Article

Transcriptomic Analysis of Dehydration Rate of Mature Rice (Oryza sativa) Seeds

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Abstract: The transcriptomic analysis of the dehydration rate of mature rice seeds was conducted to explore candidate genes related to the dehydration rate and provide a theoretical basis for breeding and utilization. In this study, we selected Baghlani Nangarhar, an extremely fast dehydration material, and Saturn, a slow dehydration material, based on the results of the previous studies of screening of 165 germplasm materials for dehydration rate phenotypes. Fast dehydration experiment on these two types of seeds was conducted. Four comparative groups were set up under control and dehydration conditions. The differentially expressed genes (DEGs) were quantified by transcriptome sequencing and quantitative real-time PCR (qRT-PCR). GO and KEGG analyses were carried out. The results showed that in Baghlani Nangarhar, 53 DEGs were screened, of which 33 were up-regulated and 20 were down-regulated. Similarly, in Saturn, 25 DEGs were screened, of which 19 were up-regulated and 6 down-regulated. The results of the GO analysis showed that the sites of action of the differentially expressed genes enriched in the fast dehydration modes were concentrated in the cytoplasm, internal components of the membrane and nucleosomes. They play regulatory roles in catalysis, binding, translocation, transcription, protein folding, degradation and replication. They are involved in adaptive responses to adverse external environments such as reactive oxygen species and high temperature. The KEGG analysis showed that the main metabolic pathways enriched are protein processing in the endoplasmic reticulum, amino acid biosynthesis, and oxidative phosphorylation. The key differentially expressed genes and the most important metabolic pathways in the rapidly and slowly dehydrated materials obtained in this study were protein processing in the endoplasmic reticulum and oxidative phosphorylation metabolism, which are presumed to have important regulatory roles in stress/defense, energy metabolism, protein synthesis/folding, and signal transduction during dehydration and drying of mature seeds. The results of this study could potentially provide a valuable reference for further research on the genes and metabolic pathways related to the dehydration rate of mature rice seeds and provide theoretical guidance for the selection and breeding of new rice germplasm that can be rapidly dehydrated at the mature stage.

Keywords: Rice seeds; dehydration rate; RNA-seq; DEGs

1. Introduction

Rice is one of the most important food crops in China. Rice produces orthodox seeds, which can be dried to low moisture content and stored at low temperatures to prolong viability. A high moisture content of orthodox seeds at the time of maturity and harvesting decreases the viability and longevity of the seeds. The rate of dehydration directly affects the safe harvesting and rapid drying of seeds. Therefore, it is especially important to select varieties with low moisture content and fast dehydration rate of seeds at maturity to ensure seed quality and reduce production costs. Some progress has been made on different types of seed dehydration tolerance and dehydration rate, such as Arabidopsis [1], maize [2-3], wheat [4], coffee [5], Trichoderma [6], and sand mustard seeds [7]. Some QTL and genes related to the dehydration rate and dehydration tolerance of maize seeds have been obtained from molecular biology perspective [8-13]. However, further studies are needed on how to activate genes

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associated with seed dehydration tolerance, how dehydration genes respond to dehydration stress signals, and how gene co-expression network mechanisms work.

Transcriptomic analysis is currently an important approach to studying gene function. It can help to reveal the functions of various cellular tissue components, specific biological processes and the molecular functions exercised. It is now widely used in the study of rice, maize, oilseed rape and other crops. Transcriptomics has made some progress in plant dehydration tolerance. It was used to dynamically study the effect of abscisic acid (ABA) on the dehydration tolerance of Arabidopsis seed germination, and the results showed that mature Arabidopsis seeds were more likely to induce germination [14]. For Arabidopsis further microarray data from time series, a network of gene co-expression was established, including two regions: early response (ER) and late response (LR) [15]. A study of Arabidopsis seeds obtained two specific transcriptional regulatory networks of dehydration tolerance (TFsSeed-subNetDT1 and TFsSeed-subNetDT2) with storage compounds and cytoprotective mechanisms [16]. This regulation of key genes for dehydration tolerance is multiple under extreme dehydration drying conditions [17]. Transcription factors related to dehydration tolerance, such as CaHSFA9, CaDREB2G, CaANAC029, CaPLATZ, and CaDOG-like, were obtained through transcriptomics during the study of intermediate seed coffee [18]. Dehydration-associated gene co-expression clusters were found to be functionally enriched in seed development, and these genes are involved in pathways such as programmed cell death inhibition and activation of ABA signaling in a trans-metabolic network [19]. The use of transcriptomics to study cork oak seed dehydration sensitivity yielded 2219 DEGs. These differentially expressed genes are mainly related to hormone biosynthesis (IAA, ABA) and signaling (ZEP, PYR, YUC, ERF1B, ABI5, etc.), stress response proteins (HSP70, LEA D-29, etc.), and phospholipase D (PLD1) [20]. Seed desiccation sensitivity may be determined by these genes and their interactions. Transcriptional studies on the effect of exogenous ABA on the rate of dehydration in maize cob position leaves and ears were used to obtain 73 differentially expressed genes related to water metabolism, and these genes were hypothesized to be exogenous ABA-regulated downstream genes involving multiple transcription factors [21]. A combined multi-omics analysis was used to identify 143 genes significantly associated with maize kernel water content and kernel dehydration rate, which are closely related to biological processes such as starch and fatty acid biosynthesis, cold stress, and salt stress [22].

However, there are few studies on the dehydration tolerance of rice seeds after harvest, and there is no report of genetic study on the dehydration rate of mature rice seeds. In this study, we selected Baghlani Nangarhar from Afghanistan, an extremely fast-dehydrating material, and Saturn from the United States, a slow-dehydrating material, from the core germplasm of rice from 82 countries and regions, and we verified the quantitative expression of the screened differentially expressed genes by transcriptome sequencing and real-time quantitative PCR during seed dehydration at rice maturity. The purpose of the study is to elucidate the molecular mechanism of seed dehydration rate at rice maturity, to uncover the expression patterns and metabolic pathways of key differentially expressed genes affecting dehydration rate, and to provide a new theoretical basis for the creation of new varieties of sun-free rice.

2. Results

2.1. Dehydration rate performance of rice core seed of the fast and slow dehydration materials

The moisture content of rice seeds at physiological maturity was generally in the range of 20-30%, and gradually decreased with increasing maturity. The average initial moisture content at harvest was 19.0% for the temperate japonica type seed Baghlani Nangarhar and 18.3% for the intermediate type seed Saturn, with little difference in moisture content between them. The rate of dehydration became slower as the time of dehydration increased, and the maximum moisture reduction was achieved after 4 hours of dehydration, with the difference reaching a highly significant level, and becoming less after 12 hours (Figure 1). This indicates that the dehydration rate of material Baghlani Nangarhar was significantly faster than that of material Saturn.

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2.2. Transcriptome sequencing data statistics

Fast-dehydrating seeds Baghlani Nangarhar and slow-dehydrating seeds Saturn at physiological maturity were selected for transcription sequencing, and three replicates of 12 samples were set up for control group CNSF5-1, CNSF5-2, CNSF5-3, CNSF75-1, CNSF75-2, CNSF75-3 and treatment group NSF5-1, NSF5-2 NSF5-3, NSF75-1, NSF75-2, NSF75-3. Good-quality RNA was obtained from the 12 samples (Schedule 1). From Schedule 1, it can be seen that the concentration of RNA in all 12 samples ranged from 418-1395 ng/μl, 28S/18S from 1.50-2.20, A260/280 from 1.88-2.19, and the RIN values ranged from 7.60 to 9.60. There are three main reasons: (1) When we extract RNA, we add DNase I for DNA digestion. (2) The principle of transcription library construction technology is to use oligodT magnetic beads to specifically enrich mRNA with polyA. DNA does not have this feature, so it will not bind. If rRNA removal technology is used to build a library (such as Lnc RNA products), there is also a step of DNA digestion in the technical process. (3) Our second-generation RNA library construction and sequencing does not require OD value, but mainly focuses on RIN value, 28S/18S, and total amount. In addition, the highest absorption peak of DNA and RNA is at 260 nm, so the OD value cannot distinguish DNA and RNA, therefore nanodrop detection is relatively inaccurate for the quantification of DNA and RNA. These results indicate that the RNAs of all samples in this experiment are intact, free of impurities, and of good quality for sequencing library construction, and are ready for sequencing experiments. The GC content distribution results showed that there was no separation of AT/GC in each sample, and the GC content was 51.12% and above in all samples (Schedule 2). The base quality value (Q30) of each sample was greater than 93.73, and the average matching rate was 93.91% when compared with the sequence of the rice reference genome (Nihon Haru), and the average matching rate was 81.94% for the unique sequence comparison (Schedule 2, Schedule 3). The above results indicated that the library construction quality of transcriptional sequencing was good. The final 12 samples of whole transcriptome high-throughput sequencing each obtained an average of 1.18 Gb of clean sequences, and a total of 5633 non-repeat differentially expressed genes were detected.

