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Morphological and Transcriptome Analysis of Flooding Mitigation of the Damage Induced by Low-Temperature Stress on Direct-Seeded Early *Indica* Rice at the Seedling Stage

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Abstract: Low temperature (LT) chilling damage often occurs at the seedling stage of rice growth, especially direct-seeded early *indica* rice, and flooding can alleviate the damage caused by LT chilling at the rice seedling stage. However, few studies have elucidated the molecular mechanism by which suitable flooding alleviates LT stress-induced damage. Therefore, LT, LT plus flooding (LTF) and control (CK) treatments were established at 8 °C for 3 days to determine the phenotype, agronomic traits and transcriptomic of direct-seeded early *indica* rice at the seedling stage. The results showed that compared with LTF, the seedling height, root number, fresh weight, dry weight and T3 (the 3rd leaf from the top) leaf length significantly decreased after LT treatment; LTF could reduce the damage of LT to the agronomic characters of rice seedlings. The physiological characteristics showed that compared with LT, LTF significantly decreased soluble protein content and CAT activity. Transcriptomic profiling showed that 5934 DEGs were identified from the rice leaves between the LT and CK comparison groups; 7658 DEGs were identified between the LTF and CK; and 2697 DEGs were identified between the LT and LTF treatment. In biological process, the 'metabolic process' was the most enriched subcategory. In cellular components, the three most enriched subcategories were 'cell', 'cell part' and 'organelle'. 'Binding' was the most enriched subcategory in molecular function. Differentially expressed genes (DEGs) were significantly enriched in photosynthesis, carotenoid biosynthesis, flavonoid biosynthesis, glycolysis gluconeogenesis, glycine, serine and threonine metabolism and plant hormone signal transduction pathways. Photosynthesis, energy metabolism and signal transduction pathway play important roles in flooding mitigation of LT stress. The results of this study may help to elucidate changes in physiological characteristics and gene expression through which flooding mitigates LT stress.

Keywords: low temperature; flooding; transcriptome; direct-seeded early *indica* rice

Citation: Wang, W.; Du, J.; Wu, Z.; Zeng, Y.; Pan, X.; Cheng, S.; Zeng, Y. Morphological and Transcriptome Analysis of Flooding Mitigation of the Damage Induced by Low-Temperature Stress on Direct-Seeded Early *Indica* Rice at the Seedling Stage. *Agronomy* **2023**, *13*, 834. https://doi.org/10.3390/agronomy13030834

Academic Editor: Leilei Liu

Received: 31 January 2023
Revised: 5 March 2023
Accepted: 8 March 2023
Published: 13 March 2023

1. Introduction

Low temperature is one of the important harmful factors affecting crop production [1]. During sowing and seedling stages, rice is often affected by LT, resulting in slow growth or cessation of growth [2]. With the gradual expansion of the area of direct-seeded rice, the possibility of rice being subjected to LT stress at seedling stage is increasing [3]. Rice is one of the most important crops grown in tropical and temperate regions, feeding more than one third of the whole population of the world [4]. However, rice yield is greatly affected by different abiotic stresses such as LT [5]. With the frequent occurrence of extreme weather, direct-seeded rice often suffers from "cold spell in later spring" disaster

at seedling stage. The seedling stage is one of the more sensitive stages of rice development, and LTs that occur after planting may seriously affect crop yield. In Jiangxi Province, LT reduced the yield of indica rice by 5–13 percent [6]. Rice seedlings under LT stress will break the homeostasis of cell osmotic pressure, substance accumulation and metabolic abnormalities, resulting in chlorosis and curl of leaves, slow growth, decline of fertility and decrease in yield [7,8]. In agricultural production, LT stress will cause seedling yellowing, seedling decay and other phenomena, affecting their development and yield, and so on. Meanwhile, although rice is a water-loving crop, long-term flooding stress can also affect its growth and development, as roots and leaves proceed with anaerobic respiration to produce alcohol toxicity, and the leaf photosynthetic system is weakened. So, it is important to investigate in depth the response of direct-seeded early rice to LT stress and control measures.

In direct-seeded rice planting, farmers often mitigate the seedling damage derived from LT mainly through irrigating a certain deep-water layer, such as 3–5 cm, improving the survival rate of emerging. With this measure, the production risk of direct-seeded rice can be effectively decreased. So far, the effects of LT and flooding stress on rice cultivation, physiological characteristics, genetic mechanism and other aspects has attracted a lot of attention [9,10]. Previous studies on flooding mitigation LT stress mainly focused on agronomic characters or related physiological characteristics [11]. However, there were few reports on the molecular mechanism involved flooding alleviated the damage to direct-seeded early *indica* rice seedlings caused by LT stress [12].

The study of functional genomics related to stress resistance in rice has always been the core content of rice research. The study of the mechanism of LT tolerance of rice under LT and LTF stress at seedling stage was helpful to analyze the physiological and molecular mechanism of rice with LT stress, and it lays a foundation for rice molecular breeding in the future, which is of far-reaching significance to agricultural economy and society. Rice can regulate the physiological process by resisting metabolic reaction and changing the content and activity of hormones in the body so that it can maintain normal physiological activities under the condition of LT [13]. The regulation of transcription level is the most important regulation mode of organisms, and has the advantages of strong pertinence and small research scope [14,15]. Therefore, it was considered to be a more accurate method of determination and analysis at the transcriptional level [16]. At present, using transcriptome sequencing technology, many genes related to LT tolerance such as transcription, disease defense, signal transduction, secondary metabolism and so on have been found in maize, barley and other plants [17–19]. In this study, high-throughput sequencing method transcriptome sequencing (RNA-Seq) was used to study the expression characteristics of differential genes responding to LT and LTF in rice seedlings at the transcriptome level so as to enhance the understanding of the mechanism of LT tolerance in rice, to clarify the molecular mechanism of flooding alleviating LT stress at seedling stage of direct-seeded early *indica* rice and to provide reference for the cultivation of rice varieties' tolerance to LT.

