed with increasing storage time (Fig. S2). These data suggested that the delayed effects of nitrogen application on pasting viscosity are likely related to the above changes in the components of starch and protein, with minimal contribution of the moisture content of rice.

3.1.3. Effects of nitrogen application rate on fat, acidity, and MDA contents in rice

In our previous study, high nitrogen application (N420) exacerbated the reduction in the pH of rice soup during storage of the rice (Liang et al., 2021). The main sources of acidic substances in rice soup are the

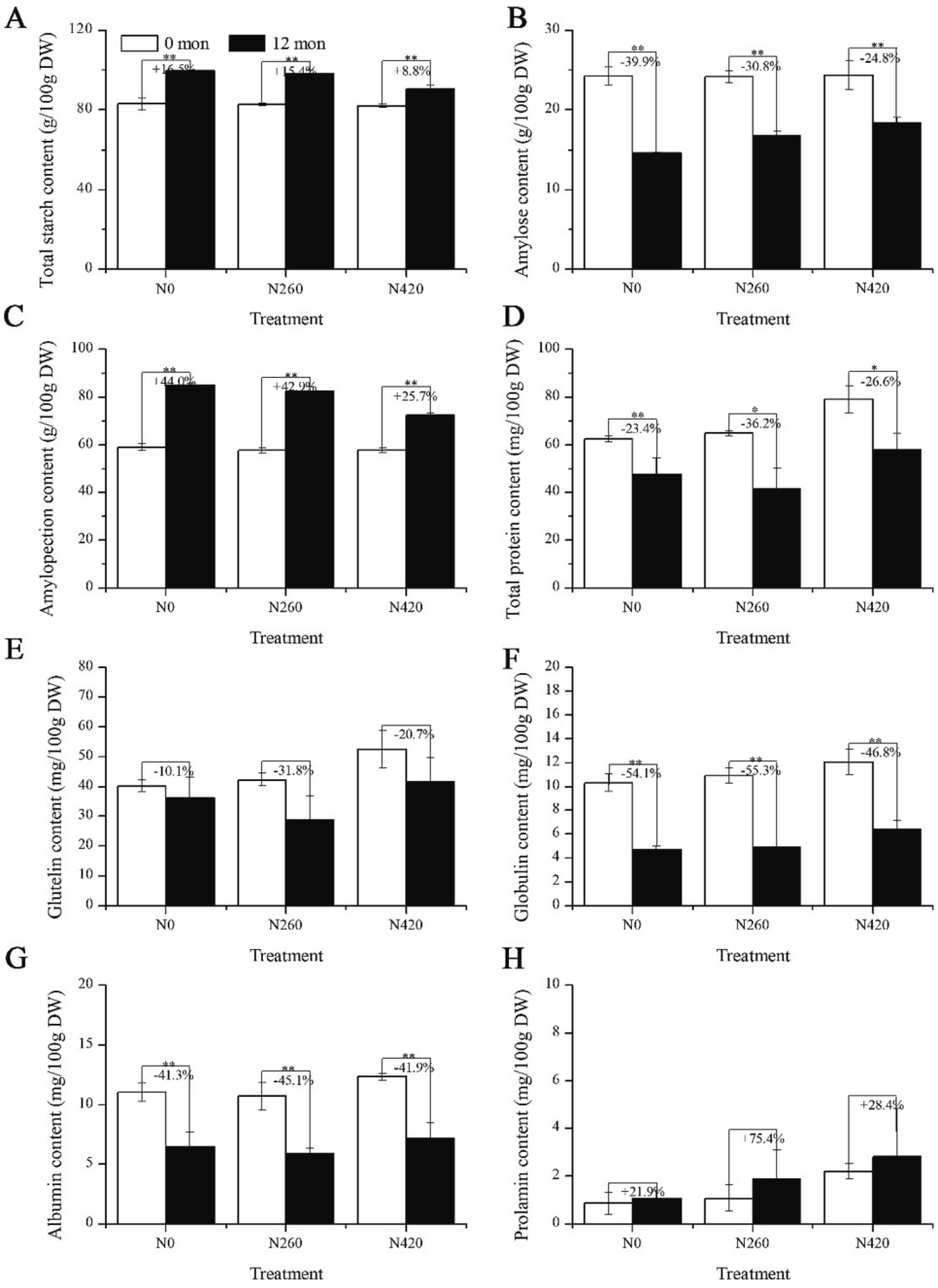

Fig. 2. Changes of the components of starch and protein during storage under different nitrogen application rates. Note: The content of all compositions was calculated by dry weight(DW) of rice.

free fatty acids produced from fat metabolism by lipase during storage. In this study, changes in the fat-related metabolites (fat content, free fatty acids, and MDA) of rice during storage were further investigated. Most previous results showed that fatty acids value and MDA gradually increased during storage. However, our results found that there were occasional fluctuations in fatty acids and MDA during the early storage period, which is also normal. Because these two components produced from lipid oxidation process, are subjected to the dynamic balance between oxidation and antioxidant systems in the rice grains during storage. They are greatly influenced by the variety, origin, quality, and storage conditions such as temperature and humidity, and are prone to changes. At the same time, rice grains have the self-repair capabilities responded to external stimuli, especially during the initial storage. Some antioxidants and aldehyde scavengers, can reduce some harmful substances produced by physiological metabolism in rice grain cells. And this result supported Tian's research (Tian et al., 2019).