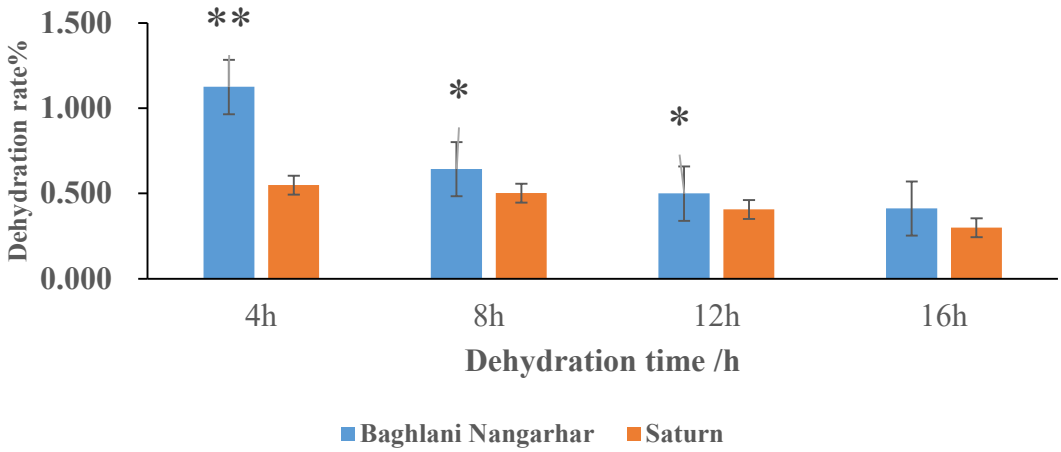

Figure 1 Variation of dehydration rate of rice seeds under fast dehydration conditions. *, Significant at P< 0.05; **, Significant at P< 0.01.

2.3. Identification of differentially expressed genes

As shown in Figure 2A, 53, 4307, 4736, and 25 DEGs were identified in the four comparative combinations CNSF5 vs. NSF5, CNSF5 vs. CNSF75, NSF5 vs. NSF75, and CNSF75 vs. NSF75, respectively. This indicates that the significant differences in gene expression levels not only occur between different treatments, but also between different varieties. In the comparison group CNSF5 vs NSF5, 33 genes up-regulated expression and 20 genes down-regulated expression were identified; in CNSF5 vs CNSF75, 1598 genes up-regulated expression and 2709 genes down-regulated expression were identified; in the comparison group NSF5 vs NSF75, 1941 genes up-regulated

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expression and 2795 genes down-regulated expression were identified; in the comparison group CNSF75 vs NSF75, 19 genes up-regulated expression and 6 genes down-regulated expression were identified (Figure 2A). Overall, NSF5 showed more up-regulated expression of DEGs compared to CNSF5 under fast dehydration conditions. In addition, more DEGs were present in the dehydrated fast material after rapid dehydration compared to the dehydrated slow rice material, so indicating that the rapid dehydration mode had a greater effect on the dehydrated fast material than the dehydrated slow material.

A total of 5633 non-duplicate DEGs were included in the four comparison groups. among them, CNSF5 vs NSF5 occupied 0.39% (22/5633), CNSF5 vs CNSF75 occupied 15.21% (857/5633), NSF5 vs NSF75 occupied 22.81% (1285/5633), and CNSF75 vs NSF75 occupied 0.089% (5/5633) (Figure 2B). Since the samples CNSF5 and CNSF75 in CNSF5 vs CNSF75 were not treated with rapid dehydration, the differentially expressed genes appearing within this combination were treated as DEGs between these two species (4307), and the differentially expressed genes in the remaining three comparison groups were treated as DEGs related to dehydration rate (1326).

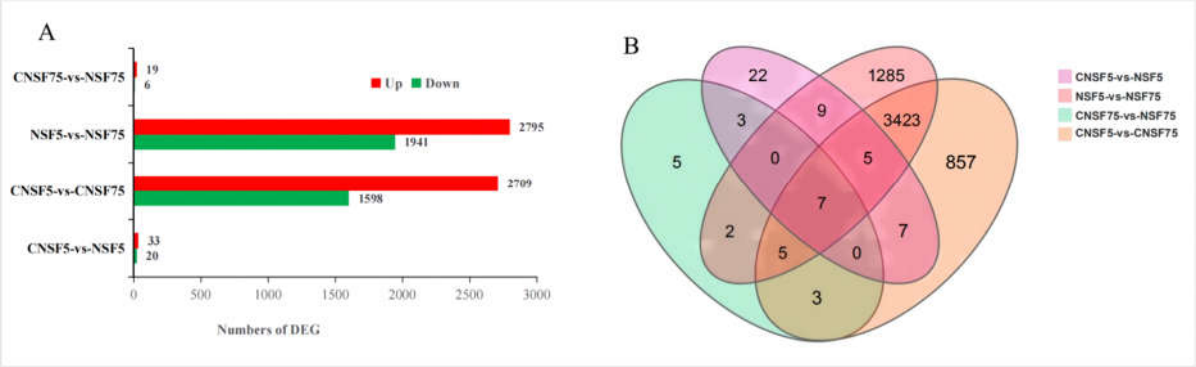

Figure 2. Overview of RNA-seq in the 4 comparison groups DEGs. A: Up and down of DEGs in four comparison groups. Red and green bars represent significantly up-regulated and down-regulated genes, respectively; B: The DEGs distribution of the Venn diagram in the 4 comparison groups.

2.4. GO enrichment analysis of differentially expressed genes

All DEGs were annotated with gene functions and classified into three major categories of Cellular component, Molecular function and Biological process by GO enrichment analysis. CNSF5 vs NSF5 has 53 DEGs, annotated into 38 entries in molecular functions, 28 entries in cellular fractions, and 46 entries in biological processes, for a total of 112 entries. The largest number of differentially expressed genes in molecular function is unfolded protein binding (GO:0051082, 7 entries); the largest number of DEGs in cellular fraction is cytoplasm (GO:0005737, 10 entries); the largest number of DEGs in biological process is pro-folded protein (GO:0005737, 10 entries). The entry with the highest number of DEGs in biological processes is protein folding (GO:0006457, 6 entries). From the GO enrichment results, it can be seen that the molecular functions performed by CNSF5 vs NSF5 are significantly enriched in (1) unfolded protein binding (GO:0051082, 7, Qvalue=7.56E-08), (2) protein self-association (GO:0043621, 4, Qvalue=8.95E-06); (3) heat shock protein binding (GO:0031072, 2, Qvalue=0.0177); the significantly enriched entries in cellular fractions are cytoplasm (GO:0005737, 10, Qvalue=7.81e-3); The entries significantly enriched in biological processes are (1) protein folding (GO:0006457, 6, Qvalue=3e-7), (2) response to heat (GO:0009408, 5, Qvalue=5.08E-07), (3) protein complex oligomerization (GO:0051259, 4, Qvalue=3.40E-07), (4) response to hydrogen peroxide (GO:0042542, 4, 6.76E-07), (5) response to reactive oxygen species (GO:0000302, 4, Qvalue=1.03E-06), (6) response to salt stress (GO:0009651, 4, Qvalue=1.02E- 04) (Figure 3 A, B, C, Schedule 4).

CNSF5 vs CNSF75 has a total of 4307 DEGs, annotated into 828 entries in molecular functions, 297 entries in cellular components, and 1109 entries in biological processes, for a total of 2234 entries. Among them, ATP binding (GO:0005524, 385) is the entry with the highest number of DEGs in molecular functions; the internal component of membrane (GO:00160, 813) is the entry with the

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highest number of DEGs in cellular components; regulation of transcription, DNA-templated, (GO:0006355, 150) are the entries with the highest number of DEGs involved in biological processes. As shown by the GO enrichment results, the significantly enriched entries in the molecular functions of CNSF5 vs CNSF75 are mainly (1) ADP binding (GO:0043531, 96, Qvalue=4.56E-05), (2) protein heterodimerization activity (GO:0046982, 31, Qvalue=0.0089), (3) UDP-glycosyltransferase activity (GO:0008194, 40, Qvalue=0.0139), (4) ligand-gated ion channel activity (GO:0015276, 11, Qvalue=0.0209). The significantly enriched entries in cellular components are nucleosome (GO:0000786, 31, Qvalue=1.75E-08), integral component of membrane (GO:0016021, 813, Qvalue=4.13E-06), plasma membrane (GO:0005886, 260, Qvalue=0.0173); entries significantly enriched in biological processes are (1) nucleosome assembly (GO:0006334, 19, Qvalue=0.0032), (2) defense response (GO:0006952, 92, Qvalue=2.61E-04), and (3) transmembrane transport (GO:0055085, 70, Qvalue=3.39E-04) (Figure 3 D, E, F).

