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Transcriptomics Analysis on Fertility Conversion in Thermosensitive Genetic Male Sterility Line Zhu1S under High Temperature

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Abstract: Zhu1S is a thermosensitive genic male-sterile (TGMS) line of rice possessing outstanding combining ability and low critical temperature, which has been extensively utilized as a female parent in two-line hybrid ricebreeding. However, the fertility of Zhu 1S during hybrid seed production is frequently affected by high temperature, thus leading to its fertility alteration and aborted hybrid seed production. To understand its fertility conversion mechanism under high temperature, we employed transcriptomics analyses on the anthers of young panicles of Zhu 1S during the fertility alternation sensitivity stage under high (Zhu 1S-H) and low (Zhu 1S-L) temperatures. The results showed that a total of 1119 differentially expressed genes (DEGs) were identified between Zhu 1S-H and Zhu1S-L anthers, including 680 up-regulated and 439 down-regulated genes. Bioinformatics analysis of these DEGs revealed that the high temperature induction caused fertility-sterility conversion in Zhu1S, mainly by decreasing the mRNA abundances of important genes closely related to plant hormone and MAPK signal pathway and transcriptional regulation factors, thereby impeding the growth and development of the anther of Zhu 1S, which ultimately affected the fertility transition of Zhu 1S under high temperature. The protein–protein interaction network analysis indicates that transcription factor OsTIFY11C possibility plays a central role in the fertility transition of Zhu 1S under high temperature.The present studies offer a theoretical foundation for further research into the molecular mechanism underlying fertility conversion in TGMS line Zhu 1S.

Keywords: TGMS line; Zhu 1S; transcriptome analysis; fertility conversion; protein-protein interaction network

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1. Introduction

A number of genetic male sterility lines are regulated by temperature; the plants are male sterile under high temperature and fertile under low temperature [1–9]. To date, several temperature-sensitive male sterile (TGMS) lines have been used in rice breeding programs, such as Zhu 1S [1–3,5,10,11]. Zhu1S is an indica male sterile line, which has low critical temperature of fertility change [1–3]. Its critical sterility induces temperatures lower than 23 °C (about 21.5 °C) and lower than those of most TGMS lines [1,3,11]. Therefore, Zhu 1S is widely applicated for two-line hybrid rice breeding, the genetic. However, the fertility of Zhu 1S during hybrid seed production is frequently affected by high temperature during young panicles stage, thus leading to its fertility alteration and hybrid seed production failure. The molecular mechanisms of fertility transition during high temperature are largely unknown.

Previous studies comparatively analyzed the young panicle proteome in Zhu 1S during fertility conversion using sodiumdodecylsulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) combined with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) [2]. A total of 20 identified differentially abundant proteins (DAPs) are mainly involved with protein synthesis, cell wall formation and other cellular processes during pollen development, thereby suggesting that these proteins play critical roles during fertility conversion in Zhu 1S [2]. More recently, the young panicle proteomes of two TGMS rice lines, Zhu 1S and Zhun S, were comparatively analyzed by proteomic approach [3]. Compared with Zhun S, the young panicles of Zhu 1S have lower levels of indole-3-acetic acid, soluble proteins and faster anther wall disintegration. These major differences may result in a more stable fertility in Zhu 1S than in Zhun S [3]. The above studies have improved our understanding of the mechanism underlying fertility alteration in Zhu 1S at the protein level under low temperature. However, until now, the molecular mechanism of fertility conversion during high temperature in Zhu 1s has not been elucidated.

Transcriptome analysis provides a powerful high-throughput method for identifying key genes involved with male sterility pathways and for revealing the relevant molecular mechanisms [4,12,13]. In the present study, we performed transcriptome analysis on the anthers of young panicles of Zhu 1S and attempted to dissect the molecular mechanism of fertility-sterility conversion during high temperature. Fertility-sterility conversions of Zhu 1S were induced when the temperature was above 21.5 °C. After comparative morphological observations and the iodine-potassium staining of pollen grains, transcriptome analysis was employed to analyze the anther transcriptome of Zhu 1S treated under high (30 °C, Zhu 1S-H) and low (21.5 °C, Zhu 1S-L) temperature. A total of 1119 DEGs were identified, and these corresponding DEGs were mainly involved in key pathways, such as signal transduction and transcriptional regulation associated with fertility transition. In addition, the protein–protein interaction network analysis indicates that the OsTIFY11C possibility plays a central role in the fertility transition of Zhu 1S under high temperature.The above results may further broaden our understanding of the mechanisms of fertility transition in the TGMS rice line.

2. Materials and Methods

2.1. Plant Materials and Growth Condition

Zhu1s rice plants were grown in the paddy field (daily temperature: 26–32 °C) at the Hybrid Rice Research Base in Hunan Normal University, Hunan, China. When the flag leaves of the plants emerged at about 10 cm (about one week before anthesis), some of the Zhu1s plants were transferred into a shallow pool containing 21.5 °C water that was circulated through an industrial chiller. The others were left in the paddy field and used as control under high temperature (30 °C). After 7 days, the anthers of young panicles of the plants were collected and immediately used, respectively. The fertility status of the anthers was determined by the number and shape of pollen grains [1].