2. Results

2.1. Phenotypic

After 3 days of LT and LTF treatment, the seedling growth of rice was significantly different under CK, LT and LTF treatments (Figure 1). After 3 d of LT stress, the leaves of the seedlings were somewhat yellow, the leaf tips became withered and the plant height was lower than that under normal conditions. The results showed that LT stress seriously inhibited the growth and development of rice seedlings, whereas flooding treatment under LT stress could play an alleviating role.

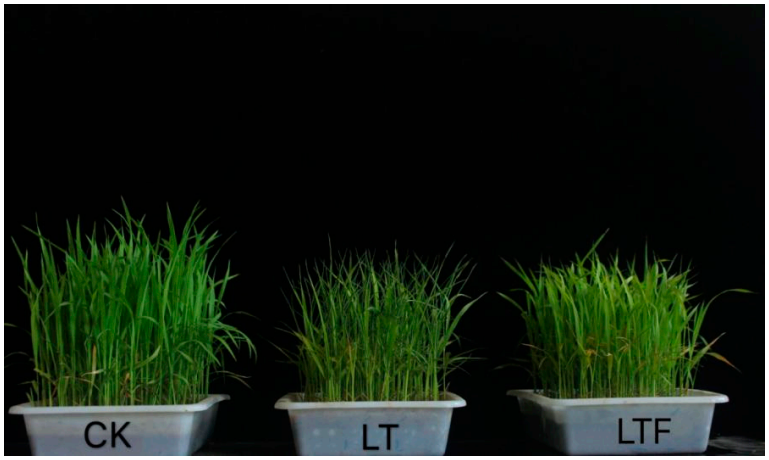


Figure 1. Zhongjiazao 17 Phenotype under different treatments. LT, low temperature treatment; LTF, low temperature flooding; CK, control.

2.2. Agronomic Characters

After 3 days of LT and LTF treatment, the seedling height of cold-sensitive cultivar Zhongjiazao 17 significantly decreased by 33.49% and 29.96% compared with CK, and the decrease in LT was greater than that of LTF treatment, reaching a significant level (Table 1). At the same time, compared with LTF treatment, the root number, fresh weight, dry weight and T3 (the 3rd leaf from the top) leaf length of cold-sensitive cultivar Zhongjiazao 17 decreased significantly after 3 days of LT treatment, and the root length, T1 (the first leaf from the top) leaf length and T2 (the 2nd leaf from the top) leaf length also decreased in varying degrees. The results showed that the LT stress affected the normal growth and development of the plant, while flooding could alleviate the effect of LT on the agronomic characters of the rice seedlings.

Table 1. Effects of LT and LTF stress on agronomic characters.

Treatment	Seedling Height /cm	Root Number /plant	Root Length /cm	Fresh Weight mg /100 plants	Dry Weight mg /100 plants	T1 /cm	T2 /cm	T3 /cm
LT	18.9 ± 0.40 c	10.7 ± 0.58 c	5.3 ± 0.77 b	186.0 ± 1.00 c	27.2 ± 0.46 c	2.4 ± 0.25 b	9.3 ± 0.20 b	7.1 ± 0.20 c
LTF	19.9 ± 0.24 b	11.0 ± 0.58 b	5.5 ± 0.11 b	212.4 ± 0.59 b	32.5 ± 0.58 b	2.4 ± 0.25 b	9.8 ± 0.55 b	8.1 ± 0.30 b
CK	28.4 ± 0.29 a	14.1 ± 1.07 a	7.9 ± 0.20 a	253.5 ± 0.50 a	36.4 ± 0.46 a	3.7 ± 0.26 a	10.6 ± 0.30 a	10.5 ± 0.22 a

LT, low temperature treatment; LTF, low temperature flooding; CK, control; T1, the first leaf from top; T2, the 2nd leaf from top; T3, the 3rd leaf from top. Different lowercase letters in the same column mean significant difference at 0.05 level.

2.3. Physiological Characteristic

The results of physiological characteristics of direct-seeded rice seedlings showed that LT and LTF treatments significantly increased the activities of SOD and POD compared to CK, and no differences were observed between LT and LTF, though those were higher in LT treatment than in LTF treatment (Table 2). In addition, the soluble protein content of LT and LTF was significantly increased compared to CK, and the CAT activity of LT was also significantly increased compared to LTF. (Table 2).

Table 2. Analysis of soluble protein content and antioxidase.

Treatment	POD Activity /μ g^{-1}·min^{-1}	SOD Activity /μ g^{-1}	CAT Activity /U·g^{-1}·min^{-1}	Cpr Content /Mg^{-1} mL
LT	288.67 ± 10.26 a	304.73 ± 9.87 a	777.79 ± 44.65 a	8.02 ± 0.34 a
LTF	269.29 ± 5.89 a	255.51 ± 3.64 a	741.34 ± 12.86 b	7.49 ± 0.07 b
CK	221.42 ± 6.50 b	234.74 ± 3.70 b	639.21 ± 40.29 c	6.76 ± 0.19 c

POD: peroxidase; SOD: superoxide dismutase; CAT: catalase. Different lowercase letters in the same column mean significant difference at 0.05 level.

