Comparison transcriptome analysis of panicle development under heat stress in two rice (*Oryza sativa* L.) cultivars differing in heat tolerance

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Abstract

Heat stress inhibits rice panicle development and reduces spikelet number per panicle. This study investigated the mechanism involved in heat-induced damage to panicle development and spikelet formation in rice cultivars differing in heat tolerance. Transcriptome data from developing panicles grown at 40°C –or 32°C were compared for two ~~different~~ rice cultivars: heat-tolerant Huanghuazhan and heat-susceptible IR36. Of the differentially expressed genes (DEGs), 4,070 heat stress-responsive genes were identified, including 1,688 heat-tolerance-cultivar-related genes (RHR), 707 heat-susceptible-cultivar-related genes (SHR), and 1,675 common heat stress-responsive genes. Gene ontology analysis showed that the DEGs in the RHR gene set ~~of genes~~ were significantly enriched in 54 gene ontology terms, some of which improved heat tolerance, including those in the WRKY, HD-ZIP, ERF, and MADS transcription factor families. DEGs in the RHR group were enriched in 15 significant metabolic pathways and some DEGs were regulated to resist heat stress, including the plant hormone signal transduction pathway. The regulation of some DEGs in the SHR group was affected by

heat stress, including those in the starch and sucrose metabolism pathway. Our ~~T~~transcriptome analysis provides insight into ~~different~~ molecular mechanisms of heat stress tolerance ~~in~~ during panicle develop~~ing rice~~ment.

Introduction

Climate change ~~–~~is predicted to increase average global temperatures by 0.3-4.8°C by the end of the 21st century (Stocher *et al*. 2013). Unusually high temperatures occur frequently during the ~~summer~~ rice ~~planting~~ growing season~~–~~ (Dwivedi *et al*. 2015; Tao *et al*. 2013) and cause reductions in ~~rice~~ yield~~s~~ and quality in ~~certain~~ several rice production regions, including China, India, and Japan. The primary cause of ~~rice~~ yield reduction in rice is a reduction in spikelet fertility due to high temperatures during the flowering period (Espe *et al*. 2017). Rice quality is also influenced by high temperature~~s~~, which cause~~s~~ carbohydrate metabolism disorders (Yamakawa & Hakata 2010). As climate change has intensified, extremely high temperatures above 40°C have become more frequent. Such high temperatures inhibit rice panicle development and reduce spikelet number by 5%-15%, ~~thereby~~ therefore aggravating rice yield losses (Wang *et al*. 2017).

High temperatures adversely affect floral ~~organ~~ development by reducing antioxidant capacity, inhibiting nutrition accumulation, and degenerating tapetal cells (Prasad *et al*. 2017). A previous study showed that high temperature (39°C) downregulated certain genes related to tapetum function, pollen adhesion, and germination, including *OsINV4* and *OsMST8*, which influenced spikelet fertilization (Endo *et al*. 2009). In addition, sugar and endogenous hormone metabolism under high temperatures reportedly plays an important role in pollen formation (Islam *et al*. 2018; Min *et al*. 2014). At the rice ripening stage, high temperature induces early termination of grain filling (Kim *et al*. 2011). Grain chalkiness increases under a mean temperature greater than 32°C, resulting in the deterioration of eating and cooking quality, which are both closely linked to starch and sucrose metabolism (Zhong *et al*. 2010). Transcriptome analysis has shown that high temperatures influence the expression of genes involved in the inhibition of sucrose degradation and starch biosynthesis while promoting starch degradation and the synthesis of storage proteins (Yamakawa & Hakata 2010; Yamakawa *et al*. 2007~~), ~~~~). Takehara *et al*. (2018) reported that upregulation of *OsSUS3*, which encodes sucrose synthase, improved high-temperature tolerance.