NSF5 vs NSF75 has a total of 4736 DEGs, annotated into 858 entries in molecular functions, 331 entries in cellular fractions, and 1176 entries in biological processes, for a total of 2365 entries. The largest number of differentially expressed genes in these molecular functions was enriched in ATP binding (GO:0005524, 419); the largest number of DEGs in cellular components was enriched in the internal component of the membrane (GO:0016021, 872); and the largest number of DEGs in biological processes was enriched in ATP binding (GO:0005524, 419); The entry with the highest number of DEGs enriched in biological processes is regulation of transcription , DNA-templated, （ GO:0006355, 174). From the GO enrichment results, we can see that (1) ADP binding (GO:0043531, 99, Qvalue=4.99E-04), (2) UDP-glycosyltranserase activity, GO:0008194, 45, Qvalue=0.00192), (3) protein heterodimerization activity (GO:0046982, 34, Qvalue=0.00192), (4) ligand-gated ion channel activity(GO:0015276, 12, Qvalue=0.00848), (5) nutrient reservoir activity (GO:0045735, 26, Qvalue=0.0186), (6) quercetin 3-O- glucosyltransferase activity (GO:0080043, 16, Qvalue=0.0258), were the most significantly enriched entries in the molecular functions of the NSF5 vs NSF75 comparison group; nucleosome (GO:0000786, 27 entries, Qvalue=1.14E-04), integral component of membrane (GO:0016021, 872 entries, Qvalue=4.59E-04) are the entries significantly enriched in cellular components; while (1) nucleosome assembly (GO:0006334, 17, Qvalue=0.123), (2) transmembrane transport (GO:0055085, 76, Qvalue=0.123), (3) cell proliferation (GO:0008283, 7, Qvalue=0.123),,(4) plant-type primary cell wall biogenesis (GO:0009833, 12, Qvalue=0.123); (5) cellulose biosynthetic process (GO. 0030244, 13, Qvalue=0.152); (6) response to water deprivation (GO:0009414, 22, Qvalue=0.720) (Figure 3 G, H, I).

CNSF75 vs NSF75 has 25 DEGs, annotated into 22 entries in molecular functions, 20 entries in cellular components, and 23 entries in biological processes, for a total of 65 entries. Among them, unfolded protein binding (GO:0051082, 10) is the largest number of DEGs in molecular function; cytoplasm (GO:0005737, 10) is the largest number of DEGs in cellular fraction; protein folding(GO:0006457, 10) are the entries with the highest number of DEGs in biological processes. From the GO enrichment results, it can be seen that the molecular functions performed by CNSF75 vs NSF75 are significantly enriched for (1) unfolded protein binding (GO:0051082, 10, Qvalue=1.9e-14), (2) protein self- association (GO:0043621, 7, Qvalue=5.2e-13), (3) DNA-binding transcription factor activity (GO:0003700, 5, Qvalue=0.0130), (4) RNA polymerase II proximal promoter sequence-specific DNA binding molecule (GO:0000978, 2, Qvalue=0.0229), (5) triose-phosphate transmembrane transporter activity(, GO:0071917, 1, Qvalue=0.0229), (6) phosphoglycerate transmembrane transporter activity (GO:0015120, 1, Qvalue=0.02361); cytoplasm (GO:0005737, 10, Qvalue=1.22E-04), cell surface (GO:0009986, 2, Qvalue=1.22E-04), protein-containing complex (GO:0032991, 2, Qvalue=1.22E-04), and perinuclear region of cytoplasm (GO:0048471, 2, Qvalue=5.51E-04) are significantly enriched entries in cellular fractions; protein folding (GO:0006457, 10, Qvalue=3.36E-16), protein complex oligomerization (GO:0051259, 7, Qvalue=8.49E-16), response to hydrogen peroxide (GO:0042542, 7, Qvalue=8.60E-15), response to reactive oxygen species (GO:0000302, 7, 1.83E-14), response to heat (GO. 0009408, 8, Qvalue=2.20E-14), response to salt stress (response to salt stress, GO:0009651, 7, Qvalue=7.88E-11), cellular response to heat (GO:0034605, 4, Qvalue=1.81E-07) were significantly enriched entries involved in biological processes (Figure 3 J, K, L, Schedule 5).

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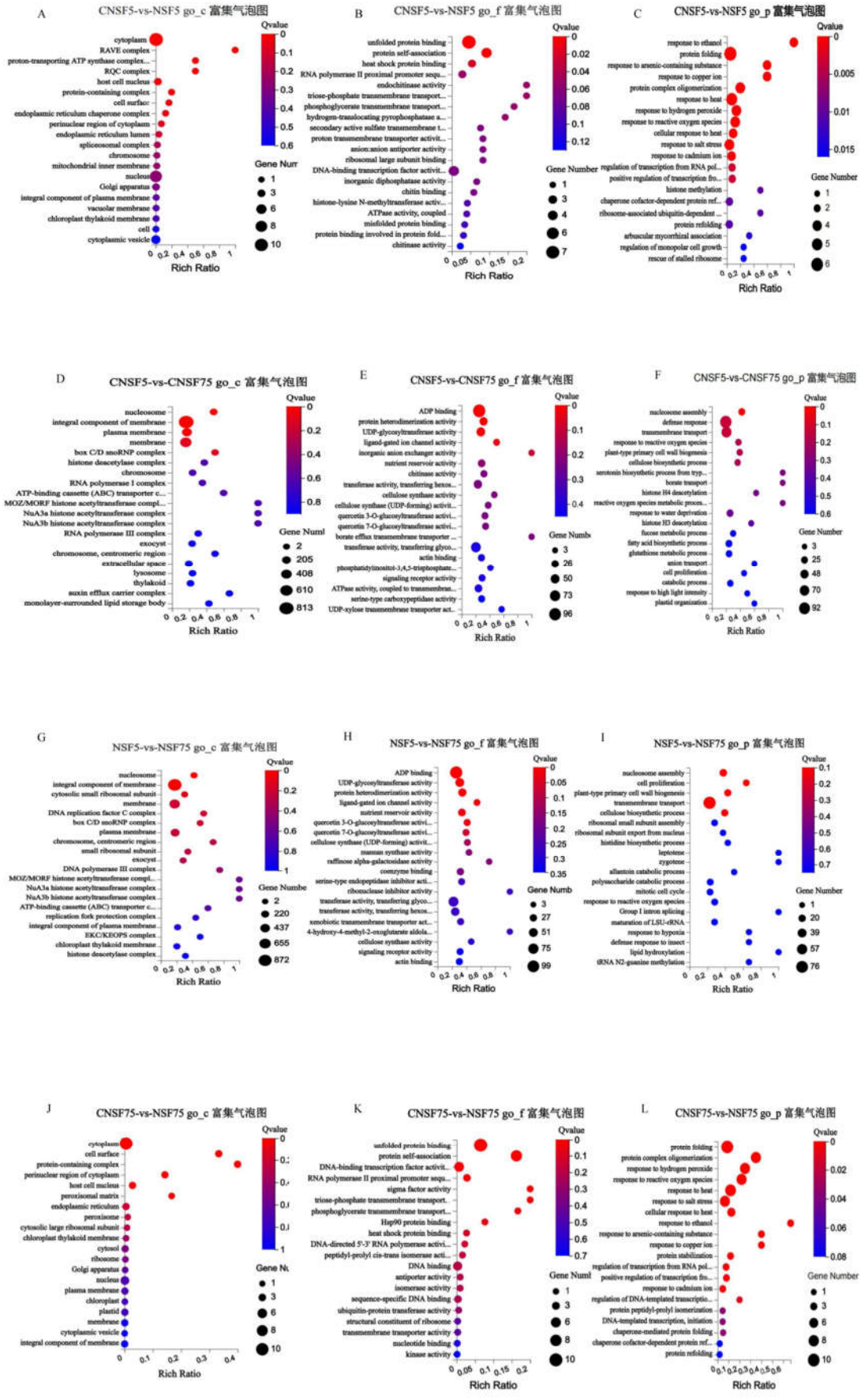

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Figure 3 Overview of GO enrichment of Four comparison groups DEGs. A, B and C denote the cell components, molecular functions and bioprocess GO enrichment of CNSF5 vs NSF5 in the comparison group; D, E and F denote the cell components, molecular functions and bioprocess GO enrichment of CNSF5 vs CNSF75 in the comparison group; G, H and I denote the cell components, molecular functions and bioprocess GO enrichment of NSF5 vs NSF75 in the comparison group; J, K and L denote the cell components, molecular functions and bioprocess GO enrichment of CNSF75 vs NSF75 in the comparison group. GO terms are listed on the Y-axis, and rich factors are shown on the X-axis. A smaller Q value indicates a higher significance level and closer proximity to the red line. A larger circle means more genes.

2.5. KEGG enrichment analysis of differentially expressed genes

In the four comparison groups CNSF5 vs NSF5, CNSF5 vs CNSF75, NSF5 vs NSF75, and CNSF75 vs NSF75, 53 DEGs could be annotated to 9 KEGG pathways, 4307 DEGs to 120 KEGG pathways, 4736 DEGs to 123 KEGG pathways and 25 DEGs could be annotated to 4 KEGG pathways, respectively. The top 20 most significantly different genes in each comparison group were selected for bubble plots (Figure 4 A, B, C, D).