2.2. RNA Extraction, Library Construction and Sequencing

Total RNA was extracted using the QIAGEN RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN). Purification of mRNA from total RNA was conducted using an Oligotex mRNA Mini Kit (QIAGEN). The mRNA was then used to construct cDNA libraries using RNA-Seq Sample Preparation KitTM (Illumina), following standard protocols.

2.3. Sequencing Data Analysis and Differentially Expressed Gene Evaluation

Preprocessing the original data (the elimination of sequencing joints and low-quality sequencing data) is necessary to produce reliable analytical results. It was performed by counting the original sequencing, effective sequencing and Q30 for a comprehensive evaluation. The raw sequence reads were uploaded to the SRA, and the code number is PRJNA838645. Differential expression genes (DEGs) were screened using the fold change >1.5 and corrected p value (padj) <0.05.

2.4. Functional Classification and KEGG Analysis of Differentially Expressed Genes

Biological process, molecular function and cell component are the three major components of the gene ontology (GO) annotation system. Two-way *p*-value and rich factor were used to analyze the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [4,12].

2.5. Protein Interaction Analysis Using STRING

The STRING database was used to identify the predicted interactions of proteins [4]. The network connections were visualized by their confidence score, where a thicker line indicates a higher interaction score. The minimum interaction threshold was set to 0.700 in this study. The rice gene IDs inputs were used for the STRING database.

2.6. Quantitative RT-PCR

The RNAs of representative DEGs were analyzed with quantitative reverse-transcription polymerasechain reaction (RT-PCR). The total RNA extraction from the anthers, the first-strand DNA synthesis and real-time polymerase chain reaction (real-time PCR) were performed according to the instructions of the corresponding commercial kits (TransGen Biotech, Beijing, China). Real-time PCR analyses were performed as described previously [14]. *OsActin1* was used as control, and all primers used for the real-time PCR assay are listed in Table S1.

3. Results

3.1. Phenotype Analysis of Zhu 1SPollen Grains during Fertility Transition

To determine the critical condition of Zhu 1s fertility conversion, we performed phenotype observations. The results showed that more than 90% normal pollen grains were deeply stained by iodine–potassium iodide under low temperature (21.5 °C), suggesting that the pollen grains were rich in starch (Figure 1A). However, the small and shrinking pollen grains were found in Zhu 1S under higher temperatures (30 °C), and the pollen grains were unstainable by iodine-potassium iodide, indicating that the pollen grains possessed almost no starch (Figure 1B). The above data showed that the fertility of Zhu 1S is affected by high temperature and were consistent with previous results [1,11]. Moreover, Zhu 1S is an ideal and reliable material for the study of fertility transition of two-line hybrid rice.

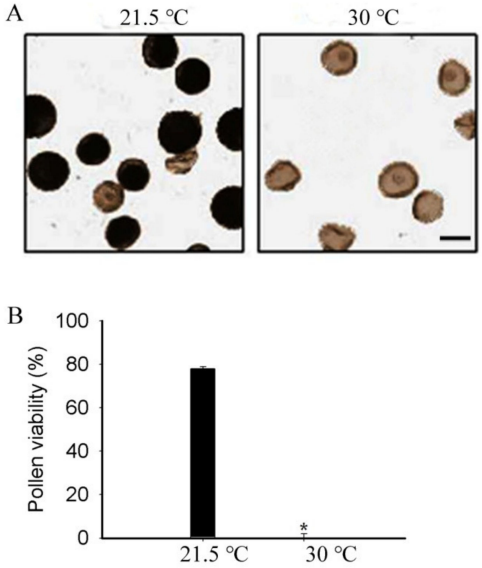

Figure 1. Pollen viability analysis of Zhu 1S under different temperatures. (**A**) Iodine–potassium staining of pollen grains from Zhu 1S under different temperatures. Bar = 100 μm. (**B**) Pollen viability of Zhu 1S under different temperatures. The experiment was repeated three times with similar results. Asterisks represent significant differences. Error bars represent ±SD. Significant difference was found between high (30 °C) and low (21.5 °C) temperature ($p < 0.01$ by Student's *t*-test).

3.2. Transcriptional AnalysisRevealed Genes Showing Transcriptional Changes in Response to High Temperature in Zhu 1S

Three RNA samples were prepared from the anthers collected under Zhu 1S-H (30 °C) and Zhu1S-L (21.5 °C) conditions, respectively. Two samples were found to be in good agreement, so we used the two samples for further study under Zhu 1S-H and Zhu1S-L conditions, respectively (Figure 2A). These samples were sequenced using the Illumina NovaSeq 6000 platform. A total of about 194 million raw sequencing reads with lengths of 100 bp were generated (Table S2). Low-quality reads, including adapters and unknown or low-quality bases, were discarded, according to bioinformatics analysis. About 190 million clean reads were eventually obtained, representing 93.3% of the total raw reads (Table S2). The average Q30 was about 94% (Table S2). The above results show that the transcriptome sequencing quality meets the criterion.

