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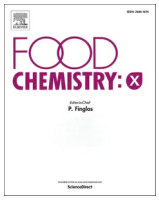

# Controlled atmosphere storage drive proteomic change in Chinese Daohuaxiang

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ABSTRACT

Although the use of a controlled atmosphere is one of the most successful storage techniques, the mechanism thereof in rice storage remains unclear. We stored aromatic rice cultivar Daohuaxiang in a package filled with 98 % $N_2$ and 35 % $CO_2$ for 3 months. We investigated 2-acetyl-1-pyrroline loss, enzyme activities, and proteomics changes of rice during storage. The results showed that the content of 2-acetyl-1-pyrroline was reduced by 37.40 %, 25.65 %, and 43.89 % during storage using 98 % $N_2$, 35 % $CO_2$ controlled atmosphere storage, and conventional storage. Controlled atmosphere storage slowed down the increase of malondialdehyde content in Daohuaxiang. The results showed that 26S proteasome regulatory particle triple-A ATPase subunit 6, superoxide dismutase, glutathione transferase, and other key proteins were upregulated during 35 % $CO_2$ regulation. This study provided a meaningful basis for exploring the regulation strategy of aromatic rice quality and strengthening the quality control of aromatic rice industry.

## 1. Introduction

Daohuaxiang (DHX) is one of the most popular varieties of aromatic rice in China. 2-Acetyl-1-pyrroline (2-AP) is a characteristic compound and key contributor of aromatic rice's aroma. The relative content of 2-AP in DHX can be as high as 10 times that of other varieties of Chinese aromatic rice (Jie, Shi, Zhang, & Yan, 2021). Because of rice's seasonality of production and the continuity of consumption, storage is required before processing. However, the loss of aroma components and off-flavor generation of aromatic rice caused by protein metabolism can lead to short-term shelf-life (Hu, Lu, Guo, & Zhu, 2020), reduced storage quality, and inability to meet consumer requirements, resulting in incalculable economic losses for enterprises (Xue et al., 2021). Controlled atmosphere (CA) storage is one of the most advanced technology of fresh food storage, which can delay their metabolism and has made an important contribution to their shelf-life extension. High $N_2$ or $CO_2$ (up to 98 %) atmosphere have been shown to create a low-oxygen environment to reduce the oxidation of protein during rice storage (Huang et al., 2020; Yang, Chen, Sun, Li, & Lin, 2020). Studies have found that a suitably controlled $O_2$/$CO_2$ atmosphere can extend the shelf-life of postharvest broccoli, limiting the occurrence of off-flavor (Li, Zhang, Guo, & Nian, 2014). Conversely, an offensive flavor was present in broccoli stored in unsuitable $O_2$/$CO_2$ atmospheres (Li et al., 2014), especially under high $CO_2$ conditions (Wang et al., 2019). Despite the successful commercial application of CA storage, the mechanisms governing protein changes during senescence and their modulation by $N_2$ or $CO_2$ during rice preservation remain incompletely understood. Therefore, comprehending disparities in protein expression at the molecular level in rice, identifying proteins linked to their physiological alterations, and analyzing the influence of CA storage conditions on rice can be highly advantageous for conducting in-depth research on CA packaging in rice preservation.

Quantitative proteomics have emerged as a approach, providing an attractive opportunity for plant biologists to screen many metabolic pathways simultaneously for alter ations at the molecular level (Baginsky, Hennig, Zimmermann, & Gruissem, 2010; Cox & Mann, 2007). Research on aging, variety identification, and nutritional quality of cereals has been carried out by proteomics (Li, Liu, Chen, & Cheng, 2018; Cao, Cai, & Liu, L.i., Zhang, M., He, X., Zhang, & Wu, 2015; Mirzaei et al., 2012). For instance, Zhao, Lin, Wang, Yousaf, Xue, & Shen (2021) used proteomics analysis and found that reactions, such as redox homeostasis and response to oxidative stress, led to the different quality of

*Abbreviations:* CA, Controlled atmosphere; DHX, Daohuaxiang; 2-AP, 2-Acetyl-1-pyrroline; TMP, 2,4,6-trimethylpyridine; MDA, malondialdehyde; AMS, alpha-amylase; POD, peroxidase; BADH, betaine aldehyde dehydrogenase; LOX, lipoxygenase.

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stored rice. According to Yang et al. (2020) research, quantitative proteomics, and molecular biology analyses reveal that CA storage could decrease the rates of carbon metabolism, protein and amino acid metabolism, and lipid and fatty acid degradation by downregulating some key proteins of Tibetan hull-less barley. To date, no proteomic analyses of rice responses to CA storage has been published.

In this study, we detected the effect of CA storage on aroma component of DHX and analyzed the proteomics of DHX under CA conditions to screen out key proteins and explore their functions and related metabolic pathways. This analysis helped further analyze the mechanism of CA storage and provided theoretical guidance for aroma quality improvement of aromatic rice.

## 2. Materials and methods

### 2.1. Chemicals

We purchased 2,4,6-trimethylpyridine (TMP) with a purity > 99 % from Changzhou Tanmo Quality Inspection Technology Co., Ltd. (Jiangsu, China). We purchased enzyme-linked immunosorbent assay (ELISA) kits for malondialdehyde (MDA), alpha-amylase (AMS), peroxidase (POD), betaine aldehyde dehydrogenase (BADHX), and lipoxygenase (LOX) from Beijing Weilaibo Biotechnology Co., Ltd.

### 2.2. Sample preparation

The samples used in this study were freshly harvested, dehulled brown rice of *Oryza sativa* L. subsp. japonica rice cultivar DHX with a moisture content of 14 % (dry basis). The rice was purchased from a certified supplier in Heilongjiang province, stored at −20 °C after collection, and analyzed within 2 weeks.

The brown rice samples were treated as follows. The rice was subjected to $CO_2$ storage, with fresh brown rice placed in a sterile packaging box with a gas ratio of 35 %. The rice was subjected to $N_2$ storage, with fresh brown rice placed in a sterile packaging box with a gas ratio of 98 %. The samples that were conventionally stored without any treatment in a self-sealed bag served as the control. Both treatment and control were stored at 30 °C for 3 months to evaluate the effect of CA storage on changes in DHX. CA storage was carried out using a modified atmosphere packaging machine (RDT320P-1, Chengdu Roadiepaul Equipment Co., Ltd., Chengdu, China).