Notably, rice with nitrogen application treatments had significantly higher acidity with 0–4 months of storage and higher MDA contents with 6–10 months of storage than those without nitrogen treatment (Fig. 1D–F). These results suggested that rice treated with high nitrogen have relatively stronger fat metabolism during 0–10 months of storage, resulting in the production of more free fatty acids and MDA in 0–4 months and 6–10 months of storage. Remarkably, a slow change in the pasting characteristics was observed in rice with high nitrogen application, rather than a more drastic change, which was inconsistent with Li et al. (2017). This discrepancy might be explained by the low fat content in rice, indicating that fat would have a relatively minimal influence on pasting characteristics during storage, in line with the

findings of Zhou et al. (2003).

3.2. DEPs in rice with different nitrogen application rates during storage

3.2.1. Proteomics overview of rice

Based on the results of the label-free proteomic quantitative analysis, the proteins of rice with N0, N260, and N420 treatments were analyzed before and after 12 months of storage, according to the following three comparison groups: A1 vs. B1, A2 vs. B2, and A3 vs. B3. Among the 16,315 unique peptides, 2,691 proteins (Table S1) and 440 DEPs (Table S2) were identified, with proteins showing a fold change ≥ 1.2 (or ≤ 0.83) and $P < 0.05$ considered to be upregulated (or downregulated) DEPs (Fig. 3A–C). These DEPs could be used to differentiate between freshly harvested and stored samples under different nitrogen application conditions (Fig. 3D–F). Further quantitative analysis revealed 126, 171, and 219 quantifiable proteins in A1 vs. B1, A2 vs. B2, and A3 vs. B3, respectively. Among these proteins, six were shared by all three comparison groups. Nine, six, and forty-nine proteins were shared by the A1 vs. B1 and A2 vs. B2, A1 vs. B1 and A3 vs. B3, A2 vs. B2 and A3 vs. B3, respectively. The specific number of proteins of A1 vs. B1, A2 vs. B2, and A3 vs. B3 were 105, 107, and 158, respectively (Fig. 3G). The detailed information of the quantitative proteins is listed in Table S2.

To screen out the DEPs involved in the effect of different nitrogen application rates on the change in pasting characteristics of rice, we further analyzed 136 DEPs (Table S3) showing different degrees of change or opposite change trends between the high-nitrogen comparison group (A3 vs. B3) and the other two comparison groups (A1 vs. B1 and A2 vs. B2). Because no-nitrogen and high-nitrogen treatments exhibited the largest differences in their effect on the rate of change of the pasting characteristics (Fig. 1), we focused on the DEPs from these two comparison groups (A1 vs. B1 and A3 vs. B3). Fourteen proteins showed significant changes in both comparison groups (NO.2-3, NO.8-11, NO.18, NO.34-35, NO.50-51, NO.79, NO.119, NO.134-136). Notably, two of the proteins (NO.2-3, NO.50-51) displayed opposite trends in the two groups (Table S3).

3.2.2. Functional annotation analysis of DEPs in rice

The top 10 items of level-2 results of the GO enrichment analysis according to significance are presented in Fig. 4. The DEPs of the three comparison groups were mainly involved in cellular, metabolic, and stress processes, distributed in cells, organelles, and cell membranes, and were associated with molecular functions such as catalytic activity, binding, transporter, and nutrient reservoir activity ($P < 0.01$).

Furthermore, the results of all levels in the high-nitrogen comparison group (A3 vs. B3) and no-nitrogen comparison group (A1 vs. B1) showed that the DEPs of the two comparison groups were mainly involved in metabolic processes involving carbohydrates, organo-nitrogen compounds, and processes associated with the cellular response to stress. For the biological process categories in particular, the DEPs in the A3 vs. B3 comparison were enriched in the response to temperature stimulus. These results indicated that the difference in the storage characteristics between the two comparison groups may be related to the metabolic processes of carbohydrates and organic nitrogen compounds (mainly involving proteins in rice) during storage. In addition, there may be a substantial difference in the stress response of rice according to the nitrogen application rate.