The panicle initiation stage is an important period for spikelet proli~~o~~feration. Dry matter accumulation is essential for panicle development; however, the pathway for carbohydrate accumulation during spikelet formation under heat stress remains

vague. The reduction in spikelet number that occurs under high temperature conditions has been associated with heat-induced phytohormone changes, especially enhanced cytokinin degradation (Wu *et al*. 2017; Wu *et al*. 2016). The number of spikelets per panicle is determined by spikelet differentiation and degeneration. Spikelet differentiation is correlated with dry matter accumulation and influenced by environmental factors (Liu *et al.* 2005). Ding *et al*. (2016) reported that hormone metabolism, stress response, carbohydrate metabolism and transport, and protein degradation were regulated to influence panicle initiation. Additionally, certain genes, such as MADS-box genes, are related to panicle initiation (Kang *et al*. 2013; Kobayashi *et al*. 2012). Quantitative trait loci –for spikelet degeneration have been identified (Yamagishi *et al*. 2004), and the genes *SP1*, *ASP1*, *TUT1*, *PAA2*, and *OsALMT7* have been found to control spikelet degeneration (Bai *et al*. 2015; Heng *et al*. 2018; Li *et al*. 2010).

However, the mechanism of panicle development under high temperature conditions is still unclear. In this study, RNA-Seq analysis was used to explore the mechanism of heat ~~damage~~ tolerance ~~to~~ in panicle development. Huanghuazhan (HHZ) is a heat-tolerant rice~~(*Oryza sativa* L.)~~ cultivar ~~that, is~~ widely grown in the middle and lower reaches of the Yangtze River in China (Cao *et al*. 2009; Zhou *et al*. 2012). ~~The inbred *indica* cultivar~~ IR36 is a heat-susceptible cultivar (Fang *et al*. 2006)~~,~~ and ~~was~~ it is a ~~n original~~ parental line ~~in the breeding pedigree~~ of HHZ. These two rice cultivars were used in the current study to ~~ascertain~~ investigate the transcriptome differences between two temperature treatments, ~~a heat-tolerant rice cultivar and a heat-susceptible cultivar grown at~~which were 40°C and 32°C ~~beginning~~ at the spikelet differentiation stage. The differentially expressed genes (DEGs) of young panicles in the two cultivars under the two temperature treatments were identified and they were further analyzed by Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to improve our understanding of the molecular mechanism of heat-induced inhibition of spikelet development and to provide an important ~~reference~~ insight for rice breeding.

Materials and methods

Plant materials and heat stress treatments

~~We used the inbred *indica* rice (*Oryza sativa* L.) cultivars~~ Rice cultivars HHZ ~~(heat-tolerant)~~ and IR36 ~~(heat-susceptible)~~were used in this study. Pre-germinated seeds were sown in seed trays filled with a ~~matrix consisting~~mixture of vermiculite, charcoal, soil, and slow-release fertilizer. After 20 days, the seedlings were transplanted into pots ~~(~~with four ~~seedlings~~ per pot~~)~~. Each pot (24 cm length × 22.5 cm width × 21.5 cm height) contained 10 kg air-dried paddy soil. The pot~~sted rice plants~~ were kept under

natural environmental conditions.

The amount of fertilizer applied to each pot was based on ~~fertilizer used in~~a field ~~rice production, which~~application rate of 14 kg nitrogen per 666.7m^2. Before transplanting into the pots, 3.5 g compound fertilizer (nitrogen: phosphorus: potassium = 15%:15%:15%) was applied to each pot. At the tillering stage, 0.6 g urea was supplemented in each pot. At panicle initiation, 0.6 g urea and 0.5 g potassium chloride were also applied in each pot. Pests, diseases, and weeds were intensively controlled.

Automatic growth chambers were used ~~to control the two~~for temperature treatments~~ used on the study~~. The high-temperature (40°C) and control temperature (32°C) treatments were imposed for eight hours each day from 9:30 to 17:30 h~~;~~ (~~the temperature~~ setting details are shown in Table 1). The humidity in the chambers was maintained at 75-80%. Rice plants were grown under natural ambient conditions during all the growth stages before and after the ~~high temperature and control temperature~~ treatments~~ were applied~~.