### 2.3. Analysis of 2-AP by HS-SPME-GC–MS

We carried out the determination of 2-AP content in DHX after storage under different conditions using the internal standard method. 2,4,6-trimethylpyridine (TMP) was the internal standard. The measurement procedure was as follows: 1 g of rice powder was weighed and 1 mL of TMP solution was added (10 mg $L^{-1}$ TMP solution was taken up to 5 mL, and then diluted with a mixture of anhydrous ethanol and dichloromethane to 100 mL to obtain a 0.5 mg $L^{-1}$ TMP solution), and then placed in a water bath at 80 °C for 3 h. After cooling to room temperature, the supernatant was transferred to an injection bottle, and an SPME fiber (DVB/CAR/PDMS, 2 cm, 50/30 μm; Supelco, Bellefonte, PA, USA) was inserted into the top space of the sample and allowed to stand for 0.5 h before measurement.

To conduct the analysis, we injected the fiber containing 2-AP into the gas chromatography–mass spectrometry (GC–MS) injector at 250 °C for 4 min (Dias, Duarte, Mariutti, & Bragagnolo, 2019). We used a DB-Wax capillary chromatographic column (0.32 mm × 30 m × 0.25 μm) and performed the GC–MS analysis using an Agilent 7890 gas chromatograph system, which was coupled with a 5977B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). We used helium gas as the carrier gas, and the initial temperature of the column oven was 50 °C, which was held for 2 min, and then ramped up to 280 °C at a rate of 10 °C $min^{-1}$, and then held for 3 min. The MS conditions were as follows: the interface and ion source temperatures were 280 °C and 230 °C, respectively, the electron energy was maintained at − 70 eV, and the mass spectrum ion scan range was 20–500 *m/z*.

### 2.4. Determination of enzyme's activity

We determined the content of MDA and the activity changes of AMS, POD, LOX, and BADH in stored DHX using assay kits.

### 2.5. Proteomic analysis

#### 2.5.1. Protein extraction

We ground the sample by liquid nitrogen into cell powder. Then we added four volumes of phenol extraction buffer (10 mM dithiothreitol, 1 % protease inhibitor and 2 mM EDTA) to the cell powder, followed by sonication three times on ice using a high-intensity ultrasonic processor (Scientz-IID, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). The lysate was centrifuged at 5,500 g at 4 °C for 10 min after adding an equal volume of tris-saturated phenol, and the supernatant was collected and mixed with five volumes of 0.1 M ammonium acetate/methanol, and then precipitated overnight. Finally, the remaining precipitate was washed with cold acetone three times. The protein was redissolved in 8 M urea, and the protein concentration was determined with a bicinchoninic acid (BCA) kit according to the manufacturer's instructions. For digestion, we reduced the protein solution with 5 mM dithiothreitol for 60 min at 37 °C and alkylated with 11 mM iodoacetamide for 30 min at 25 °C.We then diluted the protein sample by adding 100 mM triethylammonium bicarbonate (TEAB) to urea concentration less than 2 M. Finally, we added trypsin at a 1:50 trypsin-to-protein mass ratio for the first digestion overnight at 37 °C.

#### 2.5.2. TMT labeling

After trypsin digestion, the peptide was desalted using a C18 SPE column (Strata X,Phenomenex Inc., Torrance, CA, USA) and vacuum-dried. The peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for the TMT kit/iTRAQ kit. Briefly, we thawed one unit of TMT reagent, which was reconstituted in acetonitrile. The peptide mixtures were then incubated for 1 h at 25 °C and pooled, desalted, and dried by vacuum centrifugation.

#### 2.5.3. LC-MS/MS analysis

The tryptic peptides were dissolved in 0.1 % formic acid (solvent A), and directly loaded onto a homemade reversed-phase analytical column (15 cm long, 75 μm i.d.). The gradient was composed of an increase from 3.0 % to 38 % solvent B (0.1 % formic acid in 98 % acetonitrile) over 120 min, at a constant flow rate of 300 nL $min^{-1}$ on an ultra-performance liquid chromatography (UPLC) system (nanoACQUITY UPLC M−Class, Waters, Milford, MA, USA).

The peptides were subjected to ESI source followed by tandem mass spectrometry (MS/MS) in Q Exactive HF (Thermo Fisher Scientific, Waltham, MA, USA) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The *m/z* scan range was 400 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 60,000. We then selected peptides for MS/MS using normalized collision energy (NCE) setting as 36 and detected the fragments in the Orbitrap at a resolution of 45,000. A data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with 45.0 s dynamic exclusion. We set the automatic gain control at 2E5 and set the fixed first mass as 100 *m/z*.

#### 2.5.4. Database search

We processed the resulting MS/MS data using the Proteome Discoverer (version 2.2) search engine. We searched the MS/MS spectra against the Uniprot (Mythimna separata) database, which was concatenated with reverse decoy database. Trypsin was specified as cleavage enzyme allowing up to two missing cleavages. The first search range was

set to 10 ppm for precursor ions, and the main search range set to 10 ppm and 0.02 Da for fragment ions. We specified carbamidomethyl on Cys as a fixed modification and TMT 10-plex labels modification and oxidation on Met as the variable modifications. We adjusted FDR to <0.05.

#### 2.5.5. Bioinformatic analysis

We performed functional annotation of proteins and matching and enrichment analysis of gene ontology (GO) and cluster of orthologous groups (COG) of proteins ($P < 0.05$). We performed functional annotation and matching and enrichment analysis of GO and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG) for differentially expressed proteins.

### 2.6. Statistical analysis

We carried out all experiments and analyses in triplicate, and values were represented as the mean ± standard deviation (SD). Analysis of variance (ANOVA) with Tukey comparison was performed to measure the significance of differences and ($p < 0.05$).

## 3. Results

### 3.1. Effect of CA storage on 2-AP content in DHX

The changes in 2-AP content of DHX under three conditions are shown in Table 1. The initial 2-AP content in the rice was 0.2912 mg $kg^{-1}$. The content decreased with the storage time, and the 2-AP content decreased the fastest in the first month. Compared with the initial content, the 2-AP content decreased by 34.40 % in the control group, 27.20 % in the 98 % $N_2$ group, and 25.03 % in the 35 % $CO_2$ group in the first month. Subsequently, during the storage period of 1–3 months, the changes in the 2-AP content were as follows: the control group decreased by 14.45 %, the 98 % $N_2$ group decreased by 14.00 %, and the 35 % $CO_2$ group decreased by 0.01 %. The losses of 2-AP in the control and the 98 % $N_2$ group were 43.89 % and 37.40 %, respectively, during the 3-month storage period, whereas the loss in the 35 % $CO_2$ group was 25.65 %. These results indicated that storage in 35 % $CO_2$ reduced the deterioration of the 2-AP of the rice.