The results of the KEGG pathway analysis of all significantly ($P < 0.05$) enriched pathways are shown in Fig. 5. The DEPs of the three comparison groups were significantly enriched in carbon metabolism-related pathways, especially sucrose and starch metabolism and glycolysis. This confirmed that the changes in these two pathways before and after storage are likely closely related to the deterioration of rice pasting characteristics.

In addition, the DEPs of the A3 vs. B3 comparison were significantly ($P < 0.05$) enriched in genetic information processing (protein processing in the endoplasmic reticulum). The DEPs of the A1 vs. B1 comparison were significantly ($P < 0.05$) enriched in aminoacyl-tRNA biosynthesis. Endoplasmic reticulum protein processing occurs in the rough endoplasmic reticulum, including protein folding and post-translational modifications. Aminoacyl-tRNA synthesis assists in the formation of new peptide chains in the translation stage. These data thus suggested that the delayed effect on the change in the pasting viscosity of rice during storage by high nitrogen treatment might be related to the difference in the processes associated with protein synthesis (translation and post-translational modification) compared to other treatments.

Overall, these results showed that the delayed effect of high nitrogen application on rice eating quality during storage might be related to the changes of key proteins involved in starch and sucrose metabolism, protein metabolism, and the response to stress (Table S3). The specific correlations are discussed in the following sections.

3.3. Role of carbohydrate metabolism in changes in pasting characteristics during storage

3.3.1. Synthesis and decomposition of cell wall components

Notably, at the molecular level, we show new evidence for the impact of changes in the cell wall structure on the deterioration of storage quality. In this study, in the high-nitrogen group, the proteins involved in the synthesis of cell wall components, such as UDP-glucose 6-dehydrogenase 1 (UGDH1, NO.48), were upregulated after storage, while proteins involved in the decomposition of cell wall components, including glucan *endo*-1,3-β-D-glucosidase (β-1,3-glucanase, NO.50–51) and putative polygalacturonase (PG, NO.49), were downregulated. In the no-nitrogen group, β-1,3-glucanase was upregulated. UGDH1 catalyzes UDP-glucose (UDPG) to produce UDP-glucuronate and UDP-glucuronic acid, which participate in the biosynthesis of hemicellulose and pectin, the main components of the plant cell wall (Klinghammer & Tenhaken, 2007). β-1,3-Glucanase and PG hydrolyze β-dextran and polygalacturonate, which are structural components of the cell wall (Moyrand & Janbon, 2004). These data indicate that rice under high nitrogen treatment might have a more complete cell wall structure during storage, thus better maintaining cell wall elasticity, which is conducive to retaining the water molecules entering the rice grain during the pasting process. Therefore, high nitrogen can maintain a higher pasting viscosity for a longer time in rice during storage, thereby delaying the deterioration of pasting characteristics. This result confirms the previous theory that changes in the cell wall drive the deterioration of pasting characteristics during storage (Shibuya & Iwasaki, 1984), but changes in other enzymes that impact the integrity of cell wall during rice storage were also displayed in our study, such as UGDH1, β-1,3-glucanase and PG, rather than xylanase discovered in previous studies.

3.3.2. Starch and sucrose metabolism

Starch synthesis and catabolism have been reported as the main processes regulating the generation and storage of energy in seeds during storage (Lei et al., 2020). We found that starch synthesis and catabolism also had a great impact on the starch components and their contents in rice, ultimately influencing the pasting characteristics of rice. In the high-nitrogen group, three proteins involved in starch catabolism were significantly downregulated (Table S3): 17 kDa α-amylase (NO.4), 1,4-α-glucan starch branching enzyme (SBE1, NO.5–6), and isoamylase (NO.7). This finding suggested a specific transformation process between starch components occurring in rice during storage, including the degradation of the long amylose chains to short amylose chains, or even the production of amylopectin (SBE1), and the degradation of long amylopectin chains to short amylose and amylopectin chains (isoamylate), which supports previous results from the perspective of enzymes (Gu et al., 2019). In addition, the amylose content was reduced, whereas the amylopectin content was increased in rice after storage (Fig. 2A–C), which may be the result of the comprehensive action of these three enzymes. Indeed, these three enzymes were downregulated in rice of the high-nitrogen group, which could have