Plants were moved to the growth chambers on the approximate date of spikelet differentiation when the panicle length was approximately 0.2 cm (60-70 d after seed sowing). ~~Rice p~~Plants were exposed to the different temperature treatments for nine days~~ at spikelet differentiation stage (panicle length ≈ 2 mm) and then returned to ambient conditions~~. Each treatment ~~included~~ had three replicates ~~(~~with 20 pots per~~/~~ replicate~~)~~. ~~Plants were moved to the growth chambers on the approximate date of spikelet differentiation when the panicle length was approximately 0.2 cm (60-70 d after seed sowing).~~

Panicle and spikelet morphology

Ten main tillers were sampled per replicate on day 9 of treatment to ~~monitor~~ investigate young panicle development treated under 40°C and 32°C.

Spikelet differentiation or degeneration of the main tiller panicles was determined at the heading stage. The number of degenerated spikelets was calculated by counting the vestiges present on the panicles. The number of differentiated spikelets was the sum of the surviving and degenerated spikelets. The proportion of degenerated spikelets was ~~calculated as number of degenerated spikelets per number of total differentiated spikelets × 100%~~then estimated.

Spikelet morphology was observed under a stereomicroscope (Olympus SZX7, Olympus Corporation, Tokyo, Japan) and glume length (mm) and glume width (mm) were measured at 0.63x and 2.5x using Image Pro-Plus 5.1(Olympus SZX7, Olympus Corporation, Tokyo, Japan).

RNA extraction, transcriptome sequencing, and mapping

After nine days at 40 °C or 32 °C treatment, young panicles of 20 main tillers were collected from each replicate at 12:00-13:00, and immediately frozen in liquid nitrogen. In quick succession, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the young panicles according to the manufacturer's instructions. A TruSeq RNA Sample Preparation Kit (Illumina Inc., CA, USA) was used to generate 12 sequencing libraries according to the manufacturer's instructions. The sequencing libraries were then sequenced on a HiSeq platform (Illumina, Inc., CA, USA). High quality sequence reads were obtained after filtering from raw data and were then compared to the 9311 reference genome (Oryza_indica.ASM465v1.dna.toplevel.fa) obtained from http://www.ensembl.org/. The raw RNA sequence data were submitted to the NCBI Sequence Read Archive with accession number PRJNA508820.

Gene expression level and differential expression analysis

We used HTSeq (0.9.1) to statistically compare the read count values of each gene as the original expression of the gene, and fragments per kilobase of transcript per million mapped reads (FPKM) was used to standardize the expression. Next, we used DESeq (1.30.0) to analyze differential expression of genes with the following screening conditions: an expression difference of |log2foldChange| > 1 and a significant *P-value* < 0.05.

GO and KEGG enrichment analysis of DEGs

For GO enrichment analysis of DEGs, we used the Singular Enrichment Analysis tool in AgriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) with default parameters, and a *P*-value ≤ 0.05 to indicate significant enrichment. KEGG enrichment analysis of DEGs was performed using KOBAS software with default parameters and *P*-value ≤ 0.05 indicating significant pathway enrichment.

Verification of RNA-Seq by quantitative real-time PCR (qRT-PCR)

First-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The qRT-PCR analyses were performed using an Applied Biosystems 7500 Real-Time PCR system with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The primers used for qRT-PCR are listed in Supplementary Table S1. The *OsUBQ* gene was used as an internal control. Relative gene expression levels were determined from the equation $2^{-\Delta\Delta CT}$ (Czechowski *et al*. 2004), where ΔΔCT represents ΔCT (target gene of interest)–ΔCT (control gene).