The metabolic pathway with the highest number and most significant enrichment of KEGG-enriched DEGs in the comparison group CNSF5 vs NSF5 was protein processing in endoplasmic reticulum (osa4141, 6 entries, Qvalue=3.17E-05), with the following associated genes *LOC_Os01g04340* (HSP16.6, Log2FC:1.51), *LOC_Os03g16040* (HSP17.7, Log2FC:1.22),*LOC_Os03g16020* (HSP17.4, Log2FC:1.33), *LOC_Os03g16030* (HSP18.1, Log2FC:1.94), and *LOC_Os04g01740* (HSP82, Log2FC:1.54), *LOC_Os03g53340* (HSF11, Log2FC:1.16), the expression of these differentially expressed genes were significantly up-regulated in NSF5. The expression of gene *Os08g0400200* in the lysine degradation (osa00310) pathway was significantly down-regulated in NSF5 (Log2FC: -1.15); The gene *LOC107279585* in the oxidative phosphorylation pathway(osa00190) was significantly up-regulated in NSF5 (Log2FC: 1.49).In addition, chitinase 2-like (*LOC_Os05g33130*) is involved in both amino acid sugar and nucleotide sugar metabolism (osa00520) and plant MAPK signaling pathway (osa04016), and its expression level is significantly up-regulated (Log2FC: 1.40). It is hypothesized that the gene *LOC_Os05g33130* has catalytic and transport functions. The gene *Os03g0738200* participates in the messenger RNA surveillance pathway (osa03015), and its expression is significantly down-regulated (Log2FC: -1.08); the gene *Os05g0163250* is involved in the splice some pathway (osa03040), and its expression is significantly down-regulated (Log2FC: -1.03) (Figure 4A, Schedule 6). The KEGG metabolic pathway of other genes is unknown.

The metabolic pathways with the highest number of KEGG-enriched DEGs in the comparison group CNSF5 vs CNSF75 were Biosynthesis of amino acids (osa1230, 41 entries), Phenylpropanoid biosynthesis (osa940, 38 entries), Plant- pathogen interaction (osa4626, 37 entries), and Carbon metabolism (osa1200, 36 entries). From the perspective of significant enrichment, the metabolic pathways of significant enrichment were Alanine, Aspartate, and Glutamate metabolism (osa00250, Qvalue=0.120), Tyrosine metabolism (osa00350, Qvalue=0.120), and Sphingolipid metabolism (osa600, Qvalue=0.120) (Figure 4B).

In the comparison group NSF5 vs NSF75, in terms of the number of DEGs enriched by KEGG, the results showed that the most enriched metabolic pathway was Ribosome (osa3010, 64), followed by Biosynthesis of amino acids (osa1230, 40), Plant-pathogen interaction (osa4626, 39), Carbon metabolism (osa1200, 39), Phenylpropanoid biosynthesis (osa940, 38), Plant hormone signal transduction(osa4075, 32). From the perspective of significant enrichment, the metabolic pathways of significant enrichment were Galactose metabolism (osa52, Qvalue=0.0970), Homologous recombination (osa3440, Qvalue=0.0970), Ribosomes (osa3010, Qvalue=0.0970), and Fatty acid elongation (osa62, Qvalue=0.0988) (Figure 4C).

The metabolic pathway with the highest number and most significant enrichment of DEGs in the comparison group CNSF75 vs NSF75 was protein processing in the endoplasmic reticulum (osa4141, 9, Qvalue=1.71E-10), followed by Plant-pathogen interaction (osa4626, 2). ATP synthase subunit 9 (*LOC107279585*) was significantly up-regulated in NSF75 (Log2FC: 1.16, Qvalue=0.05) and significantly up-regulated in NSF5 (Log2FC: 1.49, Qvalue=7.280e-11). *LOC_Os04g01740* (HSP82, chr4:483216-486030) was significantly up-regulated in NSF75 (Log2FC:1.74, Qvalue=2.7e-11) and in

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NSF5 (Log2FC:1.54, Qvalue=0.0163);*LOC_Os03g16020* (HSP17.4, chr3: 8833703-8834481), significantly up-regulated expression in NSF75 (Log2FC:1.23, Qvalue=0.000754); *LOC_Os03g16030* (HSP18.1, chr3:8834792-8835644), significantly up-regulated expression in NSF75 (Log2FC:2.36, Qvalue=0.00484) (Figure 4 D, Schedule 7) [23]. These heat shock protein-related genes were involved in protein processing in the endoplasmic reticulum and ATP synthesis and metabolism pathways. Their upregulation of expression may be a timely positive response during dehydration and drying, adapting to the environment, and protecting cells from damage.

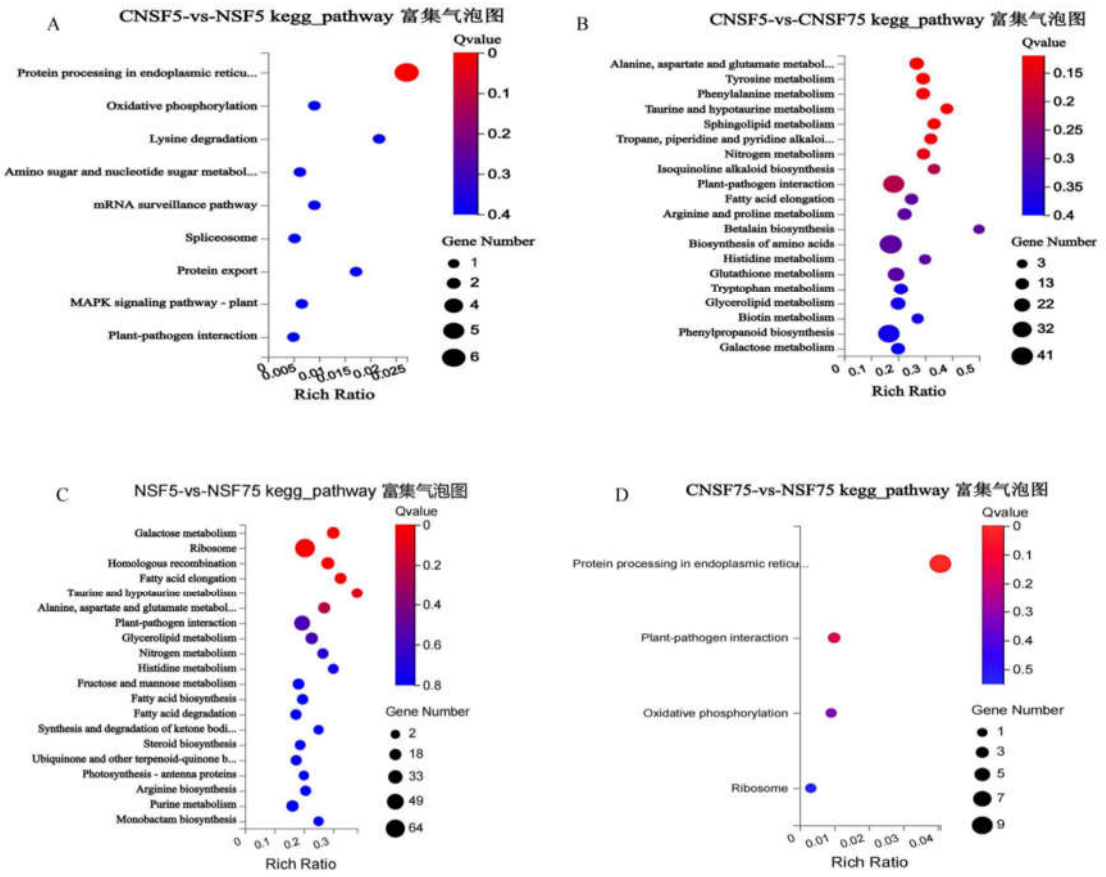

Figure 4 Analysis of differentially expressed genes by KEGG Enrichment A, B, C and D represent the most significant KEGG pathway enrichment maps of CNSF5 vs NSF5, CNSF5 vs CNSF75, NSF5 vs NSF75, CNSF75 vs NSF75 in the comparison group. GO terms are listed on the Y-axis, and rich factors are shown on the X-axis. A smaller Q value indicates a higher significance level and closer proximity to the red line. A larger circle means more genes.

2.6. Analysis of Dehydration-Related Candidate Genes in Transcriptome

2.6.1. Screening of Candidate Genes in Fast Dehydration and Slow Dehydration Materials

There were 10 significantly differentially expressed genes (padj<0.01, $|\text{Log2FC}| \geq 1$) in the fast-dehydrating material Baghlani Nangarhar and the slow-dehydrating material Saturn (Figure 5A), of which seven differed by twice or more, and all of these DEGs were consistently up-regulated in expression (Table 1), suggesting that they may play an important role in the seed dehydration process. The main metabolic pathway in which these genes are involved is the protein processing in the endoplasmic reticulum, and oxidative phosphorylation pathway (Figure 5B). The heat-stimulated transcription factor A6A, heat-stimulated transcription factor A2A, ethylene-responsive transcription factor ABR1, ATP synthesis subunit 9, small molecule heat-stimulated protein HSP20, and heat-stimulated protein HSP90 were involved.