### 3.2. Effect of CA storage on the content of MDA and the activity of enzyme in DHX

As shown in Fig. 1a, the MDA content of DHX stored under CA increased by 0.4 nmol/L from 0 d to 90 d, whereas that of DHX stored under conventional conditions increased by 1.8 nmol/L. At day 60 of storage, the MDA content of DHX stored under 35 % $CO_2$, 98 % $N_2$, and conventional conditions reached its peak, which were 3.5 nmol/L, 3.4 nmol/L, and 5.4 nmol/L, respectively, and then decreased by day 90.

As shown in Fig. 1b, the AMS activity of rice decreased with increasing storage time. During the 0- to 90-day storage period, the AMS activity of the 35 % $CO_2$ group, 98 % $N_2$ group, and control decreased by 0.4 U $mL^{-1}$, 0.3 U $mL^{-1}$, and 0.7 U $mL^{-1}$, respectively. The decrease in the CA group was significantly smaller than that in the control. According to Fig. 1c, the POD activity of rice gradually decreased with the storage time. During the 0- to 90-day storage period, the range of POD activity changes in the control was 15.80–9.99 mU/L, whereas the range of POD activity changes in the 35 % $CO_2$ group and the 98 % $N_2$ group were 15.80–14.38 mU/L and 15.80–14.18 mU/L, respectively. The POD activity under CA storage was significantly higher than that under conventional storage, indicating that CA storage can delay the oxidation of rice by maintaining the POD activity.

According to Fig. 1d, the LOX activity of rice gradually decreased during the 0–90 days of storage. The range of LOX activity changes in the control was 400.84–283.05 U $L^{-1}$, whereas the range of LOX activity changes in the 35 % $CO_2$ group and 98 % $N_2$ group was 400.84–380.27 U $L^{-1}$ and 400.84–384.52 U $L^{-1}$, respectively. As shown in Fig. 1e, the range of BADH activity change in the control was 710.16–395.03 IU/L from 0 to 90 days. The BADH activities of the 35 % $CO_2$ group and the 98 % $N_2$ group changed consistently from 0 to 90 days. The BADH activity of the 35 % $CO_2$ group decreased with a range of 710.16–656.80 IU/L and the BADH activity of the 98 % $N_2$ group decreased with a range of 710.16–708.99 IU/L from 0 to 90 days. CA storage significantly maintained BADH activity.

**Table 1**
2-AP content in DHX under different storage conditions (mg $kg^{-1}$).

| Treatment/ month | Initial | 1 M | 2 M | 3 M |
|---|---|---|---|---|
| Control | 0.2912 ± 0.009a | 0.1910 ± 0.013b | 0.1865 ± 0.017bc | 0.1634 ± 0.013c |
| 98 %$N_2$ | 0.2912 ± 0.009a | 0.2120 ± 0.009b | 0.2063 ± 0.013b | 0.1823 ± 0.006c |
| 35 %$CO_2$ | 0.2912 ± 0.009a | 0.2183 ± 0.007b | 0.2174 ± 0.006b | 0.2165 ± 0.011b |

### 3.3. Functional analysis of protein in DHX

To explore the effect of CA on protein levels of rice during the storage, proteomic analysis was conducted on CA treatments and the control. By comparing the MS data with the rice protein library in the UniProt database (*Oryza sativa*), we initially identified 10,798 peptide segments, and after credible data filtering, we obtained 8,903 peptide segments and 2,078 proteins. Based on the GO classification method, which includes biological processes, cellular components, and molecular functions, we conducted functional enrichment analysis on the 2,078 selected proteins. The main enrichment categories of DHX protein function were cellular components, such as integral component of the membrane, cytoplasm, and nucleus, and molecular functions, such as adenosine 5′-triphosphate (ATP) binding, metal ion binding, and structural constituent of ribosome.

### 3.4. Analysis of differentially expressed proteins between CA storage and conventional storage of DHX

We compared the proteins identified in the 35 % $CO_2$, 98 % $N_2$, and control pairwise, with the comparison groups being $N_2$_vs_Control, $CO_2$_vs_Control, and $N_2$_vs_$CO_2$. The protein clustering analysis of $N_2$_vs_Control and $CO_2$_vs_Control is shown in Fig. 2a–b, with samples in the same group clustering because of their similar protein features, and samples in different groups separating because of their low similarity, which indicated significant differences and good repeatability between the CA and control.

We identified 110 different proteins in $N_2$_vs_Control, including 36 upregulated proteins and 74 downregulated proteins, and identified 162 different proteins in $CO_2$_vs_Control, including 59 upregulated proteins and 103 downregulated proteins. We identified two different proteins in $N_2$_vs_$CO_2$, both of which were downregulated. The volcano plots of the differentially expressed proteins in N2_vs_Control and CO2_vs_Control are shown in Fig. 3a–b.

We compared the $N_2$_vs_Control and $CO_2$_vs_Control groups and found 98 common differentially expressed proteins between the two groups—that is, differentially expressed proteins between the CA and the control, including 30 upregulated proteins and 68 downregulated proteins. The upregulated and downregulated differentially expressed proteins ((Log2 Fold Change > 1.0) information is listed in Supplementary Data (Tables 1 and 2), respectively. The upregulated differentially expressed proteins with higher-fold changes included uncoupling protein, F-box-like protein, probable(S)-ureidoglycine aminohydrolase, protein-serine/threonine phosphatase, stress-response A/B barrel domain-containing protein, NAC-A/B domain-containing protein, cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDHX; fragment),