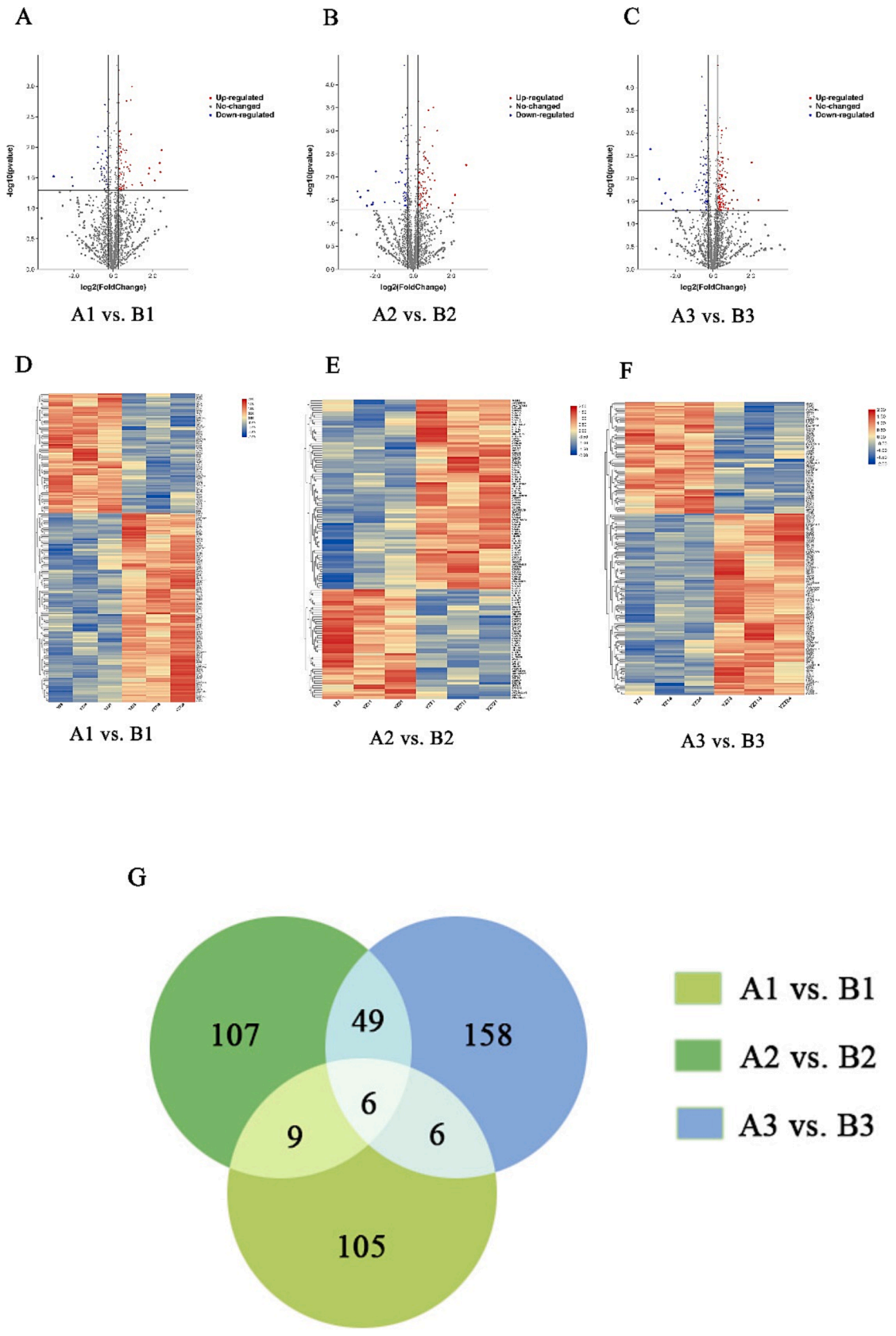

Fig. 3. The differentially expressed proteins of rice before and after storage under different nitrogen appication. A-C, Volcano plot; D-F, Heatmap; G, V enn plot.