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Further analysis showed that the differentially expressed genes *Os06g0722450* [Log2FC: 8.28] was up-regulated,*Os06g0513943* [Log2FC: 1.82] was up-regulated, and *LOC112938716* [Log2FC: -2.42] was down-regulated, which was only significantly expressed in the rapid dehydration material Baghlani Nangarhar; The differentially expressed genes *Os06g0159900* [Log2FC: 1.03], which was significantly expressed only in the slow dehydration material Saturn, was up-regulated and *LOC9266706* [Log2FC: -8.25] was down-regulated. Among them, *Os06g0722450* was an expression protein with unknown function, which was not expressed in the initial state of the rapid dehydration material Baghlani Nangarhar, and was activated and up-regulated during dehydration. Its target mRNA is novel-osa-miR194-3p [23], which was presumed to be a new gene related to the dehydration rate.*Os06g0513943* was up-regulated in the rapid dehydration material Baghlani Nangarhar, whose main function was to maintain the integrity of the membrane, reduce physical damage to the seeds, and play a self-defense role [24]. LOC112938716 was down-regulated in the rapid dehydration material Baghlani Nangarhar, indicating that its expression was inhibited during dehydration. *Os06g0159900* belongs to the U-box domain protein [25-27], which was highly up-regulated in the slow dehydration material Saturn. Its molecular function was ubiquitin protein transferase activity and kinase activity, which can regulate the internal and external pressure balance of seed cells. *LOC9266706* was an expression protein with unknown function, which was down-regulated in a large amount in the slow dehydration material Saturn, and was expressed in the initial state. After dehydration and drying, the expression amount was close to zero. It is hypothesized that its expression is inhibited during seed dehydration. However, the gene was up-regulated in the fast dehydration material Baghlani Nangarhar, and was predicted to be a new gene novel-osa-miR116-5p through targeted miRNA information.

The comparison group NSF5 vs NSF75 GO enrichment analysis yielded 45 genes related to water metabolism, including the gene PIP1;1 (*LOC_Os02g44630*) (Schedule 8), which was induced to be up-regulated in both the fast-dehydrating material Baghlani Nangarhar and the slow-dehydrating material Saturn. The expression of the gene PIP1;1 (*LOC_Os02g44630*) was 1.67 times higher in the fast-dehydrating material than in the slow-dehydrating material. It is assumed that this gene has an important role in the regulation of water transport channel activity during seed maturation and drying. This is consistent with the results of Valérie [12], who identified up-regulated expression of water channel proteins (genes) (OsPIP1, OsPIP2) associated with seed dehydration using QTL localization method to study the relationship between seed dehydration and ABA content in maize seeds.

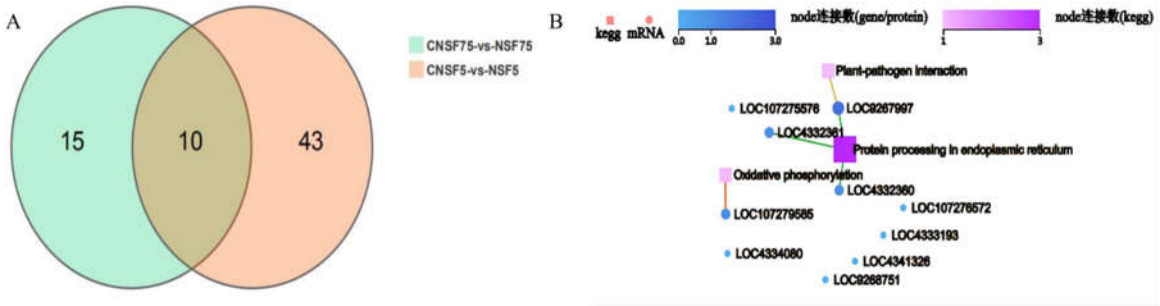

Figure 5 Overview of shared differentially expressed genes and gene network interactions A: The Venn diagram of DEGs in the groups of CNSF75 vs NSF75 and CNSF5 vs NSF5; B: Interaction map of shared significantly differentially expressed gene network in the groups of CNSF5 vs NSF5 and CNSF75 vs NSF75.

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Table 1 The shared differentially expressed genes in CNSF75 vs NSF75 and CNSF5 vs NSF5

NCBI Gene No.	MSU Gene No.	Variance multiplier log2 (NSF5 / CNSF5)	Variance multiplier log2 (NSF75 / CNSF75)	Gene annotation
LOC4341326	*LOC_Os06g36930*	4.3997	4.3444	Putative heat stress transcription factor A-6a
LOC4334080	*LOC_Os03g53340*	1.1557	1.7690	Similar to Heat stress transcription factor A-2a
LOC9268751		2.2410	1.7960	ethylene-responsive transcription factor ABR1
LOC107279585		1.4887	1.1606	ATP synthase subunit 9, mitochondrial
LOC4332361	*LOC_Os03g16030*	1.9368	2.3599	Low molecular mass heat shock protein Oshsp18.0
LOC9267997	*LOC_Os04g01740*	1.5389	1.7422	Similar to Heat shock protein 82 Heat shock protein 81-1 (HSP81-1) (Heat shock Similar to Heat shock protein 80 Non-protein coding transcript
LOC4332360	*LOC_Os03g16020*	1.3314	1.2284	Low molecular mass heat shock protein Oshsp17.3

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Table 2 The Information on the nine candidate genes

NCBI Gene No.	MSU No. or RAP No.	Function Comments	Molecular function	Biological process
LOC4334080	*LOC_Os03g53340*	Similar to Heat stress transcription factor A-2a	GO:0000978 RNA polymerase II proximal promoter sequence-specific DNA binding; GO:0003677 DNA binding; GO:0003700 DNA-binding transcription factor activity; GO:0043565 sequence-specific DNA binding	GO:0006355 regulation of transcription, DNA-templated; GO:0034605 cellular response to heat; GO:0043618 regulation of transcription from RNA polymerase II promoter in response to stress; GO:0061408 positive regulation of transcription from RNA polymerase II promoter in response to heat stress
LOC4332360	*LOC_Os03g16020*	17.4 kDa class I heat shock protein-like	GO:0043621 protein self-association; GO:0051082 unfolded protein binding	GO:0000302response to reactive oxygen species; GO:0006457protein folding; GO:0009408 response to heat; GO:0009651 response to salt stress; GO:0042542 response to hydrogen peroxide;
LOC4332361	*LOC_Os03g16030*	18.1 kDa class I heat shock protein-like	GO:0043621protein self-association; GO:0051082 unfolded protein binding	GO:0000302 response to reactive oxygen species;GO:0006457 protein folding; GO:0009408 response to heat;GO:0009651 response to salt stress; GO:0042542 response to hydrogen peroxide
LOC9267997	*LOC_Os04g01740*	heat shock protein 82	GO:0005524 ATP binding; GO:0051082 unfolded protein binding;	GO:0006457 protein folding; GO:0034605 cellular response to heat; GO:0050821 protein stabilization
LOC4331608	*LOC_Os03g05290*	probable aquaporin *TIP1-1*	GO:0015250water channel activity; GO:0015267 channel activity	GO:0006833water transport; GO:0055085 transmembrane transport
LOC4330265	*LOC_Os02g44870*	dehydrin *DHN1*-like	unknown	GO:0006950 response to stress; GO:0009414 response to water deprivation; GO:0009415 response to water; GO:0009631 cold acclimation; GO:0009737 response to abscisic acid

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LOC4326935	*LOC_Os01g50700*	dehydrin *Rab25*-like	unknown	GO:0009414 response to water deprivation; GO:0009415 response to water; GO:0009631 cold acclimation; GO:0009737response to abscisic acid
LOC4330248	*LOC_Os02g44630*	aquaporin *PIP1-1*-like	GO:0015250water channel activity; GO:0015267 channel activity	GO:0006833 water transport; GO:0009414 response to water deprivation; GO:0055085 transmembrane transport

Continued Table 2

NCBI Gene No.	MSU No. or RAP No.	Function Comments	Molecular function	Biological process
LOC4343122	*LOC_Os07g26690*	probable aquaporin *PIP2-1*	GO:0005215 transporter activity; GO:0015250 water channel activity; GO:0015267 channel activity	GO:0006810 transport; GO:0006833 water transport; GO:0055085 transmembrane transport

2.6.2. Validation of transcriptome sequencing genes by qRT-PCR

Based on the results of the previous screening of 165 germplasm materials for dehydration rate phenotypes, two extreme materials were selected: the fast-dehydrating material Baghlani Nangarhar (NSF5) and the slow-dehydrating material Saturn (NSF75), and qRT-PCR expression analysis was performed for nine candidate differentially expressed genes, and the expression profiles of these nine differentially expressed genes were verified by transcriptome sequencing. The sample RNA was the residual RNA at the time of sequencing. qRT-PCR quantification was performed on two materials with three biological replicates. The results showed that the real-time PCR expression patterns of the nine genes were consistent with the RNA-Seq expression profiles (Figure 6). This indicates that the RNA-Seq expression profile of this experiment is authentic and reliable.