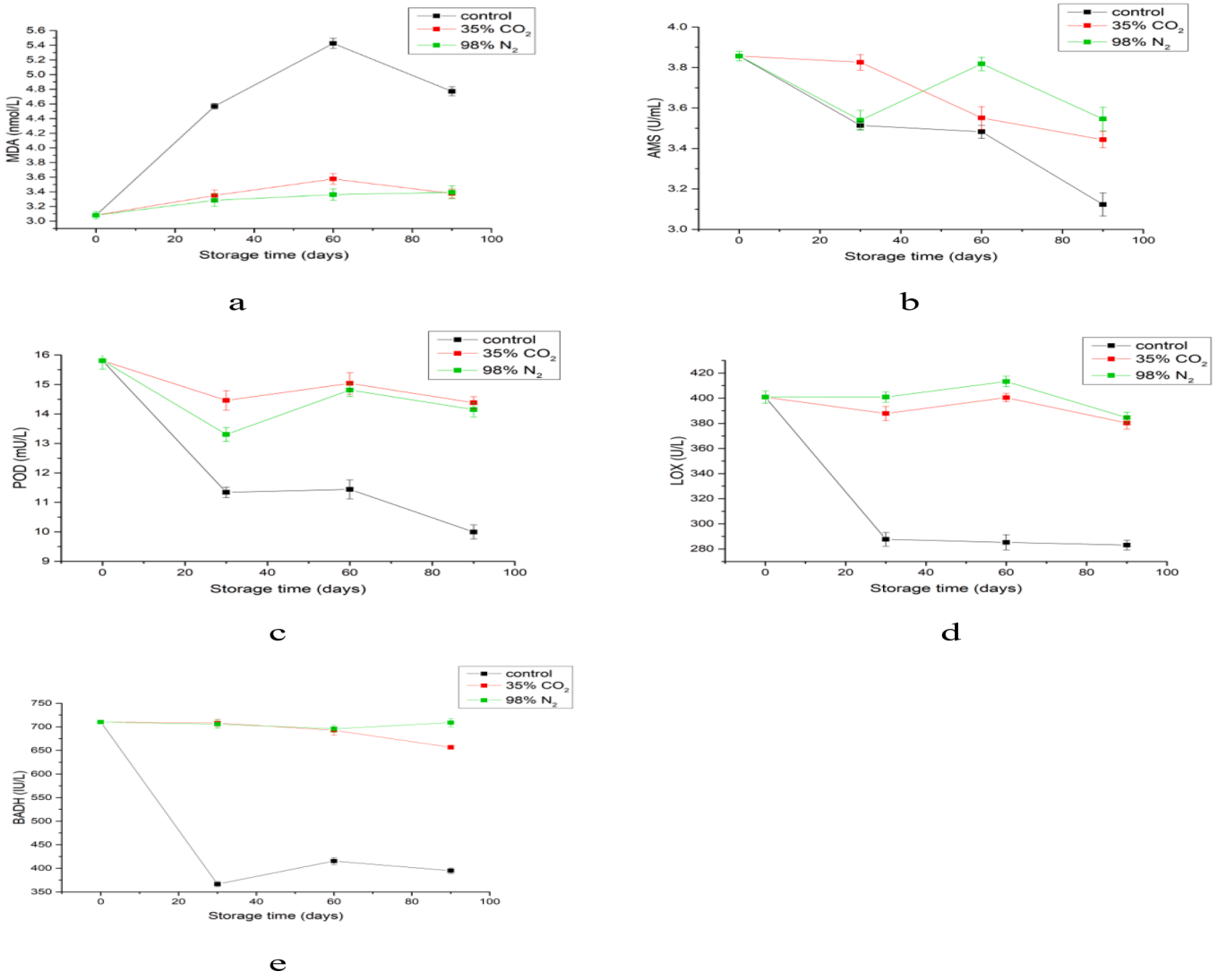


**Fig 1.** Changes in MDA content and enzyme activity of Daohuaxiang during storage. a. MDA; b. AMS; c. POD; d. LOX; e. BADH.

assimilatory sulfite reductase (ferredoxin), and superoxide dismutase, among others. The downregulated differentially expressed proteins with higher-fold changes included 60S ribosomal protein, late embryogenesis abundant (LEA) protein, nonspecific lipid-transfer protein, differentiation embryo protein, zinc knuckle domain-like protein, cold-shock domain protein, rapid alkalinization factor-like protein, glycine-rich protein, and bowman-birk type bran trypsin inhibitor, among others.

The differentially expressed proteins between $N_2$_vs_$CO_2$ were annexin protein and co-chaperone hsc20 family protein, expressed (Fragment), both of which were downregulated differentially expressed proteins. We inferred that only a little difference existed in the proteome of rice stored in $N_2$ and $CO_2$. The differentially expressed proteins in $CO_2$_vs_Control included most of the differentially expressed proteins in $N_2$_vs_Control, especially the downregulated differentially expressed proteins. Some upregulated differentially expressed proteins, however, were identified only in the $CO_2$_vs_Control. The upregulated differentially expressed proteins with higher-fold changes in the $CO_2$_vs_Control included UTP-glucose-1-phosphate uridylyltransferase, phosphoglycerate kinase, nucleoside diphosphate kinase, MPN domain-containing protein, glutathione transferase, glutathione transferase (GST) *N*-terminal domain-containing protein, and enoyl-[acyl-carrier-protein] reductase [NADHX] 1,chloroplastic-like.

We performed GO enrichment analysis on the differentially expressed proteins in the $N_2$_vs_Control with molecular function categories, including ATP binding, adenyl nucleotide binding, adenyl ribonucleotide binding, purine nucleotide binding, ribonucleotide binding, anion binding, nucleotide binding, carbohydrate derivative binding, organic cyclic compound binding, and heterocyclic compound binding (Fig. 4a). Further KEGG pathway enrichment analysis of the differentially expressed proteins in the $N_2$_vs_Control revealed that the metabolic pathways mainly involved ribosome, ubiquitin-mediated proteolysis, sulfur metabolism, terpenoid backbone biosynthesis, proteasome, phagosome, purine metabolism, and oxidative phosphorylation (Fig. 4b).

We performed GO enrichment analysis on differentially expressed proteins between $CO_2$_vs_Control, with functional categories including biological process and molecular function, such as macromolecule localization, organic substance transport, transport, establishment of localization, biological process, ATP binding, adenyl nucleotide binding, and adenyl ribonucleotide binding (Fig. 4c). Further enrichment analysis of differentially expressed proteins between $CO_2$_vs_Control using KEGG pathways revealed that the metabolic pathways primarily included ribosome, oxidative phosphorylation, ubiquitin-mediated proteolysis, biotin metabolism, folate biosynthesis, sulfur metabolism, fatty acid biosynthesis, terpenoid backbone biosynthesis, protein export, and proteasome (Fig. 4d).