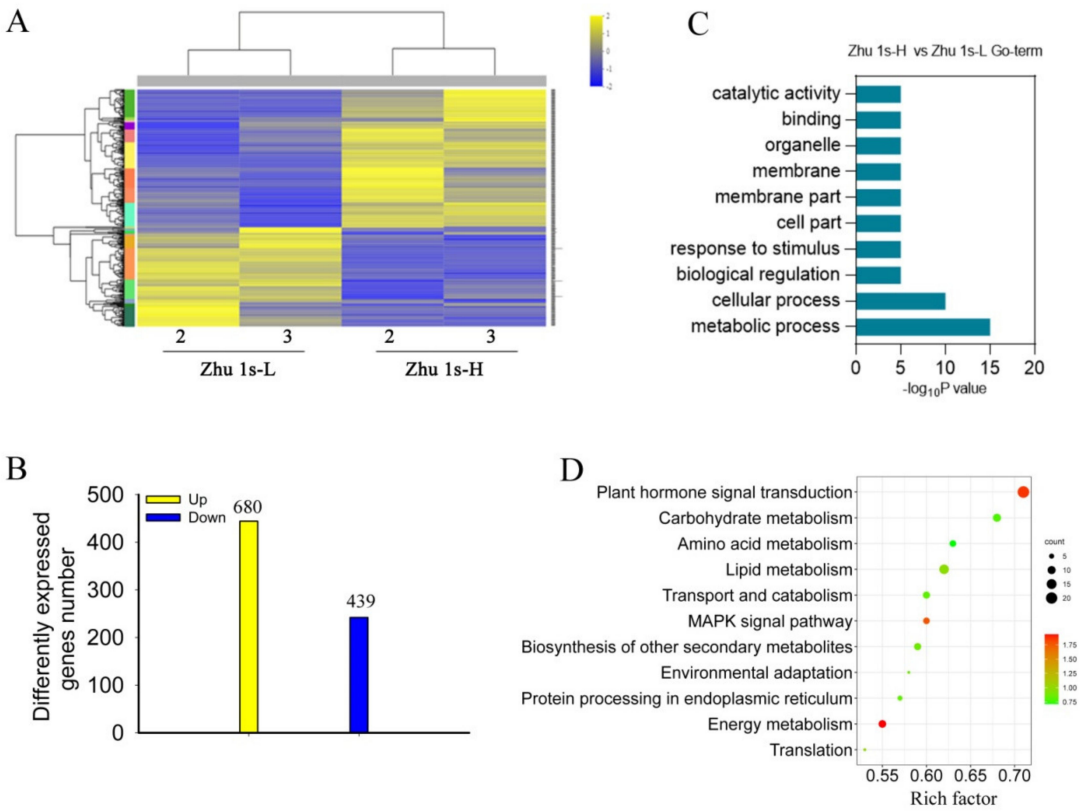

Figure 2. Transcriptome analysis on the anthers of Zhu 1S under low (Zhu 1S−L) and high (Zhu 1S−H) temperature. (**A**) Hierarchical cluster analysis of gene expression in Zhu 1S−H and Zhu 1S−L. The yellow and blue colors represent increased and decreased transcript level, respectively. (**B**) The up- and down-regulated gene numbers in Zhu 1s−H relative to Zhu 1s−L. (**C**) GO enrichment analysis for differential expression in Zhu 1S−H compared to Zhu 1S−L. (**D**) KEGG pathway analysis for differential expression in Zhu 1S−H compared to Zhu 1S−L.

Generally, genes with similar expression patterns have similar functions or are related to the same signal pathways. To identify clusters with functional enrichment, hierarchical clustering was performed based on gene expression patterns (Figure 2A). Remarkable differences were found in gene expression in Zhu 1s plants under high temperature treatment compared to the expression under low temperature treatment. Then, we filtered these differential expression genes (DEGs) with parameters fold change >1.5 and corrected *p*-value (padj) <0.05. A total of 1119 DEGs were consequently identified between the Zhu 1s-H and Zhu 1S-L samples (Figure 2A). Among them, 680 up-regulated DEGs and 439 down-regulated DEGs were detected in Zhu 1S-H samples, respectively (Figure 2B).

3.3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analysis of Differentially Expressed Genes Response to High Temperature in Zhu 1S

The GO assignment system was used to define the crucial functional categories for these identified DEGs. A large proportion of DEGs were found to be connected with the cellular process, biological regulation, membrane and cell part, metabolic process, catalytic activities, response to stimulus (Figure 2C). DEGs were also found as chiefly concerned with signal transduction and transcription regulation by KEGG (Figure 2D).