2.4. RNA-Seq Data Analysis

The transcriptional response of direct-seeded early *indica* rice seedlings to LT and LTF stress was investigated using high-throughput RNA-seq. Rice leaf samples were sequenced using an Illumina HiSeq platform. Approximately 44.48, 40.81 and 38.06 million clean reads were obtained from LT, LTF and CK treatments after adapter trimming and filtering low-quality reads (Table 3). The average of Q20, Q30 and GC content in LT, LTF and CK were 96.89%, 91.92% and 52.39%, respectively, with the clean reads of Q20 occupying over 96% of the total reads. These findings proved the excellent quality of the sequencing results. The respective mapped reads information between LT, LTF and CK samples was 52.33%, 43% and 41% multiple mapped; and 99.48%, 99.57% and 99.59% uniquely mapped, respectively. Combined with the above data, it can be shown that the transcriptome data was suitable for further bioinformatics analysis.

Table 3. Summary of RNA-seq data and reads mapping.

Sample	Total Reads	Clean Reads	Mapped Ratio	Q20 (%)	Q30 (%)	GC (%)	Multiple Mapped	Uniquely Mapped
LT	51,839,643	44,476,183	85.79%	96.82%	91.82%	52.45	232,023 (52.33%)	44,244,160 (99.48%)
LTF	47,035,894	40,810,432	86.71%	96.78%	91.66%	51.73	176,840 (43.00%)	40,633,593 (99.57%)
CK	43,688,132	38,060,065	87.08%	97.07%	92.29%	53.00%	158,285 (41.00%)	37,901,780 (99.59%)

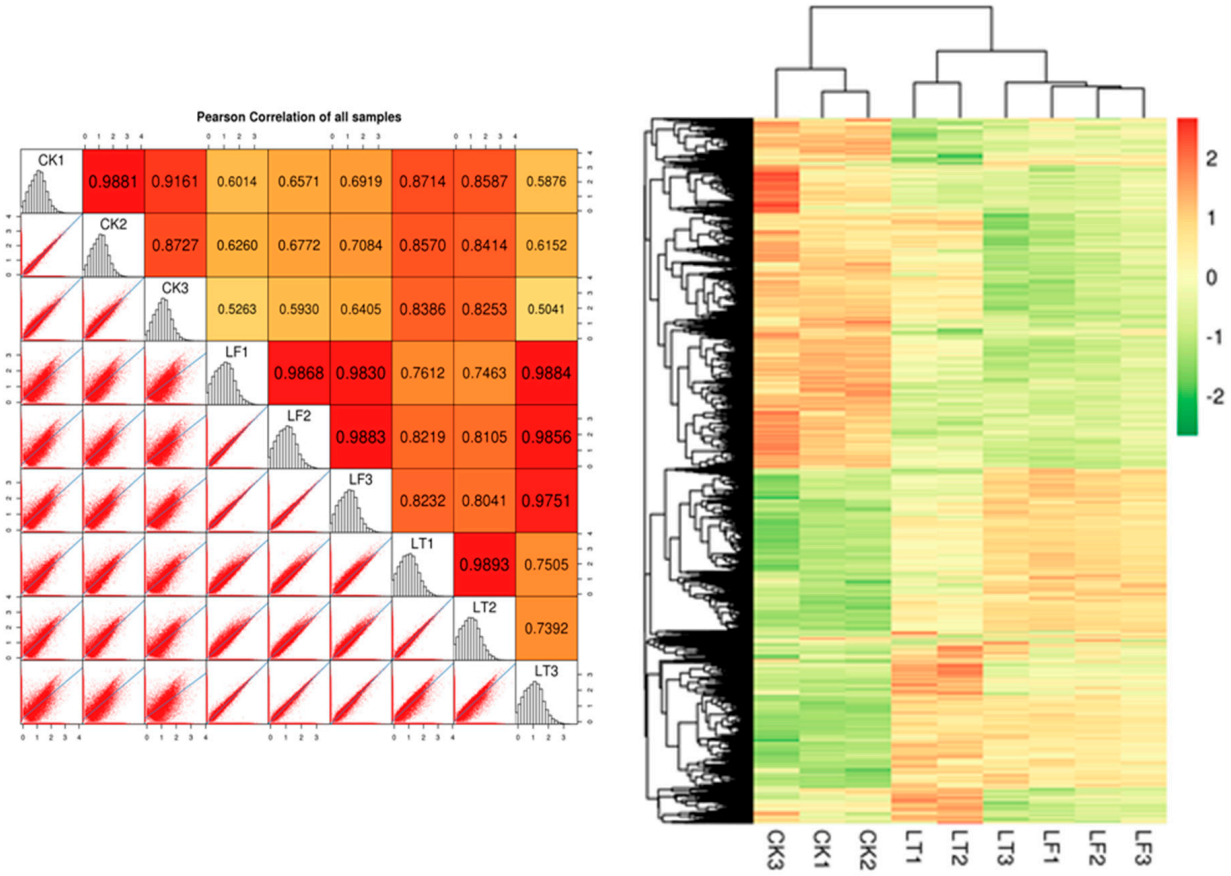
LT, low temperature treatment for 3 d; LTF, low temperature flooding treatment for 3 d; CK, control.

2.5. Differential Expression Gene Analysis

In this study, the reliability of the transcriptome data was assessed using the Pearson's correlation coefficient, that is, the closer the r is to 1, the stronger the correlation between the two replicate samples; the good reproducibility of all three biological replicates in each treatment was obtained from the correlation coefficient (Figure 2a). In addition, we visualized the expression patterns of different points in each group through cluster analysis, which further showed that the data were reliable (Figure 2b). To compare the differences on gene expression under LT and LTF, we divided the different treatments into three groups. In total, 5934 DEGs were identified from the rice leaves between the LT and CK (3207 up-regulated and 2727 down-regulated) (Figure 3a,d); 7658 DEGs were identified between the LTF and CK (3487 up-regulated and 4171 down-regulated) (Figure 3b,d); and 2697 DEGs were identified between the LTF and LT (451 up-regulated and 2246 down-regulated) (Figure 3c,d).