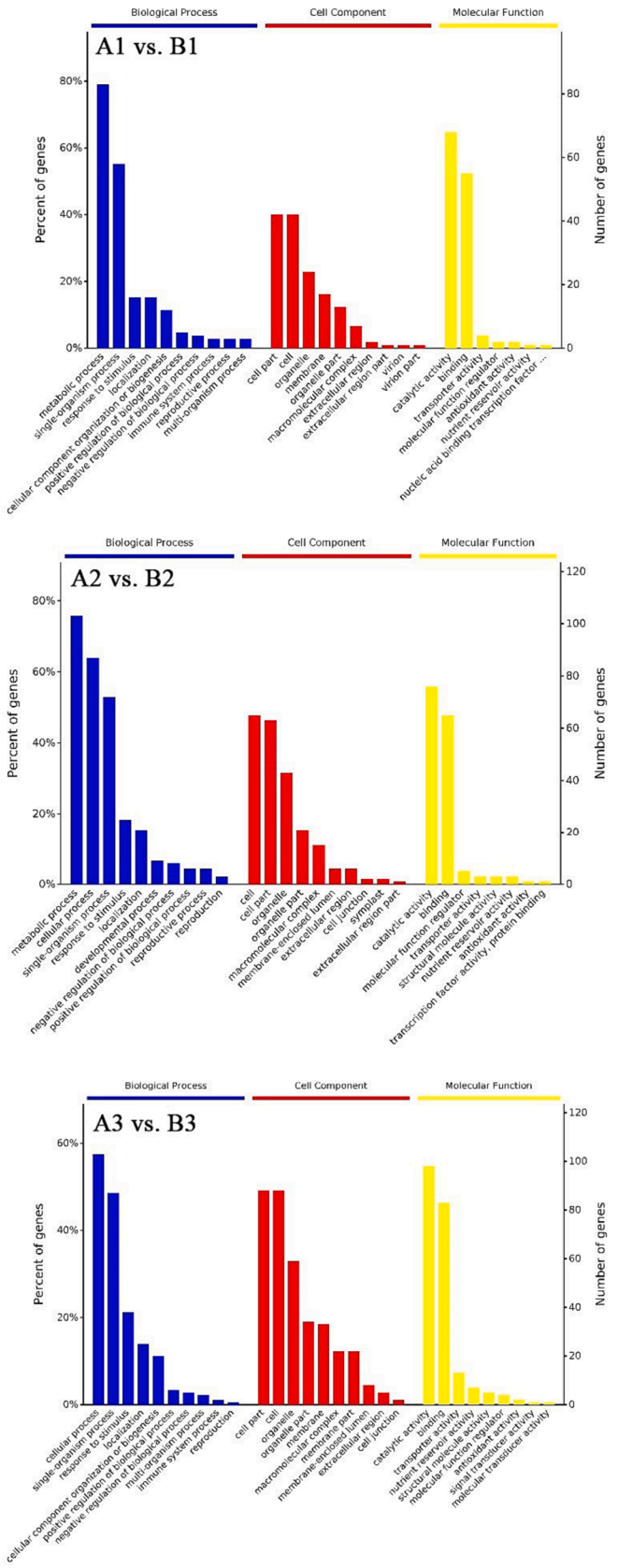

Fig. 4. GO function annotation analysis of differentially expressed proteins of rice before and after storage under different nitrogen application rates.

resulted in the smaller change in decreased amylose content and increased amylopectin content, ultimately manifesting as a smaller change in the pasting viscosity. This is supported by the fact that viscosity has been negatively correlated with the amylose content but positively correlated with the amylopectin content of rice (Lu et al., 2009).

In addition, the conversion process of starch to sucrose as an energy supply is often accompanied by the enhancement of sucrose metabolism. In this study, glucose-1-phosphate adenylyl-transferase small subunit 1 (AGPS1, NO.2) was significantly downregulated in the high-nitrogen group, whereas AGPS1 (NO.3) and likely sucrose phosphate synthase 4 (NO.1) were significantly upregulated in the no-nitrogen group. These results suggested that the sucrose metabolism of rice decreased in the high-nitrogen group but increased in the no-nitrogen group, which is consistent with the results of starch catabolism mentioned above, and indicates a reduced energy supply in the high-nitrogen group (Lei et al., 2020). Thus, we conjecture that rice treated with a high nitrogen concentration may require less energy to support the weaker physiological metabolism during storage. Interestingly, this speculation is in line with the conclusions of a previous study showing that for strongly dormant rice (N22), the expression levels of genes related to starch metabolism and glucosyltransferase were higher than those in weakly dormant rice (Q4646) (Qin et al., 2010). Moreover, Wen et al. (2017) found that nitrogen application reduced the germination rate of wheat seeds. Accordingly, we speculate that high nitrogen application in the field reduced sucrose and starch metabolism and therefore strengthened the dormancy of seeds, which was harmful to germination but beneficial for the maintenance of quality during storage due to the lowered metabolic rate, ultimately delaying the change in the pasting characteristics of rice.

3.4. Role of protein metabolism in changes in pasting characteristics during storage

As the second largest component in rice, storage proteins often undergo hydrolysis or aggregation due to oxidation during storage, thereby deteriorating the quality parameters of rice such as pasting properties (Shi et al., 2017). In contrast, we found that, from the perspective of protein metabolism, glutelin degradation and globulin aggregation may help delay the deterioration of quality in rice.

In the high-nitrogen group, glutelin (NO.99-100) abundance was significantly downregulated after storage, which was consistent with the above finding that high nitrogen elevated the decrease in glutelin (Fig. 2E). As Guo et al. (2015) suggested a relationship between the existence of glutelin and increase in pasting viscosity of rice during aging, our results indicated that high nitrogen concentration increased the rate of glutelin degradation during storage, thereby delaying the increase in pasting viscosity.

