Effects of high temperature on rice grain development and quality formation based on proteomics comparative analysis under field warming

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Abstract

With the intensification of global warming, rice production is facing new challenges. Field evidence indicates increased temperature during rice grain-filling lead to a further deterioration of grain quality. Clarifying the potential regulation mechanism of elevated temperature on rice development and quality formation will be contributed to develop suitable cultivation measures to better cope with climate warming in the future. In this study, open field warming and DIA mass spectrometry were conducted to explore the regulatory effects of high temperature on pathways related to grain development and material accumulation during the formation of rice quality. 840 differentially expressed proteins (fold change > 2, p-value < 0.05) were identified when exposed to high temperature. Among these, prolamin PPROL 14E, PSB28, granule-bound starch synthase 1 and 26.7 kDa heat shock protein were the most significantly regulated, and that ultimately affected the main substances accumulation of starch and protein in the kernel, and further degraded rice quality under high temperature. In addition, the results provided novel targets involved in regulating the metabolism of storage compounds under warming environment, and that will help us to better understand the regulation mechanism of global warming on the formation of rice quality.

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Key words: rice; global warming; proteomics; data-independent acquisition; grain quality

1.Introduction

Rice is the staple food for more than 50% of world's population. With the improvement of people's living standards, high-quality rice has become more preferred by the rice production and consumption market. However, with the rapid development of industrialization, human activities are estimated to have caused approximately 1.0°C (a likely range of 0.8°C to 1.2°C) of global warming above pre-industrial levels (IPCC, 2018). According to the fifth assessment report (AR5) completed by the IPCC (Intergovernmental Panel on Climate Change), the global temperature is expected to rise by 1.4-5.8°C in 2100 (IPCC, AR5, 2014). This abnormal high temperature would seriously affect the normal growth and development rhythm of rice and ultimately affect yield and quality of rice (Xu et al., 2020; Jagadish, 2020). In particular, results of our 10-year field trials showed that rice quality formation generally exhibits negative response when exposed to high temperature. The increase of temperature leads to the significantly increased chalkiness and decreased milling quality of rice, which could extremely reduce the purchase expectation and the market value of rice (Dou et al., 2017). Therefore, rice production will have to face the challenges of high temperatures exacerbated by the intensification of climate warming in the future.

High temperature usually affects the key processes of rice growth and development, including germination, seedling growth, leaf emergence, tillering, heading and maturity stage (Hakata et al., 2012; Lin et al., 2010; Shi et al., 2016; Tsukaguchi and Iida, 2008). Among them, as the decisive stage of rice quality formation, grain-filling is the most sensitive period to external temperature. Grain-filling is the process of grain development and accumulation of storage materials e.g. starch, storage proteins and lipids, which ultimately determines the rice quality-related indicators. An increase in temperature at this stage would induce the decline in rice quality, including reduced milled rice fraction, significantly increased chalky rate and chalky area (Shi et al., 2013 Jagadish et al., 2015). As the most abundant components in rice grain, starch had been proved to be sensitive to increased temperature. Our previous study showed that the accumulation of total starch and amylose in early grain-filling stage was accelerated under the condition of increased temperature, but the accumulation speed in later stage was significantly decreased, which resulted in the lower content of amylose and total starch in mature grain compared to normal temperature treatment (Tang et al., 2019). Furthermore, high temperature during grain-filling could increase the contents of grain storage proteins, with a significantly increased composition of glutelin and decreased prolamin, and ultimately improved the nutritional quality of rice. However, rice with high protein content is more susceptible to deterioration during storage, and the appearance and eating quality of rice could be further declined (Cao et al.,2017). In addition, high temperature can enhance the activity of protease and accelerate the protein transformation into soluble nitrogen compounds such as amino acids, which would significantly increase the total amount of amino acids and the relative content of each component in rice grain. Overall, high temperature mainly accelerates the rate of grain-filling, but shortens its active duration, resulting in the insufficient accumulation of photosynthetic substances in rice grains (Kim et al., 2011; Wahid et al., 2007). In our previous research, this change was manifested in abnormal grain development caused by high temperature and the accumulation balance change of starch and storage proteins, which coordinately determines the formation of grain quality (Tang et al., 2018; Dou et al., 2017). Although we have obtained the physiological and biochemical evidence of high temperature in regulating grain storage material accumulation through field trials, the regulatory mechanism remains to be further clarified. The anabolism of rice starch and protein is a relatively complex process, including a series of metabolic pathways, synthesis, transport, modification, accumulation and other processes. Therefore, we intend to conduct the investigation and clarification of the key regulatory factors that participate in the above process during the warming process through high-throughput methods, which would help us to further understand the regulatory effects of high temperature on the main pathways of rice quality formation.

In-depth understanding of the regulation mechanism of warming on the synthesis and metabolic pathways

of grain storage materials has important practical significance for further establishing high-quality rice cultivation methods under climate warming. Thus, the main purpose of this study is to further evaluate the critical period of temperature regulation of grain-filling and to clarify the key regulatory factors involved in grain quality formation under low-amplitude warming scene in the actual paddy field.

2. Materials and methods

2.1 Experimental site description

The experiment was conducted at the Danyang Experimental Base of Nanjing Agricultural University (31°56'39"N, 118deg59'13"E, 80m). The test site belongs to the main high-yield rice cultivation area in the lower reaches of the Yangtze River in China. The climate belongs to the subtropical monsoon climate and the soil condition is loam with a pH value of 6.04. The total soil nitrogen was 1.4 g*kg^{-1}, the available nitrogen was 7.8 mg*kg^{-1}, the available phosphorus was 20.1 mg*kg^{-1} and the available potassium was 91.7 mg * kg^{-1}. The temperature and precipitation in the experiment are listed in the supplementary data.

2.2 Plant materials and temperature treatment

The high-quality rice (*Oryza sativa L.*) variety Wuyujing 3 (W3) was used as the plant material for its sensitivity of the quality formation to the increased temperature and the large area of cultivation in the experimental region. In order to evaluate the individual and co-impact effects of the increased temperature, field treatments were conducted as follows: (i) normal temperature (CK); (ii) elevated temperature (ET). Temperature treatment was carried out with the free-air temperature enhancement system (FATE) from full heading (80% of the rice in the field starts the heading stage). Each treatment was designed with 3 replications and 80cm intervals and protection lines were set up between the treatment blocks to ensure the independence of the experiment. Rice cultivation methods were adopted by seedling raising and artificial transplanting. Field management measures including fertilization, wet-dry alternate irrigation, prevention of pests and weeds were carried out according to the local high-yielding cultivation measures.