2.6. GO Enrichment Analysis of DEGs

The main biological functions of DEGs in rice seedlings under LT and LTF stress were further to be studied using gene ontology (GO) enrichment analysis. All DEGs can mainly contain three categories such as biological process (BP), molecular function (MF) and cellular component (CC). GO enrichment analysis showed that the DEGs were involved in nine subcategories of CC (Figure 4a), eight subcategories of MF (Figure 4b) and fifteen subcategories of BP (Figure 4c) between LT, LTF and CK. In cellular components, the three most enriched subcategories were 'cell', 'cell part' and 'organelle'. The 'binding' was the most enriched subcategory in the molecular function. In biological process, the 'metabolic process' was the most enriched subcategory.



(**a**) (**b**)

Figure 2. (**a**) Repeatability test between each two samples. Pearson coefficient closer to 1 indicates that the correlation between the two repeated samples is stronger. (**b**) Results of hierarchical cluster analysis of differentially expressed genes. Different columns represent different samples, and different rows represent different genes.

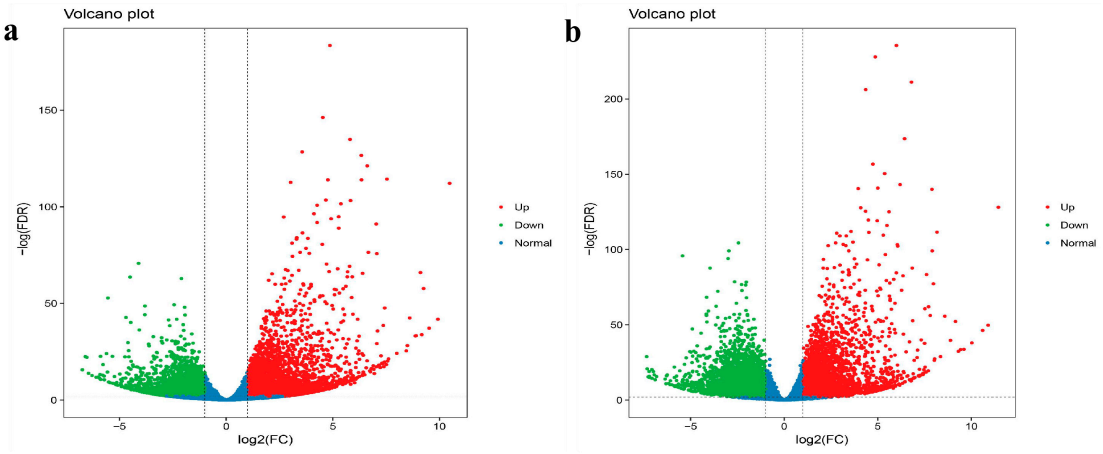


Figure 3. *Cont.*

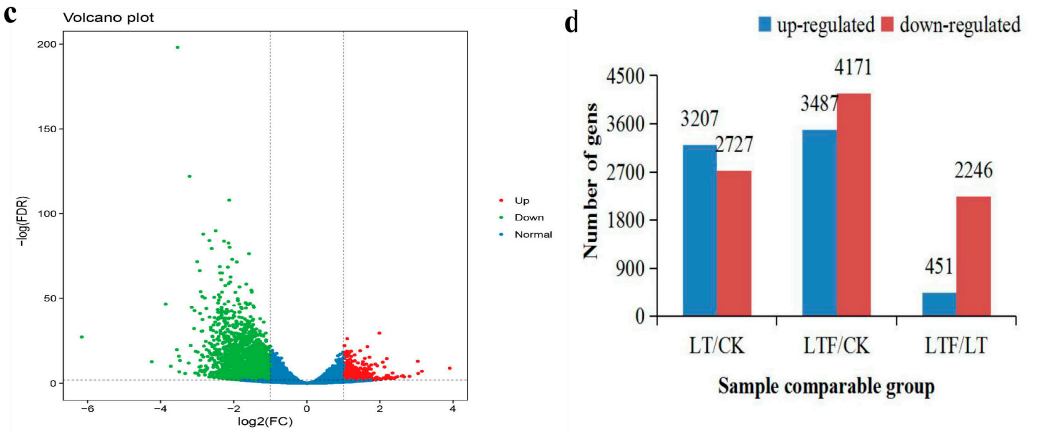


Figure 3. Volcano plot of differentially expressed gens. (**a**) LT/CK (**b**) LTF/CK (**c**) LTF/LT. LT, low temperature treatment for 3 d; LTF, low temperature flooding treatment for 3 d; CK, control. The green dots mean the down-regulated genes, the red dots mean the up-regulated genes, and the blue dots mean the non-significant differentially expressed genes. (**d**) Number of leaves genes up-and down-regulated. The blue column and red column represent the number of up-regulated and down-regulated genes, respectively.