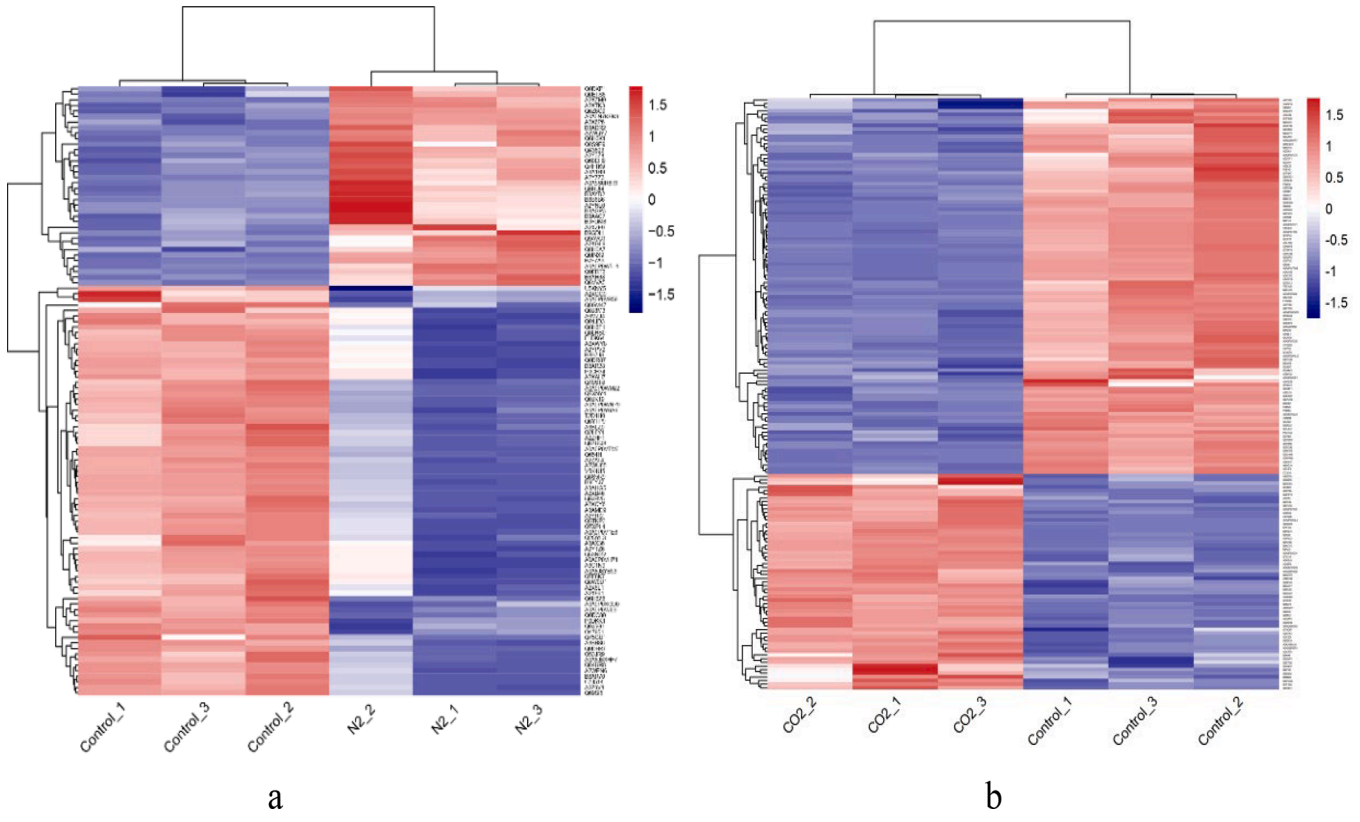


**Fig 2.** The protein clustering analysis of a. $N_2$_vs_Control and b.$CO_2$_vs_Control.

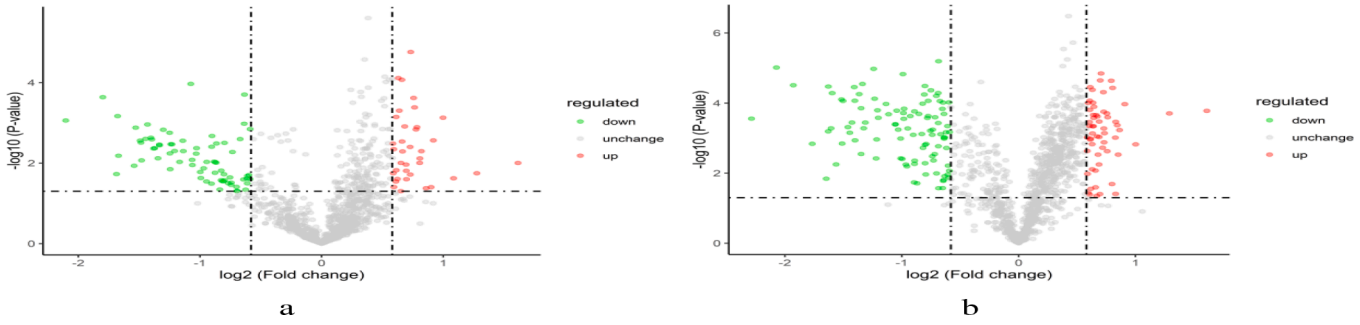


**Fig 3.** The volcano plots of the differentially expressed proteins in a. $N_2$_vs_Control and b. $CO_2$_vs_Control.

## 4. Discussion

Related research has indicated that the ratios of 2-AP can be used as aging markers of aromatic rice (Zhao, Yousaf, Xue, & Shen, 2020). The main mechanism leading to 2-AP loss during the storage of aromatic rice is related to the storage conditions. Zhao's (2020) research demonstrated that high-temperature storage could cause a decrease of 2-AP in DHX. Other studies, however, also found that the extent of 2-AP reduction was similar in the short-term storage of aromatic rice, regardless of storage conditions (partial vacuum or atmospheric pressure) or the form of rice (paddy, hulled, or milled rice) (Widjaja, Craske, & Wootton, 1996). The results of this study showed that compared with 98 % $N_2$-CA storage, 35 % $CO_2$-CA storage was more effective in reducing the loss of 2-AP in rice. The expression and composition of proteins in biological organisms constantly changed, and different environments could induce changes in gene expression products, resulting in differential proteins and leading to differences in the accumulation of secondary metabolites. Therefore, proteomics research could unravel the mechanisms underlying the regulation of aromatic rice's aroma in 35 % $CO_2$-CA storage.