2.3 Field warming treatment

In order to realistically simulate the global warming scene, we have conducted elevated temperature experiment since 2009 through the FATE (free-air temperature enhancement) system in the rice field (Rehmani et al., 2011&2014; Dou et al., 2018; Tang et al., 2019). Warming treatment was performed by FATE with 12 ceramic infrared radiator heaters (FTE-1000-240-0-L10-Y; 1000W, 240V; 245 mm long x 60 mm wide) 1.2m above the rice canopy in each experimental plot. Angles of the heater in the horizontal and vertical directions are 45deg and 30deg to ensure the consistent and stable heating of the canopy and the effective area of infrared radiation is 1.5 x 1.5 x 3.1416 = 7.1m^2, respectively (Figure 1). Two sensors (Model HOBO U23-001) were installed and located at the canopy height in each plot to record the temperature and humidity changes of rice canopy. The HOBO was set to record the data every 30 minutes, and HOBO Pro software was used for data processing. Field meteorological data during the test period were collected from the meteorological station (WatchDog 550) installed ˜100 m away from the experimental field (Tang et al., 2019). Daytime and nighttime of rice canopy temperature were increased by 1.568 and 3.089 , respectively (Supplemental figure 1). Temperature increase range and trend were in accordance with the temperature range of global warming and the rule of asymmetric warming characteristics.

2.4 Sampling

Rice superior spikelets (located at the upper 1/3 of the rice panicle) flowering on the same day were tagged and sampled at 9:00 am on the 3rd, 6th, 9th, 12th and 15th day after flowering. The collected spikelets were immediately frozen in liquid nitrogen and stored at -80 deg for further analysis. All samples collected from the ET treatment were within the effective warming area (Figure 1). At the mature stage, 10 holes were randomly selected from each plot, and the number of spikelets per hole, the number of spikelets per panicle, 1000-grain weight, seed setting rate were investigated to calculate the final yield. Harvested spikelets were air dried under natural conditions for the determination of starch and protein content.

2.5 Measurement of starch and storage protein composition

Refined rice samples were milled into flour in the liquid nitrogen, and the starch was purified according to the instructions of Tran et al. (2011). The starch molecules are completely dissolved by the protease, sodium bisulfate, DMSO/LiBr (0.5%) ethanol solution, and the proteins, fats, and non-starch polymers are removed without starch degradation. Starches were further debranched with isoamylase and dissolved in DMSO/LiBr solution and physicochemical properties of starch were identified from the milled rice. The total starches were determined by using the protocols described in our previous study by Yang et al. (2016).

According to the solubility of protein components in different solvents, albumin, globulin, prolamin, and glutelin can be extracted with distilled water, dilute hydrochloric acid, ethanol, and dilute alkali in sequence, and the extracts are collected separately. The biuret colorimetric method was used to determine the remaining species using the Coomassie brilliant blue colorimetric method (Sapan et al., 1999).

2.6 Determination of rice appearance, milling, cooking and eating quality

For rice appearance quality, chalk characteristics of brown rice were observed by the cleanliness test-bed according to our previous studies (Tang et al., 2019). Rice grains were milled using a mini universal grinder and dried over a 200-mesh sieve. Use vernier calipers to determine the length, width, and thickness of brown rice, and calculate the ratio of length to width. 300 grains of brown rice were randomly selected to detect chalky rice grains, and calculate chalky rice rate based on the percentage of chalky rice in the total number. Chalkiness area is determined by the proportion of chalky grain area to total grain area.

Rice milling quality includes three indicators: brown rice rate, polished rice rate and polished rice rate. During the maturity period, 30 rice panicles with uniform maturity were randomly harvested. After threshing, the grains were naturally dried with the moisture content to 15%. Brown rice percentage (BRP) and milled rice percentage (MRP) were determined by the processing machinery SY88-TH & SY88-TRF (Wuxi Shanglong Grain Equipment Co., Ltd., China), respectively.

Rice cooking and eating quality was determined by the RVA-4500 (a rapid viscometer developed by Newport scientific instruments, Australia). Weigh 3.00g rice flour with moisture content of about 14.0% into aluminum box, add 25ml distilled water, and stir it up and down rapidly for 10 times with an agitator to make the rice flour disperse evenly. The determination of RVA characteristic parameters for rice flour was programed as follows: the rice flour solution sample was heated at 50 for 1 min, then heated to 95 within 3.8 min, heated at 95 for 2.5 min, then cooled to 50 within 3.8 min, and finally heated at 50 for 1.4 min. The characteristic parameters of RVA spectrum include: peak viscosity (PKV), hot paste viscosity (HPV), cooling paste viscosity (CPV), breakdown viscosity (BDV), and depletion value (setback viscosity, SBV).

2.6 Protein enzymatic hydrolysis

Appropriate amount of protein sample was transferred into a 1.5ml centrifuge tube and added with 5mm steel bead and appropriate amount of Lysis Buffer 3, added PMSF with a final concentration of 1 mM, and EDTA with a final concentration of 2 mM. Vortex and let stand for 5 minutes, add DTT with a final concentration of 10 mM. Oscillate with a tissue grinder for 2 minutes (Frequency=50HZ). 25000g centrifugation at 4 degC for 20 minutes and add 10 mM DTT to the supernatant and water bath at 56 degC for 1 hour. After returning to room temperature, 55 mM IAM was added and incubated in dark room for 45 minutes. Add cold acetone by 4 times volume of the sample, and stand at -20 degC for 2h. Repeat previous step until the supernatant is colorless. 25000g centrifugation at 4 degC for 20 minutes and discard the supernatant. Add an appropriate amount of Lysis Buffer 3 to precipitate, followed by ultra-sonication to dissolve the precipitated proteins. After centrifugation at 25000 g * 4 degC for 20 minutes, the supernatant was taken for quantification.

For protein extraction quality control, standard protein (0.2 μg / μl BSA) was added sequentially to the 96-well plate A1 to A10. 0, 2, 4, 6, 8, 10, 12, 14, 16, 18μl, then add pure water 20, 18, 16, 14, 12, 10, 8, 6, 4, 2μl, add each well MAS Bright Blue G-250 Quantitative Working Solution 180 μl. The OD595 was measured with a microplate reader, and a linear standard curve was prepared based on the OD595 and

protein concentration. Dilute the protein solution to be tested several times, add 180 μl of the quantitative working solution to 20 μl of the protein solution and read OD595. The sample protein concentration was calculated based on the standard curve and sample OD595. Each 10 μg of protein solution was mixed with an appropriate amount of loading buffer, heated at 95 for 5 minutes, centrifuged at 25,000 g for 5 minutes, and the supernatant was poured into a well of a 12% SDS polyacrylamide gel. 120V constant pressure electrophoresis for 100 minutes; After electrophoresis, the gel was stained with Coomassie brilliant blue for 2 hours, then decolored with appropriate amount of decoloring solution (40% ethanol 10% acetic acid) on the shaker every 30 minutes, in total 3˜5 times.

100 μg of protein solution per sample and dilute with 50mM NH_4HCO_3 by 4 times volumes. Add 2.5 μg of Trypsin enzyme in the ratio of protein: enzyme =40:1, and digest for 4 hours at 37 °C. Add Trypsin once more in the above ratio and continue to digest for 8 hours at 37 . Enzymatic peptides were desalted using a Strata X column and vacuumed to dryness (Gillet et al., 2012; Rost et al., 2014).