Notably, we found that the high-nitrogen group had a stronger lysosomal degradation system than that of the no-nitrogen group, which might have contributed to the greater degradation of storage proteins (i.e., glutelin). Three proteins involved in the lysosomal pathway were upregulated in the high-nitrogen group: serine-type peptidase, aspartic-type endopeptidase, and Lon protease homolog (NO.82-84), with the latter showing serine endopeptidase activity. Peptidase and endopeptidase decompose the short peptides and long chains of proteins, respectively, and function in the lysosomal protein degradation pathway (Huber & Teis, 2016). These results thus indicated that rice in the high-nitrogen group had a stronger lysosomal degradation system than that of the no-nitrogen group, resulting in a greater degree of glutelin degradation (Fig. 2D, E). Because the optimal pH value of most degradation enzymes in lysosomes is in the acidic range (Xiong et al., 2023), our results indicate that high nitrogen increased fat-related metabolites in rice during storage (Fig. 1D–F), thereby exacerbating the reduction in the pH value of rice soup during storage for 4–6 months (Liang et al., 2021). At the same time, we also detected significant upregulation of fat metabolism enzymes in the high-nitrogen group after storage, such as

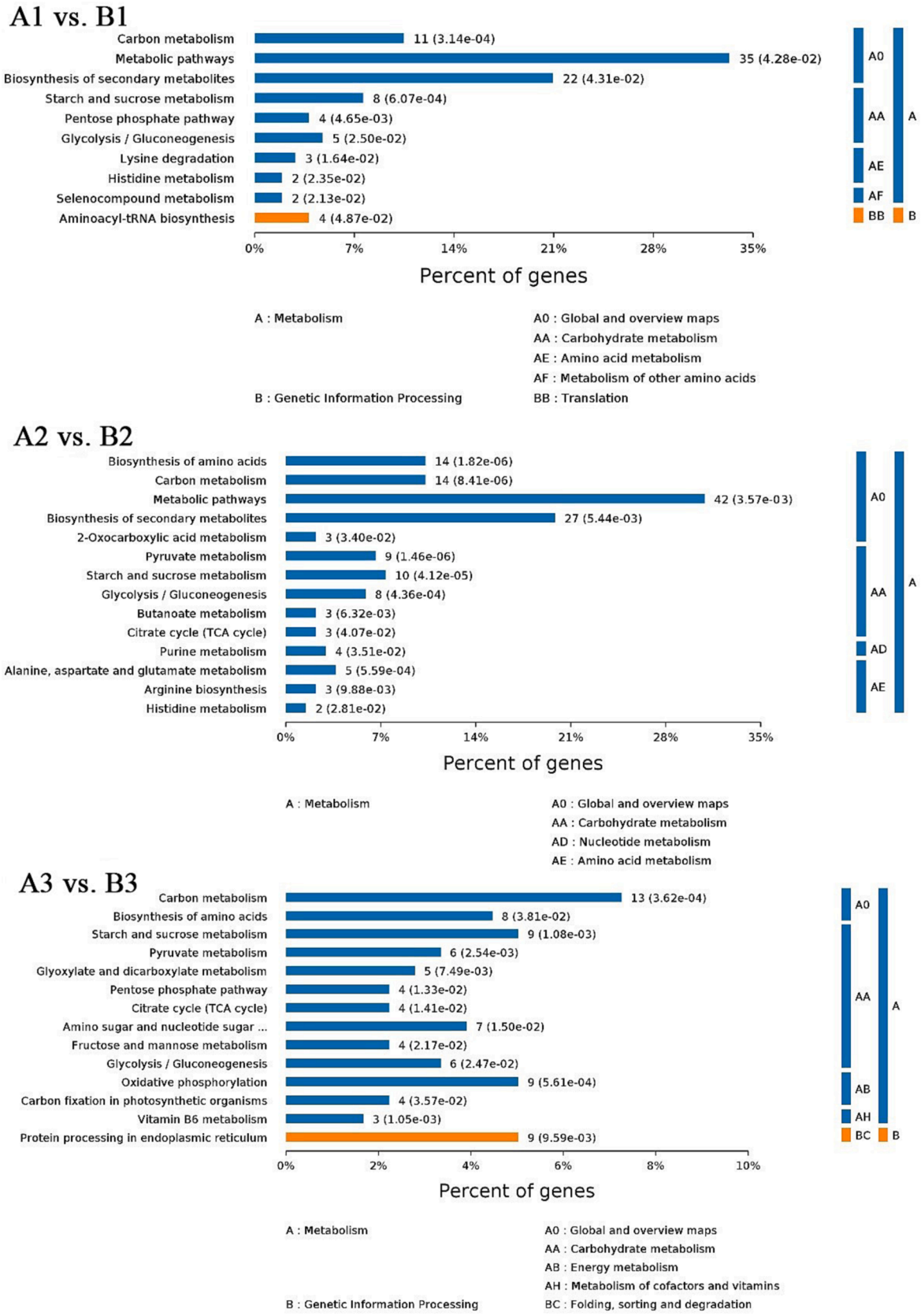

Fig. 5. KEGG pathway analysis of different proteins involved in rice before and after storage under different nitrogen application rates.