The transcriptome results showed that genes *LOC4334080*(*LOC_Os03g53340*), *LOC4332360*(*LOC_Os03g16020*), *LOC4332361*(*LOC_Os03g16030*), and *LOC9267997* (*LOC_Os04g01740*)were up-regulated in the seed transcriptome of both the fast-dehydrating material Baghlani Nangarhar and the slow-dehydrating material Saturn with highly significant differences. *LOC4332360*, *LOC4332361* and *LOC9267997* were significantly more expressed in the seeds of the fast-dehydrating material Baghlani Nangarhar than in the seeds of the slow-dehydrating material Saturn, *LOC4332360*, *LOC4332361* and *LOC9267997* were significantly more expressed in the seeds of the slow-dehydrating material Saturn than in the fast-dehydrating material Baghlani Nangarhar, while the gene *LOC4334080* was more expressed in the fast-dehydrating material Baghlani Nangarhar than in the slow-dehydrating material Saturn. The gene function annotation revealed that *LOC4334080*, *LOC4332360*, *LOC4332361*, and *LOC9267997* belonged to heat-stimulated proteins or heat-stimulated transcription factors (Table 2), and based on the experimental results, it was hypothesized that the slow dehydration material Saturn was more sensitive to external environmental heat response and induced stronger effects than the fast dehydration material. qRT-PCR quantification results further verified the reliability of the experimental results.

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The transcriptome results showed that genes *LOC4331608*(*LOC_Os03g05290*), *LOC4330265*(*LOC_Os02g44870*), and *LOC4326935*(*LOC_Os01g50700*) were up-regulated in the transcriptome of both the fast dehydration material Baghlani Nangarhar and the slow-dehydration material Saturn seeds, and the differences were highly significant.*LOC4326935* was expressed in the fast dehydration material Baghlani Nangarhar and the slow-dehydrating material Saturn, with highly significant differences between pre- and post-dehydration, and slightly higher expression in the fast-dehydrating material than in the slow-dehydrating material, consistent with the real-time quantitative PCR expression pattern, but the results showed that the expression in the fast-dehydrating material Baghlani Nangarhar was significantly higher than that in the slow-dehydrating material *LOC4331608* and *LOC4330265* were dehydration-related genes (Table 1), and the expression of these two genes in the fast dehydration material Baghlani Nangarhar was significantly higher than that in the slow dehydration material Saturn. This indicates that the sensitivity of the dehydration-related genes to water metabolism is stronger in the rapidly dehydrated material than in the slowly dehydrated material, and is presumably one of the reasons for the difference in dehydration rates between them. qRT-PCR quantification further verified the reliability of the expression results of these three genes.

The transcriptome results showed that the expression of genes *LOC4330248*(*LOC_Os02g44630*) and *LOC4343122*(*LOC_Os07g26690*) were both up-regulated in the fast-dehydrating material Baghlani Nangarhar seeds with highly significant differences, and the real-time quantitative PCR results showed that the relative expression levels of this differentially expressed genes were consistent with the transcriptome results. The expression of genes *LOC4330248* and *LOC4343122* was increased in the slow dehydrated material Saturn seeds, but did not reach a significant difference level. The results of real-time quantitative PCR showed that the expression of these two genes reached a significant difference in the slow dehydration material. It indicates that the induced expression of *LOC4330248* and *LOC4343122* was affected by the characteristics of the material itself and may be related to the material specificity.

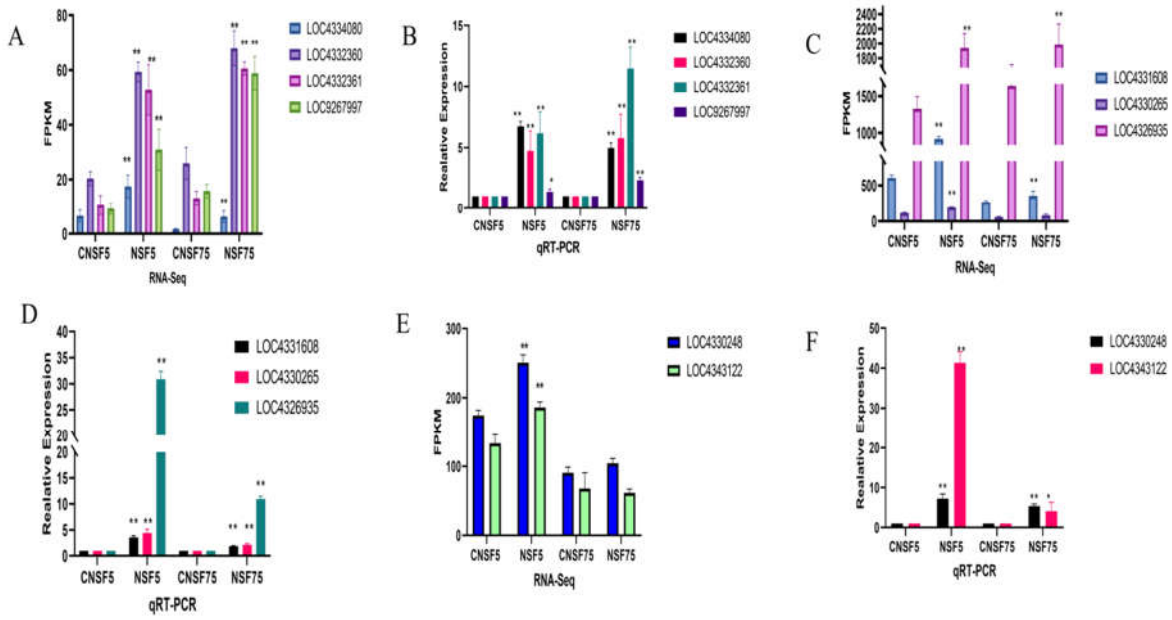

Figure 6 Validation of DEGs to RNA-seq and qRT-PCR.

3. Discussion

Dehydration of mature seeds is a complex process in which the seed dehydration tolerance is reduced by the loss of water, its metabolic activity is reduced, and the seed embryo enters a quiescent state or is metabolically inactive. Usually, dehydration causes some damage to the seeds, and the accumulated sugars, proteins, lipids and enzymes of the antioxidant system in the seeds will

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counteract the damage caused by dehydration and mitigate the effects on seed embryo viability by maintaining protein (including enzyme) stability, promoting cytoplasmic vitrification to protect subcellular stability, scavenging free radicals and other toxins, and other physiological and biochemical processes. During dehydration, the main processes involved in the seed response to dehydration are signal transduction, protein folding, sorting and degradation, amino acid metabolism, sugar metabolism, lipid metabolism, biosynthesis of other secondary metabolites, energy metabolism, environmental adaptation, growth and death, membrane transport, transcriptional regulation, translation, replication and repair, metabolism of terpenoids and polyketides, nucleotide metabolism, glycan biosynthesis and metabolism, and Aging and other metabolic pathways.

This experimental study showed that the differentially expressed gene *Os06g0159900* [Log2FC: 1.03], which was significantly expressed only in the slow dehydration material Saturn, was up-regulated and *LOC9266706* [Log2FC: -8.25] was down-regulated. Among them, *Os06g0722450* was an expression protein with unknown function, which was not expressed in the initial state of the rapid dehydration material Baghlani Nangarhar, and was activated and up-regulated during dehydration. Its target mRNA is novel-osa-miR194-3p [23], which was presumed to be a new gene related to the dehydration rate.*Os06g0513943* was up-regulated in the rapid dehydration material Baghlani Nangarhar, whose main function was to maintain the integrity of the membrane, reduce physical damage to the seeds, and play a self-defense role [24]. *LOC112938716* was down-regulated in the rapid dehydration material Baghlani Nangarhar, indicating that its expression was inhibited during dehydration. *Os06g0159900* belongs to the U-box domain protein [25-27], which was highly up-regulated in the slow dehydration material Saturn. Its molecular function was ubiquitin protein transferase activity and kinase activity, which can regulate the internal and external pressure balance of seed cells. *LOC9266706* was an expression protein with unknown function, which was down-regulated in a large amount in the slow dehydration material Saturn, and was expressed in the initial state. After dehydration and drying, the expression amount was close to zero. It was hypothesized that its expression was inhibited during seed dehydration, so the expression level was extremely low. However, the gene was up-regulated in the fast dehydration material Baghlani Nangarhar, and was predicted to be a new gene novel-osa-miR116-5p through targeted miRNA information. In this study, two new genes related to dehydration were obtained, and their functions need to be further confirmed.