2.7 High pH RP separation

All samples were taken 10 ug respectively to mix, and 200 ug mixture was diluted with 2 mL of mobile phase A (5% ACN pH 9.8) and injected. The Shimadzu LC-20AB HPLC system coupled with a Gemini high pH C18 column (5 um, 4.6 x 250 mm) was used. The sample was subjected to the column and then eluted at a flow rate of 1 mL/min by gradient: 5% mobile phase B (95% CAN, pH 9.8) for 10 minutes, 5% to 35% mobile phase B for 40 minutes, 35% to 95% mobile phase B for 1 minute, flow Phase B lasted 3 minutes and 5% mobile phase B equilibrated for 10 minutes. The elution peak was monitored at a wavelength of 214 nm and component was collected every minute. Components were combined into a total of 10 fractions, which were then freeze-dried.

2.8 DDA Spectral Library and DIA analysis by nano-LC-MS/MS

Data-Dependent Acquisition fractions and DIA samples analysis were both performed on a Q Exactive HF X mass spectrometer (Thermo Fisher Scientific) coupled with an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific). A nano-LC column (150 μm × 30 cm, 1.8 μm, 100 Å) was packed in-house for peptide separation at a flow rate of 500 nl/min. For DDA analysis, peptides were loaded to a C18 trap colullmn (300 μm × 5 mm, 5 μm, Thermo Scientific) with buffer A (2% ACN, 0.1% FA) for 5 min, then eluted with a gradient from 5% to 25% buffer B (98% ACN, 0.1% FA) for 155 min, 25% to 30% buffer B for 10 min, and 30% to 80% buffer B for 5 min. The mass spectrometry parameters were set as below: MS scan range 350-1500 m/z; loop count 30; NCE 28; MS resolution 120 000, maximal injection time (MIT) 50 ms; MS/MS HCD scans with resolution 30 000, MIT 100 ms; dynamic exclusion duration 30 s; isolation window 2.0 m/z; intensity threshold 2.0 e4; charge exclusion, exclude 1, 7, 8, >8. For DIA analysis, the same nano-LC system and gradient was used as DDA analysis. The DIA MS parameters were set as below: full scan range 400-1250 m/z at resolution 120000 with MIT 50 ms; DIA isolation window was set to 17 m/z with loop count 50 and automatic MIT, scanned at resolution 30000; stepped NCE: 22.5, 25, 27.5; AGC target 1e6.

2.9 Bioinformatics database

The proteins were classfied into three categories, biological process, cellular compartment and molecular function, by gene ontology annotation derived from the NCBI database (www.http: //www.ebi.ac.uk/GOA/). Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathway. Gene lists were analyzed using the STRING database to identify the predicted interactions of proteins.

3. Results

3.1 Effects of increased temperature on rice quality and accumulation of grain storage materials

The temperature increase of 1.6 to 3.1 during grain-filling stage induced various changes in the accumulation of storage materials during grain development, and further affected the rice yield and quality indicators. Results showed that tested rice yield in warming treatment was 21% lower than that of the normal temperature treatment (data not shown). Meanwhile, another significant change caused by warming is the reduction

in rice quality. Results indicated the increase in temperature resulted in the increased grain thickness and decreased grain length. There was no significant change in grain width, and aspect ratio of grain shape. Compared with natural temperature, warming treatment significantly increased rice chalky rate, chalky area and chalkiness. Overall, the increase in temperature leads to overall deterioration in the appearance quality of rice. In addition, elevated temperature has a significant effect on milled rice rate and head rice rate. Compared with the natural temperature, the milled rice rate and head rice rate were decreased significantly. Increased temperature has led to the deterioration of the appearance quality of rice. The increase in temperature also caused significant changes in the rice cooking quality indicators. Gelatinization characteristics peak viscosity (PKV), hot-paste viscosity (HPV), and gelatinization temperature (GT) were significant increased and the final viscosity was decreased (Table 1).

Starch and storage proteins are the main substances that constitute the grain storage material. Under elevated temperature conditions, grain total starch was increased significantly, of which amylopectin was significant increased when responded to high temperature, while amylose decreased significantly compared with normal temperature treatment, and that further led to changes in the proportion of amylopectin /amylose. The response of grain storage protein components to high temperature appears as a significant increase in glutelin and a significant decrease in prolamin. There results suggested high temperature during grain-filling stage has a general negative impact on rice quality parameters.

3.2 Quantitative expression of rice grain proteins under elevated temperature

In order to further explore the regulation mechanism of the effects of increased temperature on rice quality, we conducted the dynamic proteomic analysis of the rice grains in the early stage of grain filling and a total of 23968 unique peptides and 5872 unique proteins (Supplementary Table 1). The expression and annotation proteins identified in each period were listed and Pearson correlation analysis showed the repeatability of these protein samples was above 97%. Furthermore, we enriched all proteins with COG and GO to perform functional analysis, and results showed that the effect of elevated temperature was mainly in regulating the translation, post-translational modification, protein conversion and signal transduction during the grain-filling stage.

Differentially expressed proteins (DEPs) were defined as proteins with a FOLD CHANGE [?]2 and p VALUE < 0.05. In the present study, DEPs were identified at least twice in three biological replicates and had the same change trend. Based on these criteria, 112 DEPs were found in ET-3d (3d after heading under elevated temperature treatment) and CK-3d (3d after heading under normal temperature treatment) groups, of which 66 were upregulated and 46 were downregulated (Figure 2). In ET-6d and CK-6d treatments, 118 DEPs were identified, of which 51 were up-regulated and 67 were down-regulated. Comparing to the CK-9d, 65 proteins were up-regulated and 201 proteins were down-regulated in the ET-9d. In ET-12d and CK-12d treatments, 144 DEPs were identified, of which 59 were up-regulated and 85 were down-regulated. In addition, 200 DEPs were found during the 15d after heading, including 30 up-regulated and 170 down-regulated proteins. The volcano plots and protein annotation of DEPs in ET and CK (3d, 6d, 9d, 12d and 15d) treatments are shown in Figure 3 and Supplementary Table 2. According to the GO enrichment analysis, the prominent GO terms for CC enriched by five stages were the cell, cell part, organelle. Identified proteins were predominantly distributed in metabolic process, cellular process and single-organism process. Based on the molecular function, the DEPs were mainly classified into catalytic activity, binding, transporter activity and structural molecule activity (HT vs CK 3d, 6d, 9d 12d). The top GO MF categories that were enriched by HT-6d and CK-6d DEPs, including the catalytic activity, binding, enzyme regulator activity and transporter activity (Figure 4).