Statistical analyses

Microsoft Excel 2016 (Microsoft Inc., Redmond, WA, USA) was employed for data

collection. The panicle and spikelet morphological data collected for the 40°C and 32°C treatments (mean of three replicates) were statistically analyzed by Student's t-test ~~($P<0.05$)~~. Graphs were created using Origin 9.1 (~~Ver. 9.1,~~ OriginLab, Northampton, MA, USA).

Results

Spikelet development at high temperature

A preliminary experiment showed a significant difference in panicle development measured as spike differentiation after nine days of high-temperature treatment. The current results reported here are consistent with the~~se~~ preliminary findings. High temperature for nine days during spikelet differentiation inhibited young panicle growth; the 40°C treatment reduced young panicle length by 37.4% in the HHZ cultivar and 84.9% in the IR36 cultivar over the 32°C control treatment (Fig. 1a, b). In addition, the heat treatment reduced the dry weight of young panicles by 35.8% and 95.1%, respectively (Fig. 1c), significantly reduced spikelet survival by 22.3% and 53.6% (Fig. 2a), reduced the number of differentiated spikelets by 9.6% for HHZ and 33.2% for IR36 (Fig. 2b), and increased the proportion of degenerated spikelets by 32.3% and 67.4%, respectively (Fig. 2c). After nine days of high-temperature and control temperature treatment, approximately 15-20 days were needed for panicles to complete growth. The heat treatment reduced glume length by 10.3% for HHZ and by 16.0% for IR36 (Fig. 3b), and reduced glume width by 12.0% and 8.0%, respectively (Fig. 3c). The reductions in spikelet number and size led to reductions in panicle weight and rice yield (TableS2); the heat-susceptible IR36 ~~experienxed~~experienced a greater reduction than heat-tolerant HHZ, suggesting that high temperature had ~~a weaker~~ less effects on young panicle development in the heat-tolerant cultivar.

Transcriptome analysis

Under the 32°C control temperature, a total of 44.2 million and 48.9 million raw reads were obtained from HHZ (referred to as HHZ_32) and IR36 (referred to as IR36_32), respectively. Under the 40°C treatment, a total of 45.5 million raw reads were obtained from both HHZ (HHZ_40) and IR36 (IR36_40) (Table 2 and Table S3). More than 99.0% clean reads were obtained for downstream analyses. The results of RNA sequence mapping indicated that 85.8-88.0% of the clean reads could be mapped ~~on~~to the reference genome and most of them were uniquely mapped. ~~On average, 84.8% of the clean reads from HHZ_32 and 83.0% of the clean reads from HHZ_40 were uniquely mapped onto the reference genome, and for IR36, 83.1% of the clean reads from IR36_32 and 83.4% of the clean reads from IR36_40 were uniquely mapped onto the reference genome~~ (Table ~~S4~~2).

Identification of DEGs

To compare the differences between the two cultivars at 40°C and 32°C, we used four comparison groups: HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, IR36_40 vs HHZ_40, and IR36_32 vs HHZ_32. DEGs for the four groups were restricted to those with a |log2fold change| > 1 and a *P*-value<0.05. With these criteria, 3342, 2469, 2949, and 2461 DEGs were detected for HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, IR36_40 vs HHZ_40, and IR36_32 vs HHZ_32, respectively (Fig.4). Significantly different gene expression was observed both between cultivars and between treatments. For HHZ, 1,794 genes were upregulated and 1548 genes were downregulated in the 40°C treatment compared with the 32°C treatment (Fig.4a). Furthermore, 1140 genes were upregulated and 1,329 genes were downregulated in IR36 under the 40°C treatment compared with the 32 °C treatment (Fig.4b). For comparisons within treatments, 1,408 genes were upregulated and 1,541 were downregulated in the IR36_40 vs HHZ_40 and 893 genes were upregulated and 1,751 genes were downregulated in the IR36_32 vs HHZ_32 group (Fig.4c and d).