### 4.1. Reduce protein synthesis, degradation, and transportation of rice

Proteomics results have revealed significant differences in protein expression levels between 35 % $CO_2$-CA and conventional storage, with more downregulated differentially expressed proteins than upregulated differentially expressed proteins. Compared with conventional storage, the main functions of the downregulated differentially expressed proteins in 35 % $CO_2$-CA storage were cell differentiation, embryonic development, and protein export, including LEA protein, nonspecific lipid-transfer protein (nsLTP), differentiation embryo protein, and zinc knuckle domain-like protein (Supplementary Data Tables 1 and 2), which were involved in metabolic pathways, such as ribosomes, ubiquitin-mediated proteolysis, and oxidative phosphorylation (Fig. 4).

The main function of ribosomes is to convert genetic code into amino acid sequences and build protein aggregates from amino acid monomers (Caleb, Embree, & Guramrit, 2022). In this study, proteins were downregulated in the ribosomes, indicating that protein synthesis in

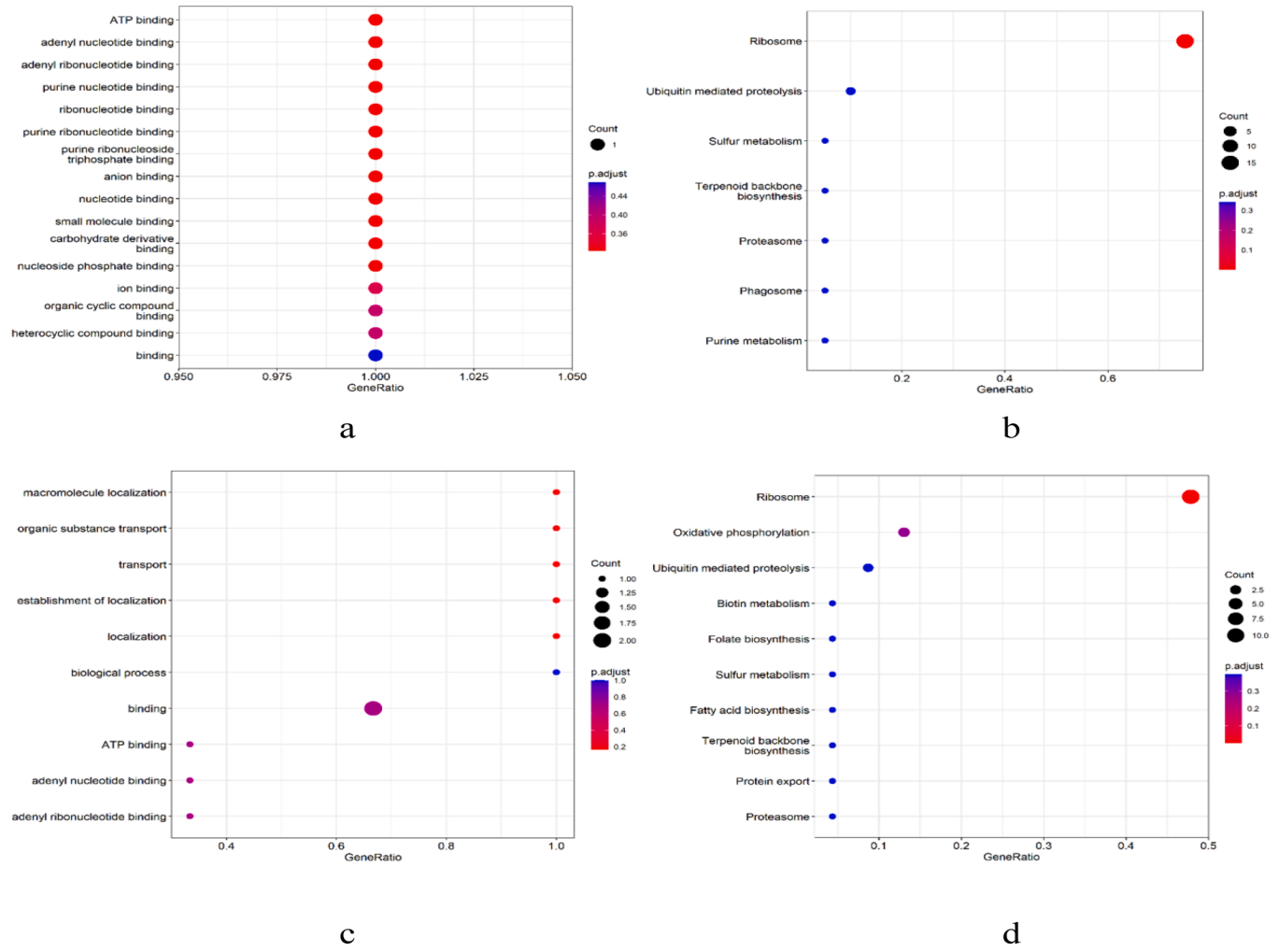


**Fig 4.** A. go function enrichment of differentially expressed proteins in $N_2$_vs_Control; b. KEGG function enrichment of differentially expressed proteins in $N_2$_vs_Control; c. GO function enrichment of differentially expressed proteins in $CO_2$_vs_Control; d. KEGG function enrichment of differentially expressed proteins in $CO_2$_vs_Control. Note:The higher the value on the abscissa, the higher the degree of differential protein enrichment in the corresponding pathway. Dot color represents the p-value of the hypergeometric test; smaller values reflect increased test reliability and greater statistical significance. The size of the dot represents the number of differential metabolites in the corresponding pathway; larger numbers indicate that more differential proteins were identified in the corresponding pathway.