The GO analysis of CNSF5 vs NSF5 showed that 53 DEGs were enriched for a total of 112 entries. The most enriched entries of these differentially expressed genes were unfolded binding proteins and folded proteins, and the sites of action of these proteins were concentrated in the cytoplasm. CNSF75 vs NSF75 GO analysis showed that 25 DEGs were enriched with a total of 65 entries, and the most enriched entries of these differentially expressed genes were unfolded binding and folded proteins, and again the cytosolic component was mainly enriched in the cytoplasm. These proteins act as catalytic, binding, translocating, transcriptional, and other regulatory roles in adaptive responses to hydrogen peroxide, reactive oxygen species, high temperature, etc. in the adversity they are exposed to. The comparison group CNSF5 vs NSF5 detected the gene for chitinase, heat shock protein, similar to the results of Chen [28]. Chen showed that the chitin receptor (*OsCERK1*) processed in the rice endoplasmic reticulum interacts with heat shock protein (Hsp90) and its accessory molecular chaperone Hop/Sti to form a complex that regulates the efficient transport of plasma membrane GTPase. The study of maize dehydration rate-related genes obtained specific genes at the transcriptional level of expression was closely associated with tissue senescence, regulation of hormone levels and hormone-mediated signaling pathways, heat, reactive oxygen species, ethanol, and dehydration response [22], and the results are consistent with the results of the biological process of differentially expressed genes GO enrichment screened in this study. The main functions of differential proteins involved in the study of the functions of differential proteins obtained during dehydration of wheat seed maturation were considered to be stress/defense, storage proteins, energy metabolism, starch and sucrose metabolism, protein synthesis/folding/degradation, lipid metabolism, signal transduction, and transcription/translation [29]. This is similar to the results of the present study. The molecular functions of the differentially expressed genes presumably obtained in

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this study that were associated with the rate of dehydration were unfolded protein binding (GO:0051082), protein self-association (GO:0043621), ADP binding (GO:0043531); while biologically important processes were protein folding, (GO:0006457), response to reactive oxygen species (GO:0000302), and response to heat (GO:0009408). It was also hypothesized that heat shock protein-related genes in the metabolic pathway (*LOC_Os03g16020*, *LOC_Os04g01740*, etc.), chitinase 2-like (*LOC_Os05g33130*) play an important regulatory role in the regulation of the formation and efficient transport of plasma membrane GTPases.

KEGG analysis showed that the most significantly enriched metabolic pathway for CNSF5 vs NSF5 was protein processing in the endoplasmic reticulum, with six differentially expressed genes involved in the up-regulated expression, mainly some small molecule heat shock proteins HSP16.6 (*LOC_Os01g04340*), HSP17.7 (*LOC_Os03g16040*), HSP17.4 (*LOC_Os03g16020*), HSP18.1 (*LOC_Os03g16030*), the large molecule heat shock protein 82 (*LOC_Os04g01740*) and the heat shock transcription factor HSF11 (*LOC_Os03g53340*) (Schedule 6). The most significantly enriched and enriched metabolic pathway of CNSF75 vs NSF75 was protein processing in the endoplasmic reticulum, with eight differentially expressed genes involved in up-regulated expression and one in down-regulated expression. The differentially expressed genes involved in up-regulated expression were mainly HSP16.9 (*LOC_Os01g04360*), HSP17.8 (*LOC_Os02g48140*), HSP17.4 (*LOC_Os03g16020*), HSP18.1 (*LOC_Os03g16030*), HSP23.2 (*LOC_Os04g36750*), HSP16.0 (*LOC_Os06g14240*), HSP21.9 (*LOC_Os11g13980*), heat shock protein 82 (*LOC_Os04g01740*), and the gene involved in down-regulated expression was HSP81-3 (*LOC_Os09g30418*) (Schedule 7). This was consistent with the results of small molecular heat shock proteins obtained in the study of dehydration tolerance of Fraxinus mandshurica seeds by Liu [30]. Liu studied and obtained seven proteins related to dehydration tolerance, one up-regulated protein, and six down-regulated proteins. The main biological functions of these seven proteins were related to energy metabolism, stress defense, and transcriptional regulation. The differentially expressed genes obtained from transcriptome analysis of Fraxinus mandshurica seeds were mainly involved in biological processes such as protein folding, hydrogen peroxide reaction, heat adaptation, drying reaction, salt stress reaction, and hypertonic reaction. Some studies suggest that small molecule heat shock proteins could repair and degrade damaged proteins, stabilize polypeptide chains, maintain normal protein activity, and help improve the resistance of seeds to adverse conditions, thereby improving storage capacity related to seed lifespan [31]. The research results suggest that there was a positive correlation between heat shock proteins and the vitality of rice seeds. The higher the content of heat shock proteins in seeds with stronger dry resistance, the stronger their seed vitality and the stronger their later storage resistance [32]. It was believed that the level of seed vigor was affected by the synthesis ability of heat shock proteins in seeds, and a large amount of synthesis of heat shock proteins could improve seed vigor [33]. It was believed that the ability of mature seeds to tolerate large amounts of water loss was related to the function of sHSPs, as sHSPs could protect cell components and avoid damage caused by water loss [34]. It is believed that the main role of small-molecule heat-excited proteins is to maintain chaperone folding activity and the folded or unfolded state, to maintain limited intracellular water in dehydrated and desiccated environments, and to enhance seed resistance [35]. Kermode concluded that normal seeds have higher dehydration tolerance under slow dehydration conditions, probably because slow dehydration induces the synthesis of protective substances associated with dehydration tolerance, the most important of which are heat-stable proteins, including LEA proteins [36]. The heat-stimulated protein HSP maintains intracellular protein stability under normal conditions and stressful environments and helps proteins to complete proper folding, translation and aggregation, which is important for enhancing drought tolerance in plants [37]. It has been suggested that HSP promotes cytoskeleton formation, protects cellular structural stability, and mitigates damage to membranes and cellular dehydration, thereby reducing cellular damage [38-39]. It is suggested that OsHSP18.2 is highly expressed during seed maturation and acts as a molecular chaperone to protect the cell by reducing ROS accumulation, achieving enhanced seed viability and storage tolerance [40]. In summary, heat shock proteins and small molecule heat shock proteins can enhance the stability of the internal protein structure of seeds, improve seed vitality and storage life, and enhance defense and resistance capabilities. Several types

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of heat shock proteins (HSP16.9 (*LOC_Os01g04360*), HSP17.8 (*LOC_Os02g48140*), HSP17.4 (*LOC_Os03g16020*), HSP18.1 (*LOC_Os03g16030*), HSP23.2 (*LOC_Os04g36750*), HSP16.0 (*LOC_Os06g14240*), HSP21.9 (*LOC_Os11g13980*), heat shock protein 82 (*LOC_Os04g01740*), HSP81-3 (*LOC_Os09g30418*)) obtained in this study are presumed to have the effects of maintaining cell structure stability, heat resistance, and compression resistance during seed dehydration and drying.

A pathway that was significantly enriched in KEGG in this study: oxidative phosphorylation metabolism, which is a coupled reaction in which eukaryotes release energy during material processing and oxidation in the inner mitochondrial membrane and supply ADP through the respiratory chain to synthesize ATP with inorganic phosphate. *LOC107279585* (ATP synthetase subunit 9) was significantly up-regulated in the comparison group CNSF5 vs NSF5 (Log2FC: 1.49, Qvalue=7.278e-11), and significantly up-regulated in the comparison group CNSF75 vs NSF75 (Log2FC: 1.16, Qvalue=0.05). The difference was that the expression of ATP synthetase subunit 9 in the dehydrated fast material Baghlani Nangarhar was significantly higher than that in the slow dehydrated Saturn, suggesting that the fast dehydrated material may maintain high ATP synthesis, maintain a high metabolic level, and provide sufficient energy for rapid dehydration. If ATP synthesis is blocked, it will inhibit normal metabolic processes to some extent, which can lead to increased reactive oxygen species and membrane lipid peroxidation, causing damage to seed viability [41]. The key differentially expressed genes and the most important metabolic pathways in the rapidly and slowly dehydrated materials obtained in this study are protein processing in the endoplasmic reticulum and oxidative phosphorylation metabolism, which are presumed to have important regulatory roles in stress/defense, energy metabolism, protein synthesis/folding, and signal transduction during dehydration and drying of mature seeds.

4. Materials and Methods

4.1. Material Handling

The experimental materials were rice germplasm materials provided by the laboratory of Mr. Wang Guoliang of Hunan Agricultural University. The fast-dehydrating material Baghlani Nangarhar (No. 19NSF5) and the slow-dehydrating material Saturn (No. 19NSF75) were selected based on the preliminary physiological maturity rice seed dehydration rate test. They were planted in Sanya, Hainan Province, in South China, on December 24, 2020. Sowing was carried out in stages based on the different seed-growing stages, with 20 plants planted for each material, a tag marking the date at the beginning of the spike, until 21 days after flowering when sampling began. Sampling method: 5 spikes of uniform maturity were selected for each sample, and the yellow ripe grains were threshed at the top 1/3 of the seeds and mixed, and the seeds were put in parchment paper bags and placed in rapid dehydration conditions (constant temperature 45±2°C dryer) for dehydration. Their initial moisture content was recorded, and the samples were set up in three replicates. The control seeds (in the state at harvest) were quickly packed into 2.0 mL Eppendorf tubes, immediately snap-frozen in liquid nitrogen, and then stored in the laboratory in an ultra-low temperature refrigerator for later transcriptome sequencing. The treated seeds were rapidly dehydrated, and the moisture content was measured every 4 hours until it was stabilized at 13%, then immediately snap-frozen in liquid nitrogen, and stored in an ultra-low temperature refrigerator at -80°C. Samples from Baghlani Nangarhar dehydrated under control versus dehydrated conditions were labeled as "CNSF5" and "NSF5", and samples from Saturn dehydrated under control versus dehydrated conditions were labeled as "CNSF75" and "NSF75". A total of 4 comparison groups were constructed using these samples.