Classification of DEGs

In all four groups, a total of 5533 unique DEGs were identified, and these DEGs could be divided into 15 disjointed subgroups (Fig. 5). Among the 15 subgroups, eight from the IR36_32 vs HHZ_32 group were excluded from the analysis because they were not influenced by high temperature. In addition, 1,157, 603, 524, and 402 DEGs were ~~specifically~~ uniquely identified in HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, IR36_40 vs HHZ_40, and IR36_32 vs HHZ_32, respectively. The DEGs in groups responsive to high temperature could be further classified into three categories: heat-tolerance-cultivar-related genes (RHR, 1,~~689~~ 688 genes), heat-susceptible-cultivar-related genes (SHR, 707 genes), and common heat stress-response genes (CHR, 1675 genes) (Table 3 and Table S5). The DEGs in the RHR category benefited from heat resistance, while the DEGs in the SHR category presented the specific heat injury in the heat-susceptible cultivar.

Analysis of GO annotation

The purpose of GO enrichment analysis is to obtain GO functional terms with significant enrichment of DEGs, thus revealing the possible functions of the DEGs. Of all DEGs, 2,307 (69.0%), 1,680 (68.0%), 1,832 (62.1%), and 1,472 (59.8%) DEGs were enriched in GO terms in HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, IR36_40 vs HHZ_40, and IR36_32 vs HHZ_32, respectively. There were 75, 11, 13, and 31 significant GO terms observed in HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, IR36_40 vs HHZ_40, and IR36_32 vs HHZ_32, respectively (Fig. 6). The maximum number of

DEGs was observed for the heterocycle biosynthetic process in the IR36_40 vs HHZ_40 group. In IR36_32 vs IR36_40 and HHZ_32 vs HHZ_40, the DEGs were enriched in response to stimulus, in response to temperature stimulus, and in response to heat in the biological process category. Within the cellular component category, the DEGs were commonly enriched in chromatin, DNA packaging complex, and nucleosome in the IR36_32 vs IR36_40 and HHZ_32 vs HHZ_40 groups. However, there were no common GO terms in the category of molecular function in the IR36_32 vs IR36_40 and HHZ_32 vs HHZ_40 groups.

We further identified GO term categories for DEGs in the RHR, SHR, and CHR categories (Fig. 7 and Table S6). Among the 1,689 DEGs in RHR, 54 significant GO terms were detected. However, no significant GO terms were observed among the ~~485~~ 707 DEGs in SHR. In CHR, 30 significant GO terms were detected. In the CHR group, eight significant GO terms were in the biological process category, including response to stimulus, response to temperature stimulus, and response to heat; 17 GO terms were in the cellular component category, and two significant GO terms were in the molecular function category. In the RHR group, 30, 14 and 10 significant GO terms were in the biological process, cellular component, and molecular function categories, respectively. The most significant GO terms, in decreasing order, were RNA biosynthetic process, nucleus, and DNA binding. In the molecular function category, 50 DEGs were specifically assigned to DNA-binding transcription factor activity, which may play an important role in heat stress tolerance.

The 50 DEGs of DNA-binding transcription factor activity could be divided into 11~~12~~ transcription factor (TF) families (Fig. 8), including HSF (1), WRKY (6), MADS (12), HD-ZIP (7), GATA (3), ERF (12), ABAI (1), b-ZIP (4), ARR-B (2), E2F (1), and NF-YA (1). Expression of the genes *BGIOSGA006348* of HSF, *BGIOSGA010835* of ABAI, *BGIOSGA010142* of HAP, and *BGIOSGA000303* and *BGIOSGA000304* of ARR-B were significantly upregulated. In addition, five genes in WRKY, eight genes in MADS, two genes in HD-ZIP, two genes in GATA, six genes in ERF, and two genes in b-ZIP were also upregulated (Table S7). These results suggest that, these 30 TF genes may play important roles in heat stress resistance.