DHX was reduced during 35 % $CO_2$-CA storage (Fig. 5). Ubiquitin-mediated proteolysis is an important degradation pathway. In this study, 26S proteasome regulatory particle triple-A ATPase subunit 6 (OsRPT6) had a significant impact on the differential protein expression between 35 % $CO_2$-CA storage and the control. The 26S proteasome is the main molecular machine responsible for protein degradation in eukaryotic cells (Jennifer, Warnock, Jobin, & Robert, 2023), which participates in almost all biological activities of organisms by selectively degrading target proteins (Jennifer et al., 2023). Ubiquitin-mediated protein degradation requires a lot of energy consumption, and therefore the degradation of protein were reduced during 35 % $CO_2$-CA storage. nsLTP in plants are a class of small, alkaline proteins with low molecular weight that exhibit in vitro activity transferring phospholipids, glycolipids, and fatty acids between membranes (Khawla et al., 2022). The downregulation of this protein under 35 % $CO_2$-CA storage would reduce processes, such as protein localization and organic substance transport. Decreased abundance of several proteins involved in protein synthesis, degradation, and folding also has been shown in strawberry stored under CA conditions (Li et al., 2015).

### 4.2. *Reduce the energy metabolism of rice*

Oxidative phosphorylation is the coupled reaction in which the energy released during oxidation in vivo is supplied to ADP and inorganic phosphate to synthesize ATP through the respiratory chain. In this study, 35 % $CO_2$-CA storage put DHX in a low-oxygen environment, which resulted in the downregulated expression of some differentially expressed proteins involved in these pathways (Fig. 5). Therefore, GAPDH was upregulated during CA storage. GAPDH is a key enzyme in the glycolytic pathway, and DHX obtained energy from the degradation metabolism of sugars through the glycolytic pathway during 35 % $CO_2$-CA storage. Wang et al. (2019) also found that ATP-dependent 6-phosphofructokinase and other proteins related to the glycolysis of broccoli head were downregulated in 50 % $CO_2$-CA storage. Additionally, they suggested that glycolysis pathway metabolism increased along with an increase in $CO_2$ concentration (Wang et al., 2019). During the low oxygen storage of DHX, the pathways involved in energy production also included sulfur metabolism, which was influenced by sulfite reductase [ferredoxin], chloroplastic-like, a major protein whose expression was upregulated during 35 % $CO_2$-CA storage. Sulfite reductase is a key enzyme in the conversion of sulfite to sulfide, which participates in assimilatory sulfate reduction, a process by which organisms obtain

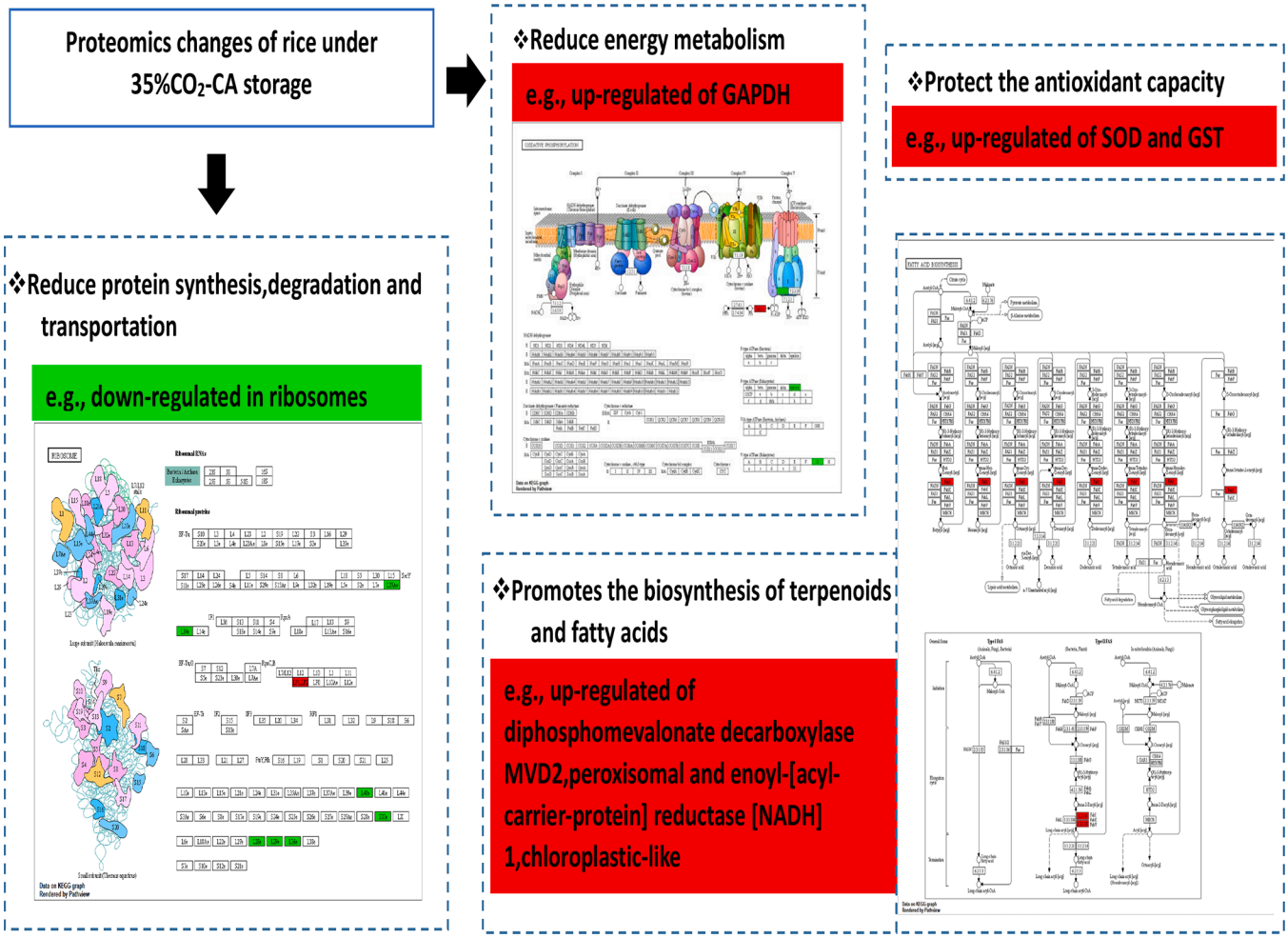


**Fig 5.** Proteomics changes of rice under 35 %$CO_2$-CA storage.

energy under anaerobic conditions (Nakayama, Akashi, & Hase, 2000). The enzyme's function is to catalyze the dehydrogenation of the substrate, and the hydrogen is then passed through the respiratory chain, ultimately being accepted by sulfate as the terminal hydrogen receptor. The coupling of this hydrogen transfer with oxidative phosphorylation generates ATP (Nakayama et al., 2000).