Differentially expressed proteins were further classified into five stages with KEGG pathway. Differentially metabolic process were defined as pathway with p VALUE < 0.05. The proteome of both the treatments revealed changes in major metabolic pathways. The major metabolic pathways in HT-3d and CK-3d were photosynthesis-antenna proteins, metabolism of xenobiotics by cytochrome P450, drug metabolism-cytochrome P450, photosynthesis, axon guidance, retinol metabolism (Figure 5). In HT-6d and CK-6d, the main pathways were homologous recombination, AMPK signaling pathway, inositol phosphate metabolism,

plant hormone signal transduction, NF-kappa B signaling pathway, ether lipid metabolism, MAPK signaling pathway-plant. Among these, the metabolic pathways enriched in HT-9d and CK-9d mainly include mannose type O-glycan biosynthesis, porphyrin and chlorophyll metabolism, tryptophan metabolism, ABC transporters, phenylpropanoid biosynthesis, isoflavonoid biosynthesis, other types of O-glycan biosynthesis, limonene and pinene degradation. Pathway enrichment analysis of HT-12d and CK-12d DEPs identified significantly enriched in ribosome, mitophagy-yeast, homologous recombination, C5-Branched dibasic acid metabolism. Moreover, two major metabolic pathways, fructose and mannose metabolism, and indole alkaloid biosynthesis were identified in HT-15d and CK-15d. Results suggested that temperature had significantly different regulatory effects on different stages of grain development.

3.3 Identification of differentially expressed proteins related to rice development and quality formation

A total of 748 unique proteins were identified based on their specific expressions when exposed to high temperature. After removing 302 proteins with lower scores and unknown functional classification, 39 proteins related to rice development and quality formation were distinguished (Table 2, Figure 6). In this study, the expression of 16.9 kDa class I heat shock protein in rice grains was significantly reduced 3 days after heating, which was down-regulated by 84% compared with CK treatment. However, the expression of heat shock factor binding protein 1 involved in heat shock protein synthesis was significantly increased. Furthermore, the expression levels of HSP70s at early grain-filling stages were only about half of normal treatment (0.47, 0.5, and 0.43 times reduction at 3d, 6d 9d after flowering, respectively). Results showed the most significant change was the 26.7 kDa heat shock protein of the sHSPs family, and its expression was increased significantly at 6, 9, and 12 days after flowering, with 6.78, 3.06, and 5.44 folds compared to the CK treatment. At the 12th day after flowering, the expressions of 18.0kda class II heat shock protein, 24.1kda heat shock protein and heat shock protein 82 were up-regulated to varying degrees (2.26, 2.01, 3.63 folds respectively) when subjected to high temperatures.

Expression of proteins related to rice quality formation were also regulated under field warming conditions. Expressions of glutelin anabolism proteins were regulated at the beginning of grain-filling. For example, the glutelin type-A 3, glutelin type-B 1, glutelin type-B 5-like expression levels of rice grains were significantly up-regulated by 2.79, 2.49, and 3.3 folds, respectively. At the same time, the expression levels of glutelin type-B 1-like and glutelin type-B 2-like proteins were decreased significantly. Furthermore, the expression levels of globulin 19 kDa globulin, globulin 1s allele and basic 7S globulin were increased significantly, which is consistent with the increase of globulin content measured in mature grains. In this study, the significant decrease in prolamin under warming conditions was mainly at the 12th day after flowering and expression analysis of prolamin PPROL 14E and prolamin PPROL 14E-like proteins showed that they were both decreased significantly (0.33, 0.38 folds) at this stage comparted to the normal temperature treatment. As the main component of rice grains, the accumulation of rice grain starch has been proved to be sensitive to elevated temperature. The synthesis of rice grain starch is mainly regulated by a series of protein families, including SSS, SBE, DBE, GBSS. In this study, the GBSS was down-regulated at the 6th day after flowering under increased temperature. While proteins related to amylopectin synthesis obtained no significant changes when compared to the CK. The expression levels of granule-bound starch synthase at 6d, 9d and 12d after flowering were significantly lower than that of the control and the expression levels of soluble starch synthase responsible for the synthesis of amylopectin SS4 and SSS2-3 were decreased under high temperatures.

Results of this study showed that another key growth and development process regulated by the increased temperature during grain-filling stage was the photosynthetic system of rice plant. In this study, the expression levels of the chlorophyll a-b binding protein 1B-21, chlorophyll a-b binding protein P4, and chlorophyll a-b binding protein 7 in chlorophyll ab binding protein were significantly up-regulated at the beginning of grain-filling (2.22, 2.03, 3.3 times), when compared to the CK. On the other hand, the expression of PSB28, which is responsible for water splitting, had a downward trend through the grain-filling period, and reaching a significant level at 12 days after flowering. Furthermore, protein TIC 62 (translocon at the inner envelope membrane of chloroplasts) responsible for the dynamic balance of the proteome was significantly downreg-

ulated at 9d after flowering. Photosynthesis is the process by which light energy is converted into chemical energy and stored, and thus it is essential for the accumulation of rice grain assimilation.

4. Discussion

4.1 Assessment of field warming and the impact on rice quality formation

Since 2009, we have continuously carried out warming experiments in rice fields based on the open-field free air temperature enhancement system (FATE), and the effects of increased temperature on rice growth and development were guardedly studied. The ultimate goal of our study is to find key regulatory factors or metabolic pathways involved in rice growth process and apply appropriate field cultivation measures to reasonably respond to the challenge of climate warming on rice. The FATE device is suspended above the field and uses 12 sets of ceramic infrared heaters to perform uniform heating in an area of $7.1m^2$. In the fully activated state, the daytime canopy temperature of rice can be increased by 2.4 on the basis of natural temperature, and the rice canopy night temperature can be increased by 5.4. This warming range is within the prediction of possible temperature increase by 1.4-5.8 °C at the end of the 21st century by IPCC (IPCC, AR5, 2014). Furthermore, the increase in night temperature is significantly greater than that during the day and that is consistent with the asymmetric trend of climate warming (Pachauri et al., 2014). Compared with closed or semi-closed warming scenario, the warming method and effect adopted in this project could closely simulate climate warming characteristics and that provides a more reliable platform for us to conduct related experiments in the actual field.

Rice quality is a complex characteristic, including appearance, milling, nutrition, cooking and eating quality. Our field evidence shows that the overall temperature increase has a relatively negative effect on rice quality, including significantly increased chalky rate, chalky area and chalkiness. Meanwhile, the milling quality indicators milled rice rate and head rice rate were decreased significantly, and that would exceedingly reduce the market recognition of rice. Our previous researches have been devoted to exploring the mechanism of the influence of increased temperature on rice quality and to our knowledge, the changes of external temperature inevitably affect the morphological composition and structure of the grain storage material, and that further induce the changes of related quality traits (Dou et al., 2017; Dou et al., 2018; Tang et al., 2018; Tang et al., 2019). Among these attributes, eating and cooking quality (ECQ) is one of the most important indicators, especially from the consumer's perspective. The eating quality refers to the sensory perception of consumers on rice, and is related to the gloss, flavor, and viscosity of rice. Although the physical and chemical properties of starch in rice endosperm can be used as an indirect indicator of ECQ, it is still a difficult task to assess ECQ through these traits. At the same time, the increase of glutelin content in rice grain is particularly obvious under the condition of increased temperature, which leads to a change in the overall balance of grain storage materials and has a negative impact on the taste and appearance quality of the rice.