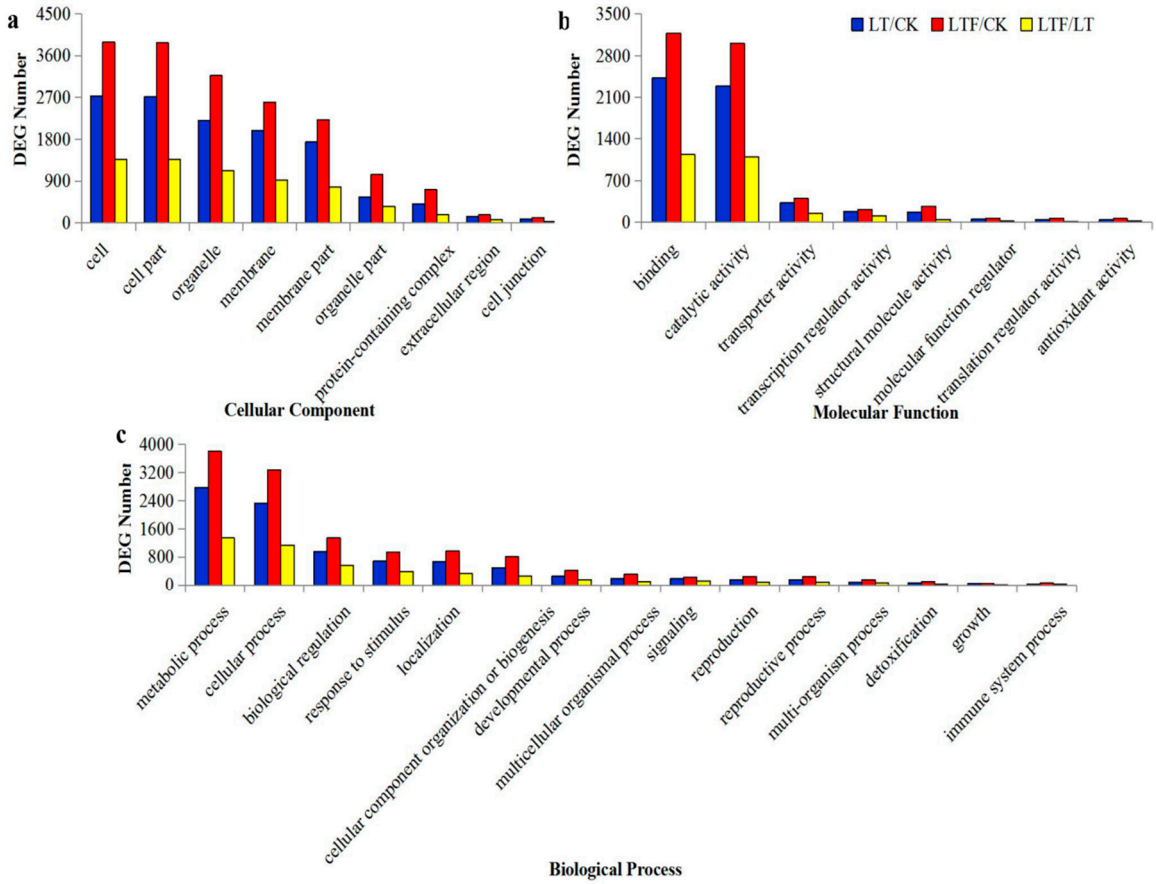


Figure 4. GO enrichment analysis of DEGs in leaves of rice seedlings. LT, low temperature treatment for 3 d; LTF, low temperature flooding treatment for 3 d; CK, control.

2.7. KEGG Pathway Analysis

KEGG overexpression pathway analysis was performed on all annotation DEGs to gain functional insight into the differences between LT, LTF and CK. The significantly ($p < 0.01$) enriched KEGG pathways are shown in Table 4. The KEGG pathways (in rank

order) are ribosome, flavonoid biosynthesis, glycosaminoglycan biosynthesis-chondroitin sulfate, tryptophan metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, plant hormone signal transduction, carotenoid biosynthesis, diterpenoid biosynthesis, indole alkaloid biosynthesis and glycolysis gluconeogenesis between LT and CK. The KEGG pathways are (in rank order): ribosome, photosynthesis, ribosome biogenesis in eukaryotes, carotenoid biosynthesis, photosynthesis-antenna proteins, flavonoid biosynthesis, glycolysis gluconeogenesis and galactose metabolism between LTF and CK. The KEGG pathways are (in rank order): photosynthesis, photosynthesis-antenna proteins, carotenoid biosynthesis, carbon fixation in photosynthetic organisms, glyoxylate and dicarboxylate metabolism, nitrogen metabolism, circadian rhythm-plant, porphyrin and chlorophyll metabolism, methane metabolism, flavonoid biosynthesis, plant hormone signal transduction, glycine, serine and threonine metabolism between LTF and LT. In the LT/CK and LTF/CK comparison groups, the co-enriched KEGG pathways were ribosome, carotenoid biosynthesis, flavonoid biosynthesis and glycolysis/gluconeogenesis.

Table 4. KEGG pathway enrichment analysis of DEGs between LT, LTF and CK.