3.4. Hormone and MAPK Signal Transduction Was Affected in Zhu 1S under High Temperature

Under biotic and abiotic stresses, signal transduction pathways transmit information at the cellular level and lead to changes of many cellular processes and metabolism pathways (Chen et al., 2019). In this transcriptional analysis, we identified 21 plant hormone and mitogen-activated protein kinase (MAPK) signal transduction-related genes in Zhu 1S under high temperature. Plant hormone signal transduction-related genes included six cytokinin-related *OsCTKs* genes (*Os03g0224200*, *Os04g0442300*, *Os12g0139400*, *Os02g0557800*, *Os07g0449700*, *Os01g0952500*), nine Auxin-responsive *Aux/IAA* genes (*OsIAA17*, *OsIAA19*, *OsSAUR8*, *OsSAUR24*, etc.) and five Jasmonic-acid-related *OsTIFYs* genes. The MAPK signal pathways include Clade A type 2C protein phosphatase-related genes (*OsPP2C*) and mitogen-activated protein kinase -related genes (*OsMAPK*) (Figure 3A). Those genes are in relation to the CTK, IAA, JA, ABA signal pathways. Among them, the expression levels of the DEGs involved in CTK, JA and MAPK signals were all down-regulated in Zhu 1S under high temperature (Figure 3A). In addition, genes related to Aux/IAA signals were also all down-regulated under high temperature, except for *OsSAUR8* and *OsSAUR24* (Figure 3A).

Due to a large group of genes enriched in signal transduction pathways under high temperature, we further categorized these genes according to their roles in signal transduction pathways. The schematics of plant hormone signal transduction and MAPK signal pathway affected by high temperature are shown in Figure 3B. The red and green squares indicate the plant hormone signal transduction and MAPK signal pathway that were up-regulated and down-regulated by high temperature, respectively. From these schematics, we found that CTK-signal-related genes (*OsARR*), JA-signal-related genes (*OsTIFY/OsJAZ*) and MAPK-signal-related genes (*OsPP2C*, *MEKK1*, *MAPKs*) also showed down-regulated transcriptional levels under high temperature (Figure 3B). The results were consistent with those in Figure 3A.

3.5. Transcriptional Regulation Process Was Affected in Zhu 1S under High Temperature

Several transcription factors are well known to be specially related to anthers and-pollen development [9,13]. A total of 29 transcription factors showed at least 2-fold transcriptional changes in Zhu 1S under high temperature (Figure 4A, Table S4). These transcription factor genes mainly belonged to the *WRKY*, *NAC*, *AP2/ERF*, *MYB*, *bHLH* and *TIFY* families (Table S3). Among them, almost all transcription factors were down-regulated, except for three *OsMYB* transcription factor genes *Os08g0157600* (*OsCCA1*), *Os06g0728700* (*OsEPR1*), *Os04g0470600* (*OsMYB80*), under high temperature (Figure 4A).

To validate the expression profiles of the transcription factor genes in our transcriptome sequencing analysis, six randomly selected genes were evaluated with real-time PCR. The expression patterns of all six genes were the same in the real-time PCR analysis as they were in the RNA-Seq analysis (Figure 4B–E), indicating the reliability of the RNA-Seq data. These results showed that transcription regulation of fertility conversion in Zhu 1S is also affected.