4.2 Overview of DIA quantitative proteomics analysis

Due to the complex mechanism of rice quality formation, this study conducted the proteomics analysis on physiological pathways such as starch synthesis and metabolism, storage protein accumulation, and plant photosynthesis under warming conditions, and to identify the key regulation factors in pathways related to temperature response. In recent years, proteomics-based mass spectrometry has made significant progress from sample preparation to liquid chromatography and instrument detection, making it possible to identify more specific expressed proteins in cells or tissues with excellent accuracy and repeatability (Tsou et al., 2015). Data independent acquisition (DIA) is widely used in proteomics analysis due to its higher protein coverage rate and reliable data acquisition ability (Renaud & Sumarah, 2016, Searle et al., 2015). Compared with iTRAQ, the advantage of DIA technology is that it can effectively measure protein molecules with extremely low abundance in complex samples, which greatly improves the reliability of quantitative analysis and has high quantitative accuracy and repeatability. In this study, samples of interest went through mass spectrometry data collection in data dependent acquisition (DDA) mode. MaxQuant was then used to carry out database search identification process and obtain all detectable non-redundant high-quality MS/MS spectral information as DIA spectral library, which contains fragment ion intensity and retention time describing the

peak characteristics of the peptide, for quantification. Here, we identified 23968 unique peptides and 5872 unique proteins, which could be specifically regulated by the increased temperature during rice grain filling. Those DEPs coordinate and execute their biological behaviors based on their metabolic pathways. Therefore, KEGG pathway-based analysis would be contributed to further understand their biological functions. From our results, these identified specifically expressed proteins have large differences in temporal and spatial characteristics, which provided the obstacles to our further identification and screening of key regulators. Therefore, returning to the essential relationship between grain-filling and quality formation, we further screened the key proteins that we believe are specifically regulated by warming during the quality formation process from the perspectives of plant photosynthesis, grain starch and storage protein accumulations.

4.3 Key regulatory factors contributed to rice quality formation under elevated temperature

Photosynthesis is the process by which light energy is converted into chemical energy and stored, and it is also the source of accumulation of rice grain assimilation. Chlorophyll content and metabolic enzyme activity are closely related to the strength of photosynthesis. In our case, chlorophyll a-b binding protein 1B-21, chlorophyll a-b binding protein P4, and chlorophyll a-b binding protein 7 were significantly up-regulated, which induced the acceleration of the synthesis and binding of chlorophyll (Ballottari et al., 2012). Meanwhile, the expression levels of photosystem I reaction center subunit VI and oxygen-evolving enhancer protein 3 from the photosystem I were also increased significantly under warming conditions, and that may explain the accelerated grain filling rate during the early grain-filling stage induced by elevated temperature, and the significant increase in the accumulation rate of grain materials compared with the normal temperature treatment. However, the expression of PSB28, which is responsible for water splitting, had a downward trend throughout the period, obtaining a significant low level at 12d after flowering under elevated temperature (0.4 folds). That may inhibit electron transfer and weakens signal transmission, thereby weakening photochemical reactions and resulting in decreased cell chlorophyll and photosynthesis (Wada et al., 2019). Previous research shows that the optical system II (PS II) is the most sensitive element to temperature in the electron transmission chain (Zhang et al., 2011). It would be interesting to further investigate whether PSB28 could be the most critical component affected by high temperature during the photosynthesis process.

To our knowledge, the contents and ratio of starch and storage protein in rice grains are the decisive factors in determining the final rice quality. Rice starch synthesis is regulated by various enzymes, including SSS, SBE, DBE and GBSS. GBSS is the main enzyme responsible for amylose synthesis. Wx protein encoded by the Waxy gene GBSS-I can tightly bind to the starch granules and promote the synthesis of amylose. High temperatures can lead to downregulation of gene expression that regulates GBSS synthesis, resulting in decreased amylose content and increased amylopectin content (Dian et al. 2005; Fujita et al. 2006). Research by Denver (1996) shows that GBSS is not only related to the synthesis of amylose, but also to the extension of amylopectin in starch granules. However, the exact effect of GBSS on the extension of normal starch granules is still unclear. Our results showed that the GBSS enzyme was down-regulated at 6d after flowering under elevated temperature. However, enzymes related to amylopectin synthesis did not change significantly. From 6d to 12d after flowering, the expression level of granule-bound starch synthase was significantly lower than that of the control. Under high temperature, the amylose content of mature rice grains was significantly lower than that of CK treatment, while the amylopectin content was significantly increased (Ahmed et al., 2015). Expression levels of the soluble starch synthase SS4 and SSS2-3, responsible for the synthesis of amylopectin, were also decreased under high temperatures (Yamakawa, 2012). This change may reduce the activities of granular starch synthase and soluble starch synthase, and lead to change in the ratio of amylose and amylopectin, which eventually affected the physical and chemical properties of starches in rice grain (Tang et al., 2019).

Rice storage proteins include albumin, globulin, glutelin and prolamin. Prolamin is directly deposited in the endoplasmic reticulum cavity in the form of intracellular protein particles, and finally buds from the endoplasmic reticulum in the form of spherical protein bodies (PBIs). While glutelin is efficiently converted into mature form by vacuolar processing enzymes, and forms irregular protein bodies II (PBII) together with

α-globulin (Krishnan et al., 1992; Kumamaru et al., 2010). The results of this study showed that warming had significant up- or down-regulation effects on the expression of storage protein family-related regulatory factors at different periods. For example, the expression of glutelin type-A and type-B proteins were either significantly up-regulated or down-regulated at 3d and 6d after flowering, and there is no obvious rule for the regulation mode of these regulatory factors under warming conditions. Based on our understanding of glutelin, its synthesis pathway is still unclear, and the presence of many unknown glutelin genes increases the difficulty in understanding the expression pattern under warming condition. Therefore, this study has not been able to essentially find the direct reasons for the changes in the final grain storage protein content.

However, several types of protein species (ribosomal protein species, superfamily II DNA and RNA helicase, and molecular chaperone IbpA) related to biosynthesis and processing of proteins was found to be affected by high temperature. Ribosomes are the primary sites for protein synthesis, and different species of ribosomal proteins play an essential role in translation, ribosome structure, and biogenesis in protein anabolism (Moin et al., 2016). In our study, the ribosomal protein species (25S, 30S, 40S, 50S and 60S) exhibited significant decreases during the middle stage of grain-filling, which may cause the reduction in the protein biosynthesis and maintain the balance between synthesis and degradation of proteins (Moin et al., 2016). The reduction in protein content related to translation, such as RNA recognition motif (RRM) domains, eukaryotic initiation factors (eIFs) and elongation factors (EFs), indicates the adverse effects of high temperature on rice protein synthesis. Furthermore, a series of molecular chaperone heat shock proteins (Hsps) were identified to be significantly up-regulated when exposed to high temperature. Heat shock proteins Heat shock protein is a highly conserved peptide in structure and could be activated and produced in large quantities when plants are subjected to abiotic stress (Timperio et al., 2008). In this study, the two most sensitive heat shock proteins are HSP70 and 26.7 kDa heat shock protein of the sHSPs (small heat shock proteins) family. Wang et al. (2014) found that overexpression of Hsp70 encoded gene could positively improve the tolerance of plants when subjected to high temperature stress. In our results, the expression level of HSP70 was decreased sharply at 9d, which in turn led to its inability to participate in the import and translocation of precursor proteins, and that further induced the disorders of rice protein synthesis in rice grains. The sHsps itself cannot refold unnatural proteins, but it has a significant effect in binding unnatural proteins, and forming a stable complex. The function of sHSP is similar to other ATP-dependent members such as Hsp70, thereby assisting the correct folding and configuration of the protein for further processing (Tabassum et al., 2020). Grain storage proteins such as glutelin precursors are synthesized in the endoplasmic reticulum, and must be folded and modified with the help of a series of molecular chaperones to form trimers, which are then transported out of the endoplasmic reticulum through vesicles, and eventually transported to protein storage vacuoles to form protein bodies (PB) II (Ren et al., 2020). During this process, the molecular chaperone heat shock protein family e.g. HSP70 / BiP in the endoplasmic reticulum can promote the correct folding of glutelin and keeps the protein stable during the folding and assembly process.