Pathway Name	Count	Percent (%)	*p*-Value
KEGG Pathways between LT and CK			
Ribosome	159	6.37	1×10^{-12}
Flavonoid biosynthesis	44	1.76	1.09×10^{-3}
Glycosaminoglycan biosynthesis-chondroitin sulfate	8	0.32	1.09×10^{-2}
Tryptophan metabolism	42	1.68	1.20×10^{-2}
Phenylalanine, tyrosine and tryptophan biosynthesis	23	0.92	1.52×10^{-2}
Plant hormone signal transduction	135	5.41	1.74×10^{-2}
Carotenoid biosynthesis	25	1.00	1.81×10^{-2}
Diterpenoid biosynthesis	25	1.00	3.58×10^{-2}
Indole alkaloid biosynthesis	10	0.40	4.47×10^{-2}
Glycolysis/Gluconeogenesis	58	2.32	4.50×10^{-2}
KEGG Pathways between LTF and CK			
Ribosome	239	7.10	5×10^{-12}
Photosynthesis	39	1.16	1.19×10^{-7}
Ribosome biogenesis in eukaryotes	61	1.81	3.88×10^{-5}
Carotenoid biosynthesis	38	1.13	2.25×10^{-4}
Photosynthesis-antenna proteins	11	0.33	4.48×10^{-4}
Flavonoid biosynthesis	54	1.61	2.46×10^{-3}
Glycolysis/Gluconeogenesis	81	2.41	7.05×10^{-3}
Galactose metabolism	51	1.52	8.56×10^{-3}
KEGG Pathways between LTF and LT			
Photosynthesis	27	2.39	7×10^{-12}
Photosynthesis-antenna proteins	8	0.71	2.21×10^{-5}
Carotenoid biosynthesis	181	1.59	3.79×10^{-4}
Carbon fixation in photosynthetic organisms	30	2.65	4.98×10^{-4}
Glyoxylate and dicarboxylate metabolism	23	2.03	7.44×10^{-4}
Nitrogen metabolism	13	1.15	1.28×10^{-3}
Circadian rhythm-plant	25	2.21	1.80×10^{-3}
Porphyrin and chlorophyll metabolism	18	1.59	7.28×10^{-3}
Methane metabolism	22	1.95	1.36×10^{-2}
Flavonoid biosynthesis	21	1.86	1.57×10^{-2}
Plant hormone signal transduction	67	5.92	1.64×10^{-2}
Glycine, serine and threonine metabolism	23	2.03	1.78×10^{-2}

LT, low temperature treatment for 3 d; LTF, low temperature flooding treatment for 3 d; CK, control.

2.8. qRT-PCR Verification

Eight genes with significantly altered expressions were evaluated using qRT-PCR to validate the results of the RNA-seq data. All of these genes were predicted to be associated

with LT, and the functional annotations of these genes are listed in Table S1. The expression patterns of Os07g0529600, Os01g0581300, Os02g0704000, Os03g0743900, Os01g0866400, Os02g0664000, Os07g0170300 and Os07g0636000 displayed a similar expression pattern in qRT-PCR analysis as in RNA-Seq analysis. This result implies that the obtained RNA-seq data are reliable (Figure 5).

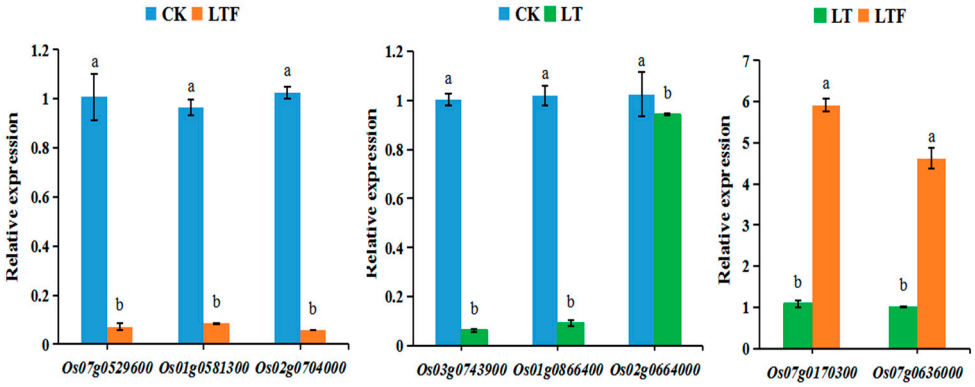


Figure 5. Comparison of genes expression levels using RNA-Seq and qRT-PCR. LT, low temperature treatment for 3 d; LTF, low temperature flooding treatment for 3 d; CK, control. Data represent the mean ± SE (n = 3), and different lowercase letters indicated significant differences at $p < 0.05$.

3. Discussion

3.1. Response to Flooding Mitigation of Low Temperature Stress by Agronomic Characters

LT chilling injury caused the most intuitive changes in the morphological indexes of rice seedlings, such as the decrease in the growth rate of seedling height, the curl of leaves and the decrease in root numbers [20]. Rice will undergo a series of physiological and ecological adaptive changes under LT stress, which will eventually show in the plant morphology. Root number, root length and leaf length can be used as identification indexes of the cold tolerance of rice [21,22]. After LT treatment, the aboveground morphological indexes and root morphological indexes of seedlings of different tolerant varieties decreased to a certain extent, but the degree of decrease was different [23]. The results showed that the growth of rice seedlings was seriously hindered under 6 °C LT stress, the growth of some rice seedlings stopped and the root activity decreased significantly [24]. Moderate flooding could reduce LT damage to early rice seedlings by increasing water temperature [11]. This study showed that LT stress affected the normal growth and development of plants. Compared with LT, the seedling height, fresh weight, dry weight and top three leaves increased significantly after 3 days of LTF treatment; flooding could alleviate the effect of LT on agronomic characters of direct-seeded early rice seedlings and play a role in protecting seedlings in deep water. Moderate flooding can stimulate changes in plant physiological characteristics, thus promoting plant height growth and allowing rice to exhibit better adaptability [25].