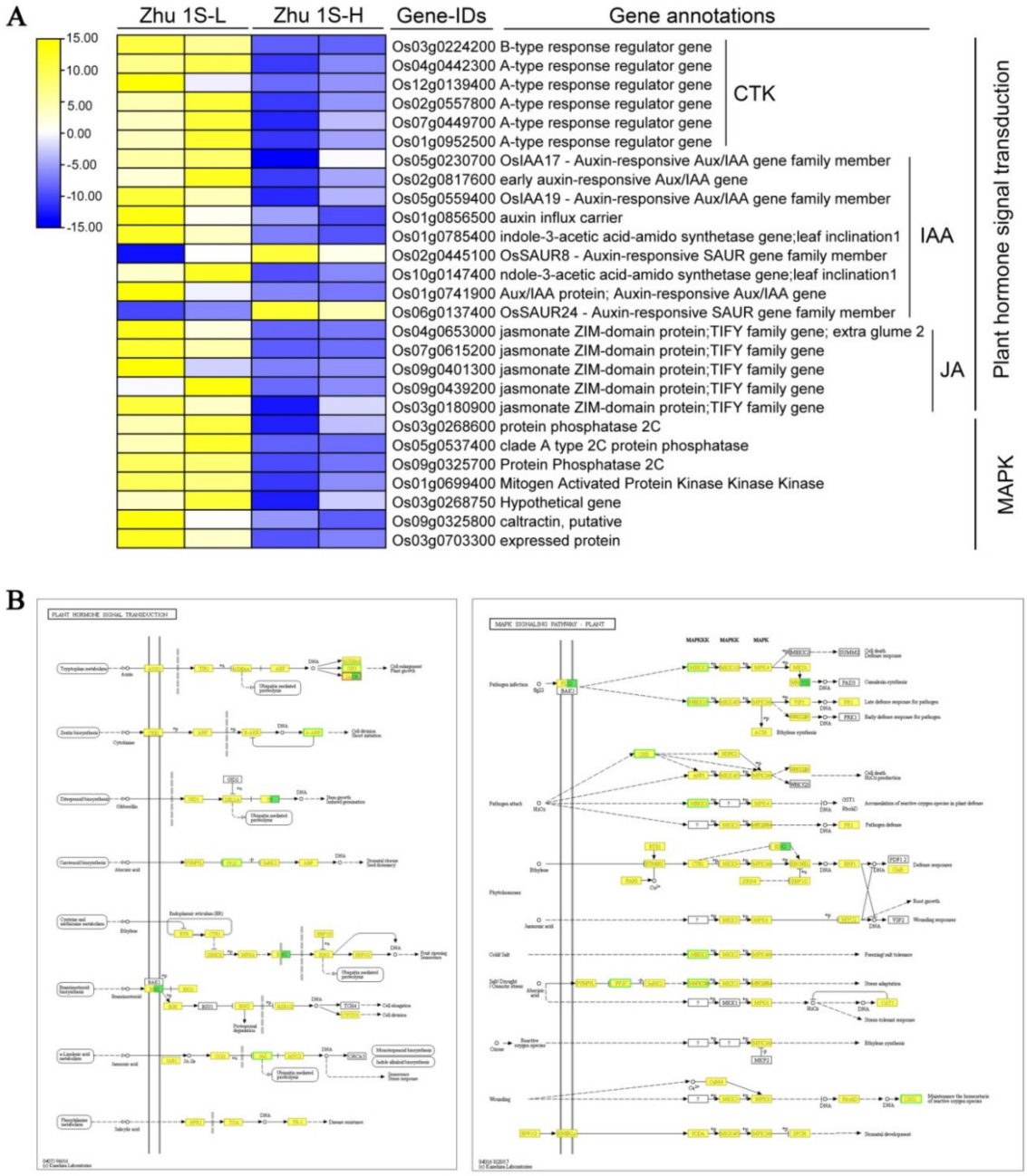

Figure 3. Differentially expressed genes involved in plant hormone and MAPK signal transduction in Zhu 1S under high temperature. (**A**) Heatmap representation of differentially expressed genes involved in the plant hormone and MAPK signal transduction in Zhu 1S under high temperature. The yellow and blue colors represent increased and decreased transcript level, respectively. (**B**) Schematic of plant signal transduction affected by high temperature in Zhu 1S. The red and green squares indicate up-regulated and down-regulated signal transduction processes under high temperature, respectively. The signal transduction schema was prepared using the KEGG web service (www.genome.jp/kegg/, 1 January 2022).

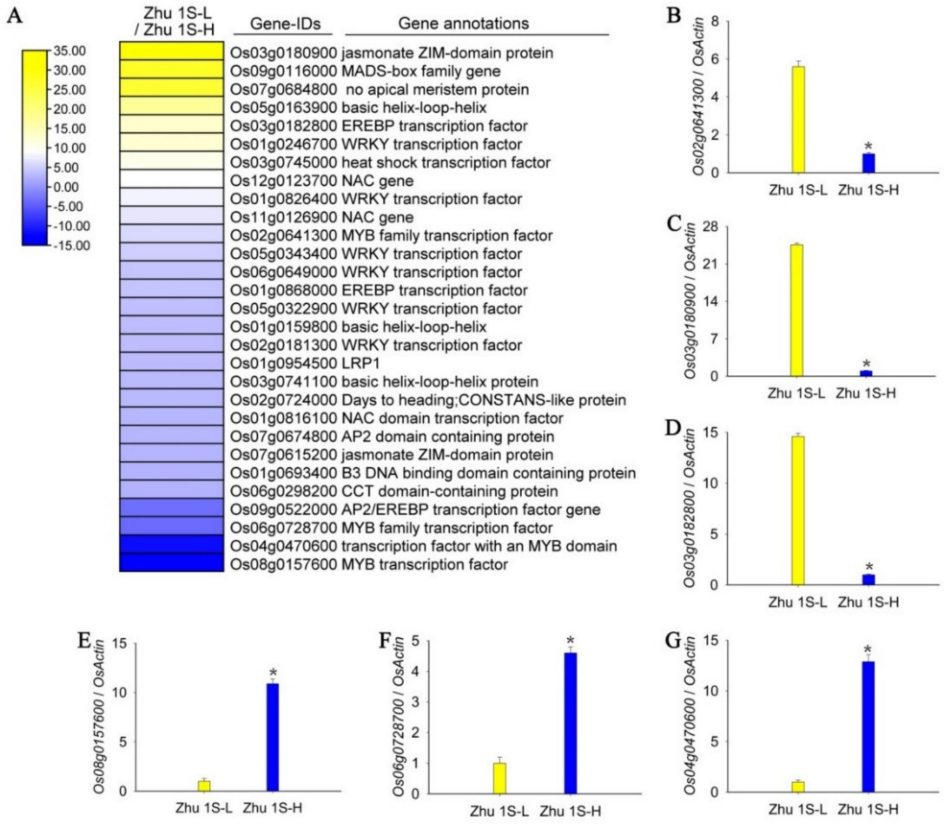

Figure 4. Differentially expressed genes involved intranscription factor in Zhu 1S under high temperature. (**A**) Heatmap representation of differentially expressed genes in Zhu 1S under high temperature. The yellow and blue colors represent up–regulation and down-regulation of genes involved in the transcription factor in Zhu 1S under high temperature, respectively. (**B**–**G**) The relative expressions of six representative genes were validated by real-time PCR. *OsActin1* as an internal control. The experiment was repeated three times with similar results. Data are means of five replicates of one experiment. Asterisks represent significant differences. Error bars represent ±SD.