4.2. Methods

4.2.1. Seed RNA extraction

An RNAprep pure Plant Kit (Tiangen, Beijing, China) was used to extract the seed RNA. Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop ND-2000

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spectrophotometer (NanoDrop Technology) were used to assess RNA concentration, integrity, and purity. We analyzed all RNA samples with OD260/OD280 ratios between 1.8 and 2.2, including a pooled RNA sample for transcriptome sequencing and RNA preparations from CNSF5, NSF5, CNSF75, and NSF75 samples for DEG sequencing. We chose cDNA libraries with RNA integrity number (RIN) values greater than 7.6 for quantitative real-time PCR.

4.2.2. Transcriptome sequencing and data assembly

After RNA extraction, each sample was analyzed for quality. Quality analysis, library construction and sequencing were performed by UW Genetics Biotechnology Co. Using SOAPnuke (v1.5.2) [42], the sequencing data were filtered by (1) removing reads that contain sequencing adapters; (2) removing reads with a low-quality base ratio greater than 20% (base quality less than or equal to 5); (3) removing reads with an unknown base ('N' base) ratio of more than 5%, and then retrieving clean reads and storing them in FASTQ format. Clean reads were mapped to the reference genome using HISAT2 (v2.0.4) [43]. Bowtie2 (v2.2.5) [44] was applied to align the clean reads to the reference coding gene set (reference genome version: GCF_001433935.1_IRGSP-1.0, https://biosys.bgi.com/assets/img/banner1.svg), and then RSEM (v1.2.12) [45] was used to calculate the expression levels of genes. The heat map was drawn using pheatmap (v1.0.8) [46] based on the gene expression in different samples.

4.2.3. Functional annotation, classification and metabolic pathway analysis

（1）Analysis of differentially expressed genes (DEGs). A total of four comparison groups were constructed: CNSF5 vs NSF5, CNSF5 vs CNSF75, NSF5 vs NSF75, and CNSF75 vs NSF75. The results of the clean reads were compared with the reference genome and stored in binary files. Gene FPKM (FPKM = total exon Fragments/mapped reads(Millions) x exon length(KB)) [47] was quantified by cufflinks [48], and the number of reads of genes in the samples was obtained using HTSeq-count [49] (California Institute of Technology, Pasadena, CA, USA) software, and the data were normalized by the software DESeq2 [50] R package in the estimation Size Factors function for data normalization, and the nbinomTest function was used to calculate Foldchange values and P-values for comparative differences to control the false discovery rate. DEGs were selected with P-value < 0.05. After correction, a rigorous threshold (Q value 0.05) was utilized by Bonferroni [51] to correct significant levels of terms and pathways.

（2）Enrichment analysis of DEGs by GO and KEGG. GO and KEGG enrichment analyses were performed on the screened differentially expressed genes using GO (http://www.geneontology.org) database [52] and KEGG (https://www.kegg.jp) database [53]. Term and pathway significance was assessed by corrected Q-value < 0.05 [51].

4.2.4. qRT-PCR validation of differentially expressed genes

Premier Software was used to design the qRT-PCR primers, and the cDNA synthesis kit from TransGen Biotech was used to reverse transcribe the RNA into cDNA, (see cDNA synthesis instructions). The reagents used in this study for quantitative PCR were Takara's quantitative PCR kits, and the total reaction system was 50 μL, including 1 μL of reverse transcription product, 1 μL of gene-specific primers, and 25 μL of 2 x Trans TaqTM HiFi PCR SuperMix II, and 3 μL of ddH2O. Amplification was performed using an ABIPRISM7500 (Applied Biosystems, USA) model real-time fluorescent quantitative PCR instrument. Genes were assessed quantitatively using the $2^{-\Delta\Delta Ct}$ algorithm [54] and significance analysis (one-way ANOVA and multiple comparisons, $p<0.05$) was performed using SPSS 13.0 to correct for differential gene expression according to the internal reference gene Actin. Three replicates were set up for each PCR. Primer-specific information is provided in Table 3.

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Table 3 Primers for qRT-PCR in this study

Serial number	NCBI Login number	Candidate genes	Primer sequences
1	*LOC4343122*	*LOC_Os07g26690*	F：TGTTTAGCCTGTACTCCCATTT R：ACGGAGGGAGTATATTCCAGAT
2	*LOC4332360*	*LOC_Os03g16020*	F：GCATTGGGCTAATCTAAAACGA R：GCACACCAAAAACACCAGTAAT
3	*LOC4332361*	*LOC_Os03g16030*	F：GGTTACCGGCTAGTAAGAAACT R：TACTGCAATTGATCACAAACCG
4	*LOC4334080*	*LOC_Os03g53340*	F：CTACGAAGGTCGATCCGGATAG R：CTTGATCGTCTTCAGGAGCTC
5	*LOC9267997*	*LOC_Os04g01740*	F：GGAGGAGGTGGACTGAATTAAA R：ACTTTCTCAACGATGGCTTAGA
6	*LOC4330248*	*LOC_Os02g44630*	F：CATTCAAGAGCAGGTCTTAAGC R：AGTTGTTCAGGGTTCAGATAGG
7	*LOC4331608*	*LOC_Os03g05290*	F：GAGTCCCAGTGGGTGTACT R：GAGATGAAGAGGACCTCGTAGA
8	*LOC4330265*	*LOC_Os02g44870*	F：GAGAAGATCGAGGGTGATCAC R：GCTTCTCCTTGATCTTGTCGAG
9	*LOC4326935*	*LOC_Os01g50700*	F：CAGTCGTGTTTCAGTTCGTTAA R：GGATACACCGTACATGCATAGA

5. Conclusions

Transcriptomic sequencing analysis of the two extremely rapid and slow dehydration materials in mature rice seeds showed that the number of up-regulated expression difference genes of the two types of materials after dehydration treatment was significantly more than that of the initial material, and the number of differential expression genes of the rapid dehydration material was significantly more than that of the slow dehydration material. Through GO enrichment analysis, it was found that the proteins encoded by these differentially expressed genes are mainly unfolded protein binding, heat shock protein binding, protein self-association, ADP binding, protein heterodimer active protein heterodimerization activity, UDP-glycosyltransferase activity, and ligand-gated ion channel activity. It might be surmised that these proteins are closely related to seed dehydration rate. The results of this study provide a valuable reference for further research on the genes and metabolic pathways related to the dehydration rate of mature rice seeds and provide theoretical guidance for the selection and breeding of new rice germplasm that can be rapidly dehydrated at the mature stage.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table Schedule 1: Results of RNA quality in twelve experimental seeds; Table Schedule 2: Statistical table of seed transcriptome sequencing quality of twelve dehydrated and dried samples; Table Schedule 3: Comparative analysis of the transcriptome of seeds from twelve dehydrated samples and the genome of Charybdis Japonica; Table Schedule 4: CNSF5 vs NSF5 GO enrichment-related genes; Table Schedule 5: CNSF75 vs NSF75 GO enrichment-related genes; Table Schedule 6: CNSF5 vs NSF5 KEGG Metabolic pathway-related genes; Table Schedule 7: CNSF75 vs NSF75 KEGG Metabolic pathway-related genes; Table Schedule 8: NSF5 vs NSF75 GO：0009414 Enrichment of water metabolism-related genes. Table Schedule 9: RNA-seq data(The RNA-seq data will be uploaded to the journal website as IJMS-2366161 supplementary data file,https://susy.mdpi.com/user/manuscripts/resubmit/.).

Author Contributions: Haiqing Zhang and Jiwai He designed experiments. Zhongqi Liu and Yuntao Yan performed experiments. Zhongqi Liu and Jinxin Gui analyzed the data. Zhongqi Liu wrote the manuscript.

Haiqing Zhang, Jiwai He and Zhongqi Liu critically commented on and revised it. All authors have read and approved the manuscript.

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NCBI geneID	MSU or RAP ID
LOC4325694	LOC_Os01g04340
LOC4332360	LOC_Os03g16020
LOC4332363	LOC_Os03g16040
LOC4332361	LOC_Os03g16030
LOC9267997	LOC_Os04g01740
LOC4334080	LOC_Os03g53340
LOC4345509	OS08g0400200
LOC107279585	Not found
LOC4338718	LOC_Os05g33130
LOC4334045	Os03g0738200
LOC4337900	Os05g0163250
LOC9267323	Os06g0722450
LOC9268035	Os06g0513943
LOC112938716	Not found
LOC4340201	Os06g0159900
LOC9266706	Not found
LOC4330248	LOC_Os02g44630
LOC4331608	LOC_Os03g05290
LOC4330265	LOC_Os02g44870
LOC4326935	LOC_Os01g50700
LOC4343122	LOC_Os07g26690
LOC4325696	LOC_Os01g04360
LOC4330496	LOC_Os02g48140
LOC4335956	LOC_Os04g36750

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LOC4340661	LOC_Os06g14240
LOC4350180	LOC_Os11g13980
LOC4347405	LOC_Os09g30418

Figure Two types of rice germplasm at maturity stage A was slowly dehydrated rice seeds 19NSF75, and B was rapidly dehydrated rice seeds 19NSF5.