5. Conclusion

The formation of rice quality is a complex process. Our previous research found that elevated temperatures could deteriorate the rice quality under field warming condition mainly through inducing the imbalance ratio of starch and protein components. However, due to the complexity of the accumulation process of storage materials, which involves multi-level interactions between gene transcription, translation, protein folding and degradation, thus it is difficult to fully elucidate the mechanism of the effect of elevated temperature on rice grain formation. In this study, we identified a certain number of key proteins and metabolic pathways that are related to quality formation and sensitive to elevated temperature through field warming simulation and DIA quantitative proteomics method. These proteins could possibly be the key candidates in elucidating the potential regulation mechanism of high temperature on rice development and quality formation. Therefore, in-depth study on these targets will be contributed to clarify the mechanism of elevated temperature impact on rice quality, and provide new coping strategies for the deterioration of rice quality under climate warming.

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8. Conflicts of Interest

The authors declare that they have no conflict of interest.

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Table1 Effects of temperature on grain yield and quality traits of rice.

Treatment	Brown rice rate (%)	Milled rice rate (%)	Head rice rate (%)	——	——	——	——	
CK	85.9a	76.3a	75.61a	——	——	——	——	
ET	83.4b	73.31b	71.47b	——	——	——	——	
Treatment	Total starch (%)	Amylopectin (%)	Amylose (%)	Amylopectin /Amylose	Albumin (μg/g)	Globulin (μg/g)	Prolamin (μg/g)	Glutelin (μg/g)
CK	66.38b	54.95b	11.43a	4.81b	69.7a	65.4a	128.5a	836.3b
ET	70.17a	60.03a	10.14b	5.92a	68.2a	63.8a	113.4b	1092.7a
Treatment	Spikelets per panicle ($\times 10^4 \cdot hm^{-2}$)	Panicles per panicle	1000-grain weight (g)	Seed setting rate (%)	yield	——	——	——
CK	471.13a	114.17a	25.03a	95.45a	12.85a		——	——
ET	457.11b	104.53b	23.22b	91.47b	10.15b		——	——

Note: Values with different letters in the same column are significantly different with $p < 0.05$; CK, natural temperature; ET, increased temperature.

Table 2 Differentially expressed proteins under elevated temperature

	Protein_ID	PG_Cscore	Description	Days after flowering	fo
Molecular chaperone	1002227700	5.589271	16.9 kDa class I heat shock protein 3	3	0.
	1002274378	5.026661	heat shock factor-binding protein 1	3	2.
	1002249168	5.638757	26.7 kDa heat shock protein, chloroplastic	6; 9; 12	6.
	1002279871	5.834035	heat shock 70 kDa protein 16 isoform X1	9	0.
	1002296675	5.888584	heat shock 70 kDa protein, mitochondrial	9	0.
	1002244395	5.738268	heat shock 70 kDa protein 17	9; 12	0.
	1002231095	5.419388	18.0 kDa class II heat shock protein	12	2.
	1002244087	5.593509	24.1 kDa heat shock protein, mitochondrial	12	2.
	1002259909	5.077947	heat shock protein 82	12	3.
Storage protein	1002256234	5.534615	glutelin type-A 3	3	2.
	1002242479	5.804829	glutelin type-B 1	3	2.
	1002269601	5.323533	19 kDa globulin	3	2.
	1002238885	5.933567	glutelin type-B 5-like	3	3.
	1002239810	5.454091	glutelin type-B 1-like	3; 6	0.
	1002245900	5.419344	glutelin type-B 2-like	3; 6	0.
	1002239619	5.638689	glutelin type-B 2-like	6	0.
	1002248312	5.772556	globulin-1 S allele	9	3.
	1002288955	5.518718	glutelin type-A 1-like	9	9.
	1002268046	5.309461	prolamin PPROL 14E	12	0.
	1002268263	5.446809	prolamin PPROL 14E-like	12	0.
	1002266396	4.918256	basic 7S globulin	12	2.
Starch synthesis	1002273855	4.53732	alpha-amylase isozyme 2A	3	2.
	1002296409	5.016673	beta-amylase 2, chloroplastic isoform X1	3	0.
	1002280365	5.764317	granule-bound starch synthase 1	6	0.

	Protein_ID	PG_Cscore	Description	Days after flowering	fo
	1002230293	4.951311	probable starch synthase 4	6	0.
	1002282772	5.383006	alpha-amylase/trypsin inhibitor	6	0.
	1002282035	5.501471	alpha-amylase/trypsin inhibitor	6	0.
	1002284731	5.634974	alpha-amylase inhibitor 5	6; 9	0.
	1002292698	5.531977	alpha-amylase isozyme 3E	9	3.
	1002285817	5.765962	granule-bound starch synthase 1b	9; 12	0.
	1002279853	5.673613	soluble starch synthase 2-3	12	0.
Photosynthesis	1002280024	5.588999	chlorophyll a-b binding protein 1B-21	3	2.
	1002272608	5.337404	photosystem I reaction center subunit VI	3	2.
	1002284550	5.787986	oxygen-evolving enhancer protein 3	3	2.
	1002290793	4.661148	chlorophyll a-b binding protein P4	3	2.
	1002286185	4.714122	chlorophyll a-b binding protein 7	3; 9	3.
	1002298841	5.009246	protein TIC 62	9	0.
	1002224946	4.238946	photosystem II reaction center PSB28 protein	12	0.
	1002303125	5.539293	protochlorophyllide reductase B	15	0.

FIGURE LEGENDS

Figure 1 Actual field warming scene based on Free-air temperature enhancement (FATE) system. A: Aerial image of test field block B: Real scene of warming community C: System configuration diagram (The yellow rectangle is an infrared heating device; The white circle is HOBO; The red area is the warming range).