phospholipase D alpha 1, Os05g0576700 protein, and Os06g0232000 protein (Table S3, NO.109-111). Therefore, in this study, high nitrogen may have enhanced fat metabolism, providing a suitable pH for lysosomal hydrolases and enhancing their ability to degrade proteins. This conjecture was also supported by previous results (Banerjee & Kane, 2020; Tan & Finkel, 2022), wherein phosphatidylinositol lipids were indicated to indirectly affect the pH value of organelle through interactions with other membrane transporters, as well as with a phosphoinositide signaling pathway that mediates rapid lysosomal repair.

Moreover, the change in globulins from a low-molecular-weight (19 kDa globulin, NO.101) to a high-molecular-weight (63 kDa globulin-like protein, NO.102) form was also observed in the high-nitrogen group. We also previously reported that a high nitrogen concentration significantly increased the content of sulfur-containing amino acids in globulin, which is the main source of sulfhydryl groups and disulfide bonds in proteins (Liang et al., 2022). Collectively, these findings suggest that high nitrogen may exacerbate the formation of disulfide bonds in globulin from oxidation during storage, so that more globulin aggregates to form high-molecular-weight globulin. Guo et al. (2015) found that the presence of globulin enhanced the decrease in pasting viscosity during storage, which was ascribed to an enhanced correlation between globulin and starch. Consistently, this change in globulin detected in our study was often accompanied by lower solubility, thus reducing the correlation between globulin and starch, which may ultimately have delayed the decline of pasting viscosity.

3.5. Role of the stress response in the change of pasting characteristics during storage

ROS, are potent and harmful agents, causing oxidative damage to biological macromolecules, such as DNA, proteins, lipids, and carbohydrates, and resulting in quality deterioration in rice during storage, including pasting characteristics (Li et al., 2017). The antioxidant system, as a defense system for preventing the excessive accumulation of ROS, is composed of a non-enzymatic system (antioxidant substances) and antioxidant enzyme systems. With applied proteomics, a sophisticated technique, and experimenting with a single variety of rice treated with different nitrogen rates, we discovered that flavonoid synthesis was enhanced in rice during storage and other new proteins involved in non-enzymatic antioxidant systems were upregulated. Moreover, we observed that aldehyde-scavenging system related proteins were upregulated. These newly discovered proteins are suitable targets for improving stress resistance in rice during storage in the future.

High nitrogen application was found to stimulate flavonoid synthesis in rice during storage. After storage, the level of transaldolase (NO.16) was significantly increased in the high-nitrogen group. Transaldolase catalyzes erythritol-4-phosphate (E4P) synthesis (Maxime & W., 2005). E4P and phosphoenolpyruvate (PEP) enter the phenylpropanoid biosynthesis pathway to synthesize flavonoids, which can scavenge ROS (Zheng et al., 2020). However, phosphoenolpyruvate/phosphate translocator 1 (PPT, NO.133) and chalcone-flavonone isomerase (CHI, NO.132) levels were significantly increased in the high-nitrogen treatment group. PPT transports PEP from the cytoplasm to the plastid and participates in the phenylpropanoid biosynthesis pathway. CHI is responsible for the isomerization of chalcones into naringin, a ROS scavenger (Dao et al., 2011). Therefore, the three upregulated proteins in the high-nitrogen group may enhance flavonoid synthesis to reduce the accumulation of ROS in rice during storage.

Moreover, the three other proteins participating in the non-enzymatic antioxidant system (glutathione metabolism) were upregulated in the high-nitrogen group, including thioredoxin H4-2, thioredoxin H, and thioredoxin DsbH domain-containing protein (NO.124–126). This showed that high nitrogen application increased the amount of the proteins involved in the glutathione metabolic pathway (Cheng et al., 2020), which might help improve the antioxidant capacity of rice.