### 4.3. *Protect the antioxidant capacity of rice*

Superoxide dismutase (SOD) is an important component of the antioxidant enzyme system in living organisms, the primary function is to regulate oxidative metabolism and to remove free radicals and slow down oxidation. GST is a key enzyme in the glutathione binding reaction and is critical in the defense system of living organisms. The upregulation of SOD and GST suggested that a 35 % $CO_2$ atmosphere played an important role in protecting the antioxidant capacity of rice and was beneficial for maintaining enzyme activity, which was consistent with the small changes observed in the activities of MDA, AMS, LOX, POD, and BADH during the CA storage (Fig. 1). MDA is one of the most abundant and representative active aldehydes among the secondary products of lipid oxidation during food storage, originating from various unsaturated fatty acids, with strong protein oxidation denaturation-inducing ability and is a marker of lipid oxidation stress (Chen, Han, McClements, & Decker, 2010). The increase and subsequent decrease in MDA content of DHX during storage was due to the large amount of free fatty acids produced during storage, which rapidly degraded to form MDA, resulting in an increase in its content. Because aldehyde are volatile, the MDA generated later exist in the form of volatile odor, resulting in a decrease in its content. In this study, 35 % $CO_2$-CA storage inhibited MDA increase and effectively delayed lipid oxidation reactions. AMS is an *endo*-enzyme that hydrolyzes straight-chain starch into glucose and maltose, and hydrolyzes branched-chain starch into glucose, maltose, and dextrin. The activity level of AMS is the main factor affecting grain quality (Huang et al., 2020). Additionally, it has been found that 2-AP in rice exists in both free and bound forms with amylopectin, and high activity of AMS can hydrolyze rice starch to produce 2-AP and generate aroma (Yoshihashi, Huong, Surojanametakul, Tungtrakul, & Varanyanond, 2005). During storage, however, the activity of AMS in grains decreased because of their own respiration and metabolic activities (Huang et al., 2020). In this study, 35 % $CO_2$-CA storage reduced the respiration of the DHX and thus delayed the decrease in AMS activity, thereby reducing the loss of 2-AP. The reason why the 2-AP content in this study could be maintained also may have been because BADH activity was stably maintained in a CA environment. The high BADH activity of the badh gene (a key gene for 2-AP synthesis) in aromatic rice catalyzed the oxidation reaction of betaine aldehyde in the cytoplasm, thereby promoting the accumulation of the 2-AP (Prodhan & Qingyao, 2020). Qu et al. (2023) also found that MDA levels were reduce and activities of antioxidant enzymes were improved under CA storage.

### 4.4. *Promote the biosynthesis of terpenoids and fatty acids of rice*

In our study, we found OsRPT6 to be upregulated in molecular functions, such as carbohydrate-derived compound binding, organic cyclic compound binding, and heterocyclic compound binding. Therefore, diphosphomevalonate decarboxylase MVD2, peroxisomal, was

upregulated under CA storage (Fig. 5). Diphosphomevalonate decarboxylase MVD2, peroxisomal, was involved in the mevalonic acid pathway, which is a metabolic pathway that uses acetyl-CoA as a raw material to synthesize isopentenyl diphosphate and dimethylallyl diphosphate. Isopentenyl diphosphate is a precursor to the synthesis of volatile carotenoids, monoterpenes, and diterpenes. The upregulation of this protein during 35 % $CO_2$-CA storage can increase the synthesis of terpenoid compounds in rice.

Enoyl-[acyl-carrier-protein] reductase [NADHX] 1, chloroplastic-like, which participates in the synthesis of long-chain fatty acids, such as acetylation, butyrylation, octanoic acid, decanoic acid, lauric acid, myristic acid, and palmitic acid, was a significantly differentially expressed protein between 35 % $CO_2$-CA and control (Fig. 5). The expression of this protein was upregulated during 35 % $CO_2$-CA storage, which led to an increase in the accumulation of long-chain fatty acids. LOX activity in this study was not rapidly inhibited as in other literature (Shen, Zhang, Devahastin, & Guo, 2019). The LOX enzyme can hydrolyze unsaturated fatty acids with a *cis*-cis-1,4-pentadiene structure, producing small volatile compounds that play an important role in the defense system and flavor formation of plants (Li et al., 2015). The metabolic pathway of LOX is relatively complex, however, and the hydrogen peroxide generated by catalysis is unstable and begins to decompose only when its concentration reaches a certain level, further generating unpleasant taste compounds, such as hexanal (Hu et al., 2020). In the initial stage of storage, the LOX pathway of fatty acid β-oxidation metabolism is a key source of precursor compounds for producing aromatic volatile esters in many foods (Yue et al., 2022).

In addition, under conditions of $CO_2$ storage, UTP-glucose-1-phosphate uridylyltransferase expression was upregulated. This enzyme was involved in sugar metabolism and synthesized UDP-glucose from glucose-1-phosphate. A decrease in this enzyme may have led to a decrease in the soluble solid content of stored rice, and therefore, a 35 % $CO_2$ atmosphere could reduce the loss of soluble solids during storage, slowing down the decline in the quality of rice for consumption. The same result was also found in the CA storage of strawberry (Li et al., 2015).

## 5. Conclusion

CA storage can reduce the loss of 2-AP of rice, especially for 35 % $CO_2$, and the loss rate of 2-AP was 50 % lower than that of conventional storage. We conducted an analysis of the proteomics change of DHX. The mechanism of this change regulates the oxidative metabolism of rice, 26S proteasome regulatory particle triple-A ATPase subunit6, superoxide dismutase, and glutathione transferase; other proteins are upregulated, thereby improving the antioxidant capacity of rice, maintaining enzyme activity, and reducing the loss of characteristic aroma substances. By regulating terpene main chain biosynthesis and fatty acid synthesis, the expression of UTP-glucose-1-phosphate uridyltransferase, hydroxyvalerate diphosphate decarboxylase MVD2, peroxisomal, enoyl-[acyl carrier protein] reductase [NADH] 1, chloroplast protein, and other proteins was upregulated. This process promoted the synthesis of soluble solids, terpenes, and long-chain fatty acids in rice.

## CRediT authorship contribution statement

**Yu Jie:** Conceptualization, Methodology, Writing – original draft. **Tianyu Shi:** Project administration, Writing – review & editing. **Zhongjie Zhang:** Supervision.

## 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101005.

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## Further reading

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