3.2. Potential DEGs Play Important Roles in Flooding Mitigation of Low Temperature Stress

Abiotic stresses in plants induce drastic molecular responses. The response of plant molecular mechanisms to abiotic stresses has attracted much attention [26,27]. To explore the molecular mechanism of rice seedlings' response to flooding mitigation of LT stress, we performed transcriptome analysis. An amount of 5934 differentially expressed genes were obtained from the rice leaves between the LT and CK (3207 up-regulated and 2727 down-regulated), 7658 DEGs were identified between the LTF and CK (3487 up-regulated and 4171 down-regulated) and 2697 DEGs were identified between the LT and LTF (451 up-regulated and 2246 down-regulated). The results of this study indicated that the gene expression in rice seedlings was greatly altered under LT and LTF stress. GO enrichment analysis helps to highlight the main biological processes in response to adversity stress. For example, 'signal transduction', 'energy and carbohydrate metabolism', 'biological regulations' and

'photosynthesis' were mostly enriched in *indica* rice under LT [28]. 'Secondary metabolites', 'tryptophan biosynthesis', 'protein phosphorylation' and 'signaling pathways' were enriched under flooding conditions [29]. Our results detected that genes involved in 'metabolic process', 'cellular process' and 'biological regulation' highly enriched in rice leaves subjected to LT and LTF stress. DEGs associated with these processes may play an important role in crop response to flooding and chilling [30,31]. Thus, the response of these related genes enhances the adaptation to flood mitigation LT stress.

3.3. Photosynthesis, Energy Metabolism and Signal Transduction Pathway Play Important Roles under Flooding Mitigation of Low Temperature Stress

The KEGG pathway has been used to describe the network of molecular interactions, reactions and relationships of the identified genes and gene products [25]. Previous studies have found that many genes are involved in cold damage resistance conditions through various hemylpropanoid biosynthesis [32,33]. For example, DEGs associated with plant hormone signal transduction pathway were mostly represented in medicago falcata roots under LT stress [34]. Genes encoding WRKY33, APX1, PYLs, CAM5, ribosomal proteins and HSPs acted as potential candidates for improving cold storage tolerance in sweet potato [35]. In this study, genes related to 'photosynthesis', 'photosynthesis-antenna proteins', 'carotenoid biosynthesis' and 'flavonoid biosynthesis' were also identified both in LTF and CK and in LT and LTF. These results were consistent with previous findings that these pathways can form a tight signaling network and play an important role in flooding mitigation of LT stress [36,37].

3.4. Some Candidate Genes for Plant Low Temperature stress Tolerance Breeding

When rice was subjected to LT stress, the balance of intracellular oxygen metabolism was disturbed, leading to ROS generation [38–40]. The accumulation of ROS scavenging enzymes is considered as one of the most important responses during LT stress [41]. Moderate flooding improves the antioxidant capacity of plants as it restrains membrane peroxidation and decreases the aggravation of peroxidative injury in leaves [25]. In this study, we found that the expression of key genes of the antioxidant pathway (Os02g0664000) was significantly up-regulated in LT treatment compared to the control. Additionally, the expression of key genes of the antioxidant pathway (Os07g0170300 and Os07g0636000) was significantly up-regulated under LTF treatment, and the present study was consistent with the previous findings [42]. LT leads to the production of reactive oxygen species (ROS), and the accumulation of ROS may cause oxidative stress. To survive LT cold damage, plants induce antioxidant enzyme systems to scavenge reactive oxygen groups in order to prevent these groups from damaging cells and improve their stress tolerance [43,44]. Furthermore, this study concluded that the expression of peroxidase gene (Os01g0866400) was significantly downregulated in LTF treatment compared to LT treatment. These genes might play a vital role in flooding to alleviate direct stress to seedlings caused by LT, and shallow flooding improves the antioxidant enzyme protection system of live seedlings of early *indica* rice under LT [45].

4. Conclusions

Phenotype, agronomic traits and transcriptomic analyses were employed to investigate the molecular mechanism by which flooding mitigates LT stress. LT caused a significant decrease in seedling height, root number, fresh weight, dry weight and T3 leaf length. Compared with LTF, the seedling height, root number, fresh weight, dry weight and T3 leaf length significantly decreased after 3 days of LT treatment. LTF could reduce the damage of LT to the agronomic characters of rice seedlings. In addition, LTF significantly reduced soluble protein content and CAT activity compared to LT. Transcriptomic profiling showed that 5934 DEGs were identified between the LT and CK; 7658 DEGs were identified between the LTF and CK and 2697 DEGs were identified between the LT and LTF treatment. DEGs were significantly enriched in photosynthesis, carotenoid biosynthesis, flavonoid

biosynthesis, glycolysis gluconeogenesis, glycine, serine and threonine metabolism and plant hormone signal transduction pathways. The expression of peroxidase gene was significantly downregulated in LTF treatment compared to LT treatment. Photosynthesis, energy metabolism and signal transduction pathway play important roles under flooding mitigation of LT stress. The results of this study may help to elucidate the changes in physiological characteristics and gene expression to alleviate LT stress through flooding. We will further clone the functional genes in the future.

5. Materials and Methods

5.1. Plant Materials and Growth Conditions

Zhongjiazao 17 (ZJZ17) is the dominant inbred early *indica* rice cultivar, which was widely planted in the Jiangxi province. The pots experiment was conducted at the Key Laboratory of Crop Physiology of Jiangxi Agricultural University (JXAU) Nanchang, Jiangxi Province of China in 2019. Uniformly germinated seeds were selected and directly seeded in plastic pots (15.0 cm height, 25.0 cm length and 23.0 cm wide), 40 germinated seeds were placed in each of the pots. The soil was obtained from the upper soil layer (0–20 cm) of the rice experiment paddy field. The physical and chemical properties of the experimental soil are shown in Table 5. Each pot contained approximately 6 kg of dry soil, which was soaked in water two weeks prior to direct seeding, and 3 g compound fertilizer (N-P-K = 15–15–15%) was applied to each pot as base fertilization. During the rice seedling growth period, other management measures were in accordance with local recommendations.

Table 5. Physical and chemical properties of the experimental soil.