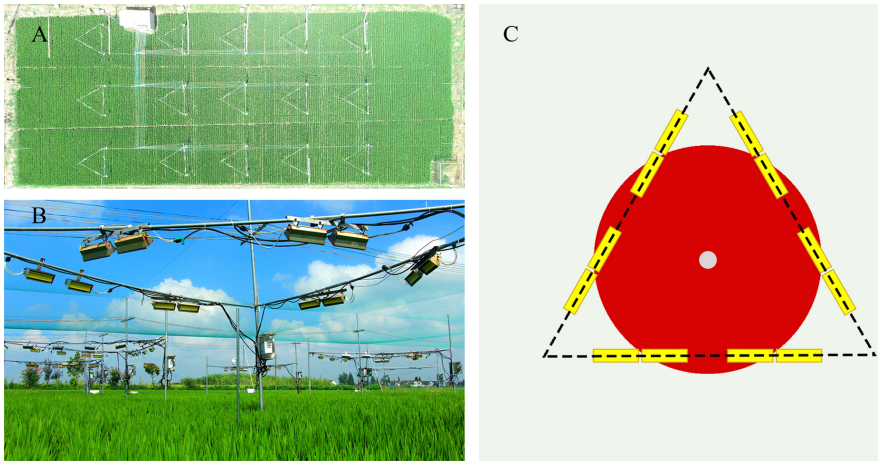
Figure 2 Proteins identified to be responsive to high temperature treatment. (A)Proteins distributed on the basis of their mass; (B) Numbers of unique peptides that were matched to proteins; (C) Pearson correlation coefficient; (D)GO function classification; (E)COG function classification.

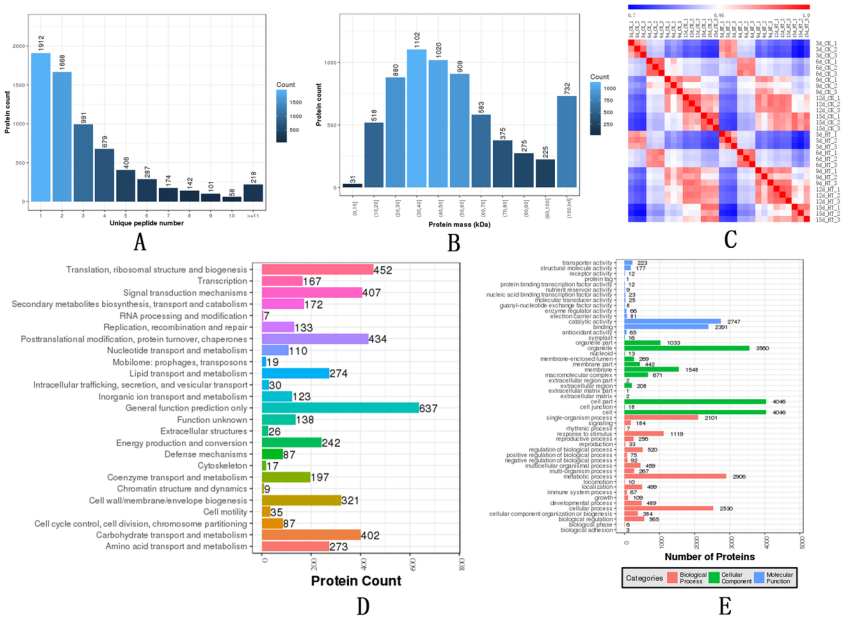
Figure 3 Volcano plots of proteins with differential expression under elevated temperature. (A) Number of DEPs in different periods. B, C, D, E, F are volcano plots of different expressed proteins on the 3rd, 6th, 9th, 12th and 15th days after flowering, respectively.

Figure 4 Gene ontology (GO) term enrichment for differentially expressed proteins under elevated temperature. A, B, C, D, and E,GO terms (including biological process, cellular component, and molecular function) enriched by proteins with differential expression in HT-3d VS CK-3d (A), HT-6d VS CK-6d(B), HT-9d VS CK-9d (C), HT-12d VS CK-12d(D), and HT-15d VS CK-15d (E) groups, respectively.

Figure 5 Enrichment of differentially expressed proteins in KEGG pathway under elevated temperature. A, B, C, D and E are the 3rd, 6th, 9th, 12th and 15th days after flowering, respectively.

Figure 6 Expression analysis of proteins related to quality formation pathways. A: Storage proteins; B: Molecular chaperone; C: Photosynthesis; D: Starch synthesis.