3.6. The Protein-Protein Interaction Network of the Differential Expression Transcription Factor Genes

To further clarify the inter-connections among the differential expression transcription factor genes, a Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis was performed to establish the interaction network. The co-expression patterns revealed that Os01g0826400 (OsWRKY24), Os05g0343400 (OsWRKY53), Os02g0181300 (OsWRKY71), Os06g0649000 (OsWRKY28), Os01g0816100 (OsNAC4), Os03g0180900 (OsTIFY11c), Os07g0615200 (OsTIFY10B), Os03g0741100 (OsbHLH148) are possibly involved in fertility conversion in Zhu 1S under high temperature (Figure 5). The protein-protein interaction network analysis by STRING database showed that Os03g0180900 (OsTIFY11c) potentially plays a central role in fertility conversion through directing the Jasmonic acid signal pathways in Zhu 1S under high temperature (Figure 5).

Then, we conducted real-time PCR analysis to confirm the validity of the changes observed in the above eight transcription factor genes. For all eight genes, the transcript levels determined by real-time PCR analysis were lower in Zhu 1S under high temperature, which was similar to those identified using RNA-Seq (Figure 6).

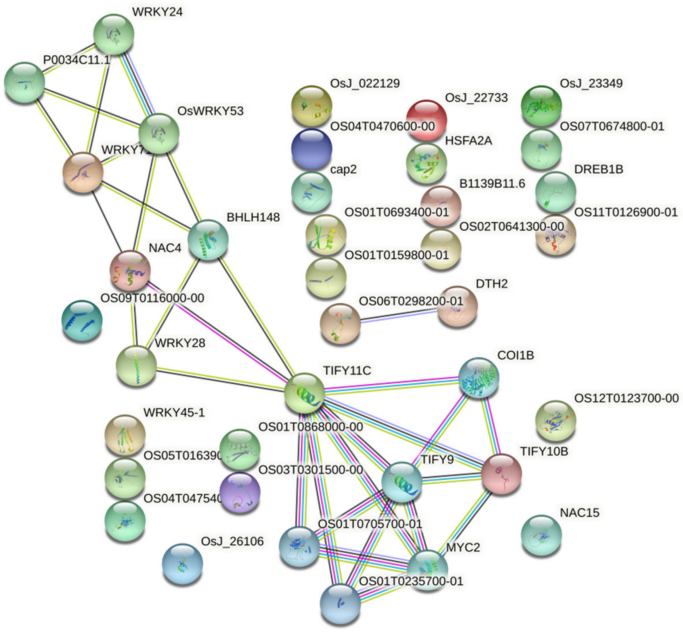

Figure 5. The protein–protein interaction network of the transcription factor genes coding proteins analyzed by STRING software. The color-coded transcription factor proteins are represented by nodes. Different colored lines represent co-expression evidence between proteins. Red line: gene fusion evidence; dark blue line: co-occurrence evidence; black line: co-expression evidence; yellow line: text-mining evidence; green line: neighborhood genome evidence; light-blue line: database evidence; and pink line: experimental evidence.