Soil pH	Organic Matter ($g \cdot kg^{-1}$)	Total Nitrogen ($g \cdot kg^{-1}$)	Available Phosphorus ($mg \cdot kg^{-1}$)	Available Potassium ($mg \cdot kg^{-1}$)
6.1	30.35	2.4	25.17	84.02

5.2. Experimental Design

Selected uniform seeds were sterilized with ethanol for one minute, then washed three times with water and soaked in water for 24 h and placed in a 25 °C artificial climate chamber for 24 h without light. Select seeds with uniform germination, sow them directly in plastic pots and place them in an artificial climate chamber (the diurnal temperature was set at 27/23 °C). After 20 days of direct seeding, the seedlings were transferred to different conditions in an artificial climate chamber. Low temperature (LT): temperature period is 10/6 °C (day/night) and potting soil remained moist. Low temperature flooding (LTF): temperature period is 10/6 °C (day/night) and the rice plants were maintained in a 5–6 cm flood water layer. Control (CK): temperature period is 27/23 °C (day/night) and potting soil remained moist. The light period for all was 12 h (7:00–19:00), the light intensity for all was 100 $\mu mol \cdot m^{-2}s^{-1}$ and the relative humidity (RH) of the artificial climate chamber for all was 75%. Each treatment was applied to 20 pots and 3 replicates. After 3 days of treatments, leaves were sampled from CK, LT and LTF rice seedlings, the tin foil was wrapped and immediately placed in liquid nitrogen, and later placed in a −80 °C refrigerator until the extraction of RNA.

5.3. Agronomic Characters

After 3 days of LT and LTF treatment, the plant height, root length, root number, leaf length and fresh weight were sampled and measured according to the conventional method; for example, to obtain the height of the plants, they were taken out of the soil, washed and measured between the base of the seedling stems and the top of the leaves. After the drying process at 110 °C for 15 min, the dry weight of the ground portion was determined after oven drying at 80 °C to achieve a constant weight.

5.4. Physiological Characteristic

Enzymatic activities were measured on the third day of LT and LTF treatments. For each treatment group, three pots of plants were selected and 3 g of leaf samples were taken, placed in tin foil and immediately put into liquid N_2 and then stored at −80 °C until extraction. Peroxidase (POD) activity, superoxide dismutase (SOD) activity, catalase (CAT) activity and soluble protein content were determined using solution configuration [46–48].

5.5. Isolation of RNA, Library Construction, and Illumina Sequencing

Total RNA was extracted from different groups of leaf tissue samples, and the concentration and purity of the extracted RNA were examined using a NanoPhotometer® spectrophotometer and a Qubit® 2.0 Flurometer, and agarose gel electrophoresis to detect RNA integrity. The mRNA in Total RNA was sorted and purified and specifically enriched for mRNA. mRNA was randomly broken into small fragments of 200 bp by adding fragmentation buffer. The cDNA was synthesized using reverse transcription with primers using mRNA as the template and sequenced using the Illumina platform. After the raw sequencing data was acquired, the high-quality sequencing data (clean data) obtained after filtering the raw sequencing data was statistically and quality assessed to ensure the accuracy of the subsequent bioinformatics analysis.

5.6. GO and KEGG Enrichment Analysis of DEGs

Differential expression analysis of the two groups was performed using the DESeq2 R package. The method of Benjamini and Hochberg was used to produce p-values < 0.01 and genes with fold changes >2 found using DESeq2 were considered to be differentially expressed. GO enrichment analysis of differentially expressed genes was implemented using the GOseq R package, which was corrected for gene length bias. GO terms with corrected p-values less than 0.05 were considered significantly enriched by differential expressed genes. The KEGG database was further consulted to explore the high-level function and utility of flooding to alleviate seedling chilling in rice from large-scale molecular data generated using genome sequencing and other high-throughput experimental techniques (http://www.genome.jp/kegg/, accessed on 1 February 2021). Statistical enrichment of differentially expressed genes in the KEGG pathway was tested using KOBAS software.

5.7. qRT-PCR Verification

Total RNA from three tissue samples was isolated using an RNA Extraction Kit (Tiangen Biotech, Beijing, China). The cDNA was prepared using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Kyoto, Japan). The qRT-PCR primers were designed using Primer 5.0 (Table S2). A total of eight genes in the LT/CK, LTF/CK and LTF/LT comparison groups were selected for qRT-PCR analysis with specific primers. qRT-PCR detailed implementation steps refer to previous literature [49]. Three biological and technical replicates were conducted separately to ensure data reliability.

5.8. Statistical Analysis

Data on agronomic traits and physiological characteristics in this study were shown as the means ± standard deviations (SD) of three biological replicates. All data analyses were conducted with SAS version 9.4. The means were analyzed using ANOVA, followed by Tukey's honest significant difference (HSD).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030834/s1, Table S1: Primers used for qRT-PCR verification of differently expressed genes. Table S2: Genes for qRT-PCR.

Author Contributions: Y.Z. (Yanhua Zeng) and S.C. conceived and designed the experiments. W.W. and J.D. conducted experiments and analyzed the data. Z.W. and Y.Z. (Yongjun Zeng) contributed new reagents or analytical tools. W.W. wrote the manuscript. X.P. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The authors thank the financial support provided by the National Natural Science Foundation of China (32272212, 31760366), the Key Project of Jiangxi Province Natural Science Foundation (20202ACBL215004), the Earmarked Fund for Jiangxi Agriculture Research System (JXARS-19-02, JXARS-02-03), the Youthful Innovation Research Team of Jiangxi Agricultural University (JXAUCXTD004), and Jiangxi Natural Science Foundation of China (20161BAB214171).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed in this study are included in this published article, and RNA sequencing data are available upon request from the corresponding author.

Acknowledgments: We thank the workers who have been at the forefront of the fight against the COVID-19 pandemic, allowing us to focus on writing this paper.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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