A
B
C


Protein count
Unique peptide number
1912
1669
991
679
406
287
174
142
101
88
218
A
Protein count
Protein mass (kDa)
31
518
880
1102
1020
909
583
375
275
225
752
B
Count
C
Translation, ribosomal structure and biogenesis
Transcription
Signal transduction mechanisms
Secondary metabolites biosynthesis, transport and catabolism
RNA processing and modification
Replication, recombination and repair
Posttranslational modification, protein turnover, chaperones
Nucleotide transport and metabolism
Mobilome: prophages, transposons
Lipid transport and metabolism
Intracellular trafficking, secretion, and vesicular transport
Inorganic ion transport and metabolism
General function prediction only
Function unknown
Extracellular structures
Energy production and conversion
Defense mechanisms
Cytoskeleton
Coenzyme transport and metabolism
Chromatin structure and dynamics
Cell wall/membrane/envelope biogenesis
Cell motility
Cell cycle control, cell division, chromosome partitioning
Carbohydrate transport and metabolism
Amino acid transport and metabolism
452
167
407
172
7
133
434
110
19
274
30
123
637
138
26
242
87
17
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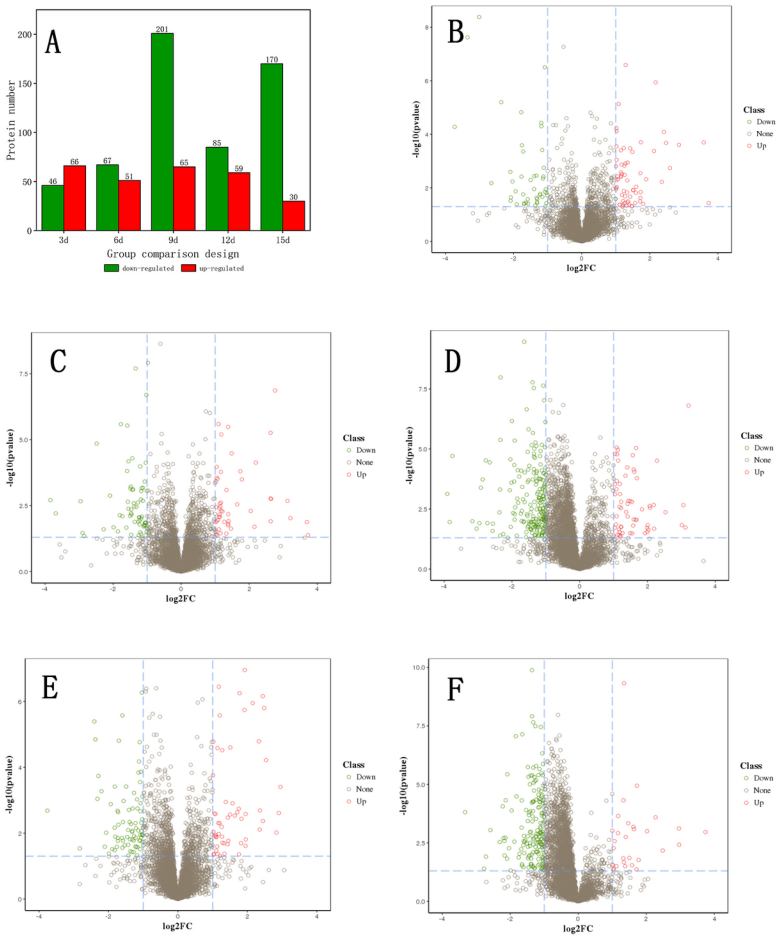
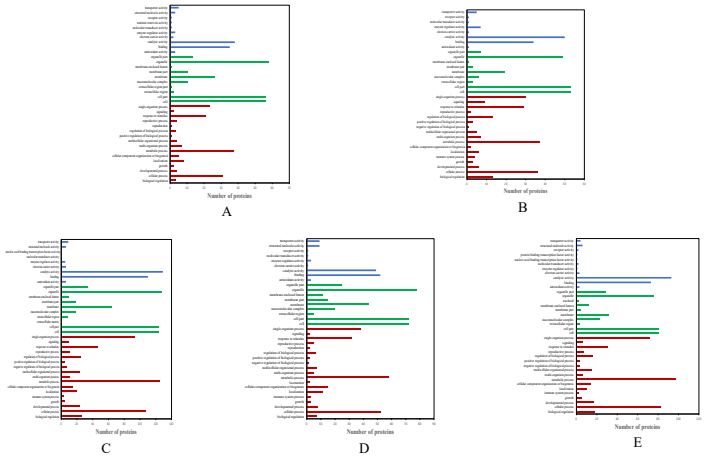
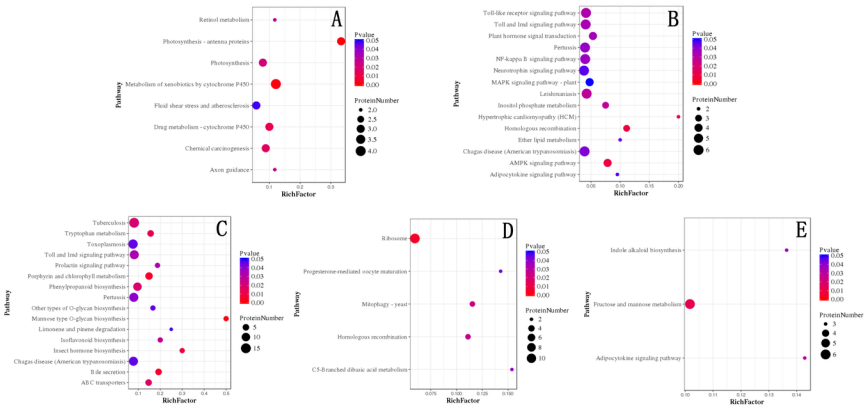
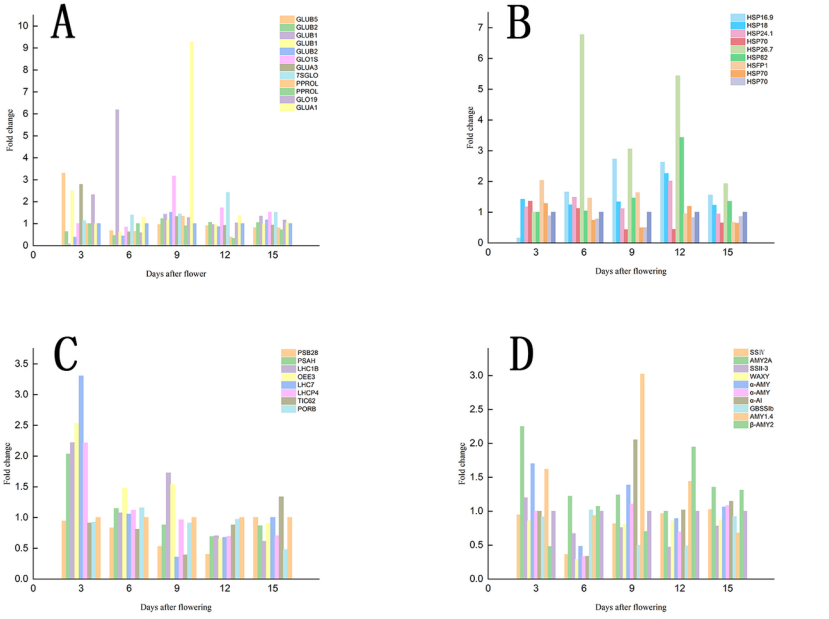
321
35
87
402
273
Protein Count
D
Number of Proteins
Categories
Biological Process
Cellular Component
Molecular Function
E


A
Protein number
Group comparison design
3d
6d
9d
12d
15d
46
66
67
51
201
65
85
59
170
30
down-regulated
up-regulated
B
C
D
E
F
-log10(pvalue)
log2FC
Class
Down
None
Up


Number of proteins
A
B
C
D
E


A
Retinol metabolism
Photosynthesis - antenna proteins
Photosynthesis
Metabolism of xenobiotics by cytochrome P450
Fluid shear stress and atherosclerosis
Drug metabolism - cytochrome P450
Chemical carcinogenesis
Axon guidance
Pathway
RichFactor
Pvalue
ProteinNumber
B
Toll-like receptor signaling pathway
Toll and Imd signaling pathway
Plant hormone signal transduction
Pertussis
NF-kappa B signaling pathway
Neurotrophin signaling pathway
MAPK signaling pathway - plant
Leishmaniasis
Inositol phosphate metabolism
Hypertrophic cardiomyopathy (HCM)
Homologous recombination
Ether lipid metabolism
Chagas disease (American trypanosomiasis)
AMPK signaling pathway
Adipocytokine signaling pathway
C
Tuberculosis
Tryptophan metabolism
Toxoplasmosis
Toll and Imd signaling pathway
Prolactin signaling pathway
Porphyrin and chlorophyll metabolism
Phenylpropanoid biosynthesis
Pertussis
Other types of O-glycan biosynthesis
Mannose type O-glycan biosynthesis
Limonene and pinene degradation
Isoflavonoid biosynthesis
Insect hormone biosynthesis
Chagas disease (American trypanosomiasis)
Bile secretion
ABC transporters
D
Ribosome
Progesterone-mediated oocyte maturation
Mitophagy - yeast
Homologous recombination
C5-Branched dibasic acid metabolism
E
Indole alkaloid biosynthesis
Fructose and mannose metabolism
Adipocytokine signaling pathway


A
GLUB5
GLUB2
GLUB1
GLUB1
GLUB2
GLO18
GLUA3
TSGLO
PPROL
PPROL
GLO19
GLUA1
Fold change
Days after flower
B
HSP16.9
HSP18
HSP24.1
HSP70
HSP26.7
HSP82
HSFP1
HSP70
HSP70
Fold change
Days after flowering
C
PSB28
PSAH
LHC18
OEE3
LHC7
LHCP4
TIC62
PORB
Fold change
Days after flowering
D
SSIV
AMY2A
SSII-3
WAXY
α-AMY
α-AMY
α-AI
GBSSIb
AMY1.4
β-AMY2
Fold change
Days after flowering