Besides, the scavenging system of aldehydes produced downstream of oxidative injury acts as a defense system in the oxidative stress response. We found that the scavenging systems for aldehydes in the high-nitrogen group appeared to be stronger than those in the no-nitrogen group. For the high-nitrogen group, three proteins related to aldehyde scavengers (Zhang et al., 2016) were upregulated: lactoylglutathione lyase (glyoxalase I), betaine aldehyde dehydrogenase, and succinate-semialdehyde dehydrogenase (NO.129–131). In the no-nitrogen group, only acetaldehyde dehydrogenase (NO.127) was downregulated, and the Os06g0531200 protein (involved in glyoxal removal, NO.128) was upregulated. These data suggested that rice with high nitrogen treatment had a stronger aldehyde-scavenging system than rice with no nitrogen treatment, helping to reduce the damage caused by aldehydes (produced from the oxidation of fats and proteins) in rice during storage.

3.6. PRM results

To verify the conclusions obtained by the label-free proteomics analysis, 20 DEPs were randomly chosen from two comparison groups (A1 vs. B1 and A3 vs. B3) for further targeted PRM assays. The results showed that 13 proteins (Table 1) had quantitative information and good consistency ($R^2 = 0.919$, $P < 0.01$) with the label-free results, suggesting that the proteomics data were reliable.

4. Conclusion

This is the first study to elucidate the mechanisms by which nitrogen application in the field delays the changes in pasting viscosity of rice during its storage. We found that high nitrogen application reduced the decomposition of cell wall components by downregulating β-1,3-glucanase and reduced the mutual transformation of starch components by downregulating the 17 kDa α-amylase, SBE1, and isoamylase. It also enhanced glutelin degradation by upregulating serine-type peptidase, aspartic-type endopeptidase, and Lon protease homolog. These results were concordant with our hypothesis. Moreover, high nitrogen treatment enhanced flavonoid synthesis by upregulating transaldolase, PPT, and CHI; besides it improved the stress response ability of rice by upregulating several proteins involved in non-enzymatic antioxidant and aldehyde scavenging, which delayed changes in the pasting characteristics of rice during storage. Meanwhile, our results also showed that changes in starch and protein metabolism might be the main reason for the deterioration of the pasting characteristics of rice, and that this could be eliminated by the enhancement of the non-enzymatic antioxidant system, which provide new references for understanding and controlling rice quality deterioration during storage. Nonetheless, we only revealed the key proteins involved in these pathways may play an essential role in the delaying effects of high nitrogen application on rice. Future research focusing on characterizing the biological significance of

Table 1
PRM verification results of differential proteins.

No.	Protein names	Ratio(B1/A1)	Ratio(B3/A3)
1	Citrate synthase	1.18	1.32
2	Glucan *endo*-1,3-beta-D-glucosidase	0.85	0.72
3	Phosphoenolpyruvate carboxylase	1.11	1.34
4	Delta-1-pyrroline-5-carboxylate synthase 1	1.14	1.23
5	Heat shock cognate 70 kDa protein	1.03	1.27
6	LEA protein	0.91	1.22
7	Chaperone protein ClpC1	1.04	1.28
8	Aldehyde dehydrogenase	0.78	0.98
9	Thioredoxin h	0.96	1.3
10	Lactoylglutathione lyase	0.95	1.65
11	Os05g0576700 protein (lipid storage)	1.01	1.31
12	NADH-cytochrome b5 reductase	1.15	1.75
13	Betaine aldehyde dehydrogenase 1	1.12	1.55

these key proteins and exploring this regulation in other variety rice and even other crop seeds will be highly valuable for designing molecular breeding or engineering programs to improve the storage tolerance of seeds.

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CRediT authorship contribution statement

Hanling Liang: Data curation, Writing – original draft, Conceptualization, Methodology, Investigation, Software. **Baiyu Gu:** Methodology, Software, Investigation, Visualization. **Wentao Sun:** Supervision, Conceptualization, Methodology. **Bo Li:** Conceptualization, Methodology, Software. **Hang Qu:** Conceptualization, Methodology. **Dongbing Tao:** Methodology, Investigation. **Qi Zhang:** Methodology, Investigation. **Tianyu Wang:** Methodology, Investigation, Software. **Yichao Ma:** Methodology, Software, Investigation, Visualization. **Yajie Wang:** Methodology, Software, Validation. **Zhaoxia Wu:** Supervision, Writing – review & editing. **Qinghai Zhang:** Conceptualization, Supervision, Writing – review & editing.

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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Appendix A. Supplementary data

(Figure S1) Changes of pasting characteristics of rice during storage under different nitrogen application rates; (Figure S2) Changes in moisture content in rice during storage under different nitrogen application rates; (Table S1) Total proteins identified via label-free quantitative proteome; (Table S2) The DEPs quantified in rice under different nitrogen application rates during storage; (Table S3) The DEPs with different degrees of change or opposite change trends between the high-nitrogen comparison group (A3 vs. B3) and the other two comparison groups (A1 vs. B1 and A2 vs. B2). Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101018.

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