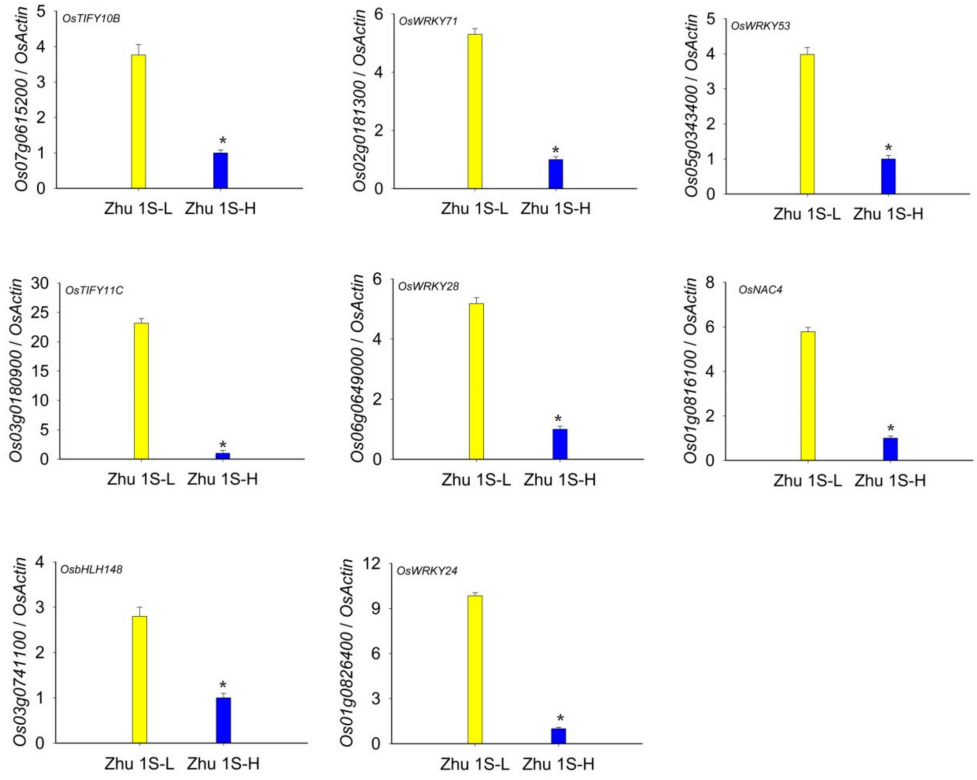

Figure 6. Differential expression levels of nine transcription factors validated by quantitative real-time PCR. *OsActin1* as an internal control. The experiment was repeated three times with similar results. Data are means of five replicates of one experiment. Asterisks represent significant differences. Error bars represent ±SD.

4. Discussion

In the present study, we used transcriptome sequencing to analyze the dynamic profiles of gene expression in Zhu 1S young rice panicles during fertility transition under high temperature. The results showed that the fertility transition of the TGMS rice line Zhu 1S mainly connects with plant hormone and MAPK signal transduction and transcriptional regulation, and so on. Thus, it is a complicated network regulation.

4.1. Plant Hormone and MAPK Signal Transduction

Plant hormones have strong effects on fertility. Plants can sense environmental signals and transmit them to the cells that produce plant hormones to adapt to temperature stress. Disruption of genes associated with hormone biosynthesis, transport and signaling leads to abnormal flowers or sterile pollen [13,15,16].

In barley and Arabidopsis anthers, heat stress significantly reduced the endogenousauxin level in developing pollen mother cells and tapetal cells, which led to premature abortion of microspore development [13,17,18]. Our results showed that the expression levels of 9 Auxin-responsive *Aux/IAA* genes (*OsIAA17*, *OsIAA19*, etc.) were almost all down-regulated in Zhu 1S under high temperature (Figure 3A). The differential expression of these genes in Zhu 1S-H and Zhu 1S-L might be related with fertility transition. As a growth regulator, Jasmonic acid (JA) acts as a signal for pollen fertility [13,19–21]. For example, in Arabidopsis and maize tassel, JA is identified as a factor affecting anther pollen maturation [19–21]. In this study, the expression levels of four JA-related genes (*OsTIFY11c*, etc.) were all down-regulated in Zhu 1S under high temperature (Figure 3A). The down-expression of *OsTIFY* genes in Zhu 1S under high temperature would lead to changed JA level. This deficiency might contribute to fertility transition in Zhu 1S. Further study looking into the roles of these *OsTIFY* genes on sterility could be worth conducting. Abscisic acid (ABA), as a key hormone, which is involved in abiotic stress responses, plays a vital role in male fertility during reproductive stress [13,22,23]. In rice, ABA regulates the expression level of monosaccharide transport-related genes and tapetum cell-wall-bound related invertases, resulting in disturbed carbohydrate metabolism in the anther and pollen sterility [22,23]. Our analysis showed that the expression levels of A type 2C protein phosphatase-related genes (*OsPP2C*, etc.) and mitogen-activated protein kinase-related genes (*OsMEKK1*, etc.) were all down-regulated in Zhu 1S under high temperature (Figure 3A), suggesting that the change of ABA signals might be involved in fertility transition in Zhu 1S under high temperature. Further studies would provide useful information of ABA signals for the fertility transition study in Zhu 1S.

The above results suggest that decreased hormone and MAPK signal transduction would alter fertility of Zhu 1S, and the above signal-related genes possibly participate in the fertility alteration process of Zhu 1S under high temperature inducement.

4.2. The Role of Transcription Factors in Fertility Conversion in Zhu 1S

Several transcription factors have been reported to be specially related to anthers and pollen development [13,15]. In this study, 29 transcription factors majorly involved in WRKY, TIFY families were identified, being involved in fertility and differentially expressed in Zhu 1S under high temperature (Figures 4A and 5, Table S3).

WRKY transcription factors have been reported to mediate in diverse signaling pathways as activators or repressors [24,25]. Our results showed that *WRKY* genes, including Os01g0826400 (OsWRKY24), Os05g0343400 (OsWRKY53), Os02g0181300 (OsWRKY71), Os06g0649000 (OsWRKY28), may regulate the fertility conversion in Zhu 1S under high temperature by down-regulating their transcription level. The TIFY family is involved in the regulation of diverse plant-specific biologic processes. The expression level of *OsTIFY* genes was more strongly induced by JA [26]. A previous study showed that Jasmonic acid regulates spikelet development in rice, and OsTIFY3/OsJAZ1can regulate pollen development [27]. However, itis not clear whether the *OsTIFY* transcription factor plays an essential role in fertility transition in Zhu 1S under high temperature. In this study,

the transcript levels of five *OsTIFY* genes (*OsTIFY11c*, etc.) were lower in Zhu 1S under high temperature (Figures 4 and 6). The protein–protein interaction network analysis showed that Os03g0180900 (OsTIFY11c) potentially plays a central role through directing the Jasmonic acid signal pathways during fertility conversion in Zhu 1S under high temperature (Figure 5). Our results showed that OsTIFY11c would be a candidate gene for regulating the fertility conversion in Zhu 1S under high temperature. The protein-protein interaction network analysis also displayed that OsTIFY11C potentially interacts with OsCOI1b andOsMYC2 (Figure 5). A previous study showed that OsJAZ1/OsTIFY3 interacts with the putative JA receptor, OsCOI1b, and with the transcription factor OsMYC2 to repress OsMYC2's function inactivating the E-class gene, *OsMADS1*, in spikelet development [27]. Further study could be performed to determine whether and how OsTIFY3 interacts OsCOI1b, OsMYC2 in regulating fertility conversion in Zhu 1Sunder high temperature.

4.3. The Complex Molecular Responses Network in Zhu 1S during Fertility Transition

The key to fertility conversion of temperature-sensitive male sterile (TGMS) lines under high temperature is a complex regulatory network involving plant hormone and MAPK signal transduction, and so on. Based on our analysis results, we proposed a model that might explain the fertility transition in Zhu 1S under high temperature (Figure 7).

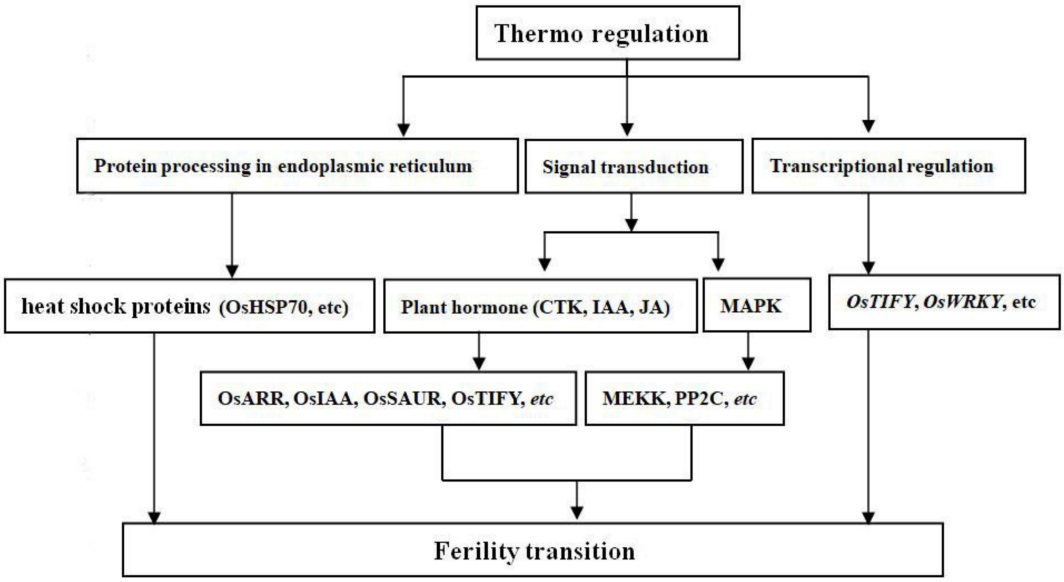

Figure 7. The proposed model of fertility transition mechanism in the TGMS rice line Zhu 1S.

First, plant hormone signal transduction and MAPK signal pathways might be involved in the development of the anther of the young panicle of Zhu 1S under high temperature. The expression levels of the genes involved in CTK, IAA, JA, ABA signal pathways were almost all down-regulated in Zhu 1S under high temperature (Figure 3A), suggesting that decreased hormone and MAPK signal transduction would alter the fertility of Zhu 1S under high temperature inducement. Second, the co-expression patterns and protein-protein interaction network analysis showed that Os03g0180900 (OsTIFY11C) could potentially play a central role in fertility conversion through directing the Jasmonic acid signal pathways in Zhu 1S under high temperature (Figure 5). Third, the metabolic pathway and starch and sucrose metabolism were involved in the development process and energy supply. Our study showed that the starch and sucrose metabolism and TCA cycle were almost all decreased in Zhu 1S under high temperature. They might not be able to offer enough energy to support pollen development (Supplementary Figure S1 and S2). In addition, lower levels of ROS scavengers in Zhu 1S under high temperature could

also result in the abortion of the pollen (Supplementary Figure S3). Taken together, the regulatory interactions described above might lead to fertility transition in Zhu 1S under high temperature.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12061255/s1, Figure S1: Schematic of starch and sucrose metabolism affected by high temperature in Zhu 1S; Figure S2: Schematic of TCA cycle affected by high temperature in Zhu 1S, Figure S3. Schematic of peroxisome affected by high temperature in Zhu 1S. Table S1. List of PCR Primers; Table S2. Preprocessing results of sequencing data quality; Table S3. Differentially expressed genes involved in transcription factor in Zhu 1S under high temperature.

Author Contributions: Y.C., D.M. and L.C. conceived and designed the experiments. Y.C. (Yan Chen), Y.C. (Yi Chen), Z.X., J.L. and H.C. performed the experiments. Y.C. (Yan Chen), D.M. and L.C. analyzed the data. Y.C. (Yan Chen) and D.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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