Analysis of KEGG pathway enrichment

In the KEGG analysis, 1158 DEGs were classified into 225, 191, 239, and 211 functional pathways in HHZ_32 vs HHZ_40; 838 DEGs in IR36_32 vs IR36_40; 732 DEGs in IR36_40 vs HHZ_40; and 539 DEGs in IR36_32 vs HHZ_32, respectively. A total of 79 pathways were significant (P <0.05) (Fig. 9). Among these pathways, the phenylpropanoid biosynthesis pathway was common in HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, and IR36_40 vs HHZ_40, which suggests that heat stress impaired phenylpropanoid biosynthesis.

Based on further analysis of the three categories with different heat-stress responses, 146 DEGs in RHR were involved in 15 overrepresented pathways, including purine metabolism, pyrimidine metabolism, and amino sugar and nucleotide sugar metabolism; 45 DEGs in SHR were involved in 11 overrepresented pathways, including arginine biosynthesis, starch and sucrose metabolism, and polyketide sugar unit biosynthesis; and 184 DEGs in CHR were involved in 29 overrepresented pathways (Fig. 10 and Table S8).

A previous study showed that plant hormones are important for panicle development. Among the 15 KEGG pathways in RHR, 21 DEGs were involved in plant hormone signal transduction, of which 14 DEGs were upregulated in HHZ; three DEGs were involved in cytochrome P450 metabolism, which plays a role in brassinosteroid (BR) biosynthesis, and two were upregulated (Table 4).

In SHR and CHR, there were three common pathways: the starch and sucrose metabolism pathway, the NOD-like receptor signaling pathway, and the estrogen signaling pathway. Carbohydrate accumulation was essential for panicle development. In the KEGG analysis, seven DEGs involved in starch and sucrose metabolism were observed in SHR and 18 DEGs involved in starch and sucrose metabolism were observed in CHR. In SHR, the genes in HHZ were not different between HHZ_40 and HHZ_32. However, genes (*BGIOSGA010570*, *BGIOSGA026140*) encoding sucrose synthase (EC 2.4.1.13), genes (*BGIOSGA026976*, *BGIOSGA009181*, *BGIOSGA030796*) encoding trehalose-6-phosphate synthase (EC 2.4.1.15), and a gene (*BGIOSGA000509*) encoding trehalose-6-phosphate phosphatase (EC 3.1.3.12) were significantly down-regulated in IR36_40 compared with IR36_32. However, the gene (*BGIOSGA031385*) encoding beta-amylase (EC 3.2.1.2) was significantly upregulated in IR36_40 compared with IR36_32 (Table 5).

qRT-PCR verification

To confirm the accuracy of the RNA-Seq results, ten representative DEGs each in HHZ_32 vs HHZ_40 (a) and IR36_32 vs IR36_40 (b), as well as five DEGs each in IR36_40 vs HHZ_40 (c) and IR36_32 vs HHZ_32 (d) were chosen to determine relative expression. Among the ten DEGs in HHZ_32 vs HHZ_40, five DEGs were in RHR: *BGIOSGA022020* is related to BR synthesis, *BGIOSGA006348* encodes a heat shock factor (Hsf), *BGIOSGA017088* is involved in the ETH TF family, *BGIOSGA006285* participates in ethylene responsive regulation, and *BGIOSGA024710* is an auxin-responsive gene involved in plant hormone transduction. Among the ten DEGs in IR36_32 vs IR36_40, five were in SHR and encoded cytokinin oxidase/dehydrogenase (*BGIOSGA005140*), sucrose synthase (*BGIOSGA026140*), trehalose-6-phosphate synthase (*BGIOSGA026976*), trehalose-6-phosphate phosphatase (*BGIOSGA000509*), and catalase (*BGIOSGA007252*). Four DEGs were in CHR for HHZ_32 vs HHZ_40

and IR36_32 vs IR36_40, and two common genes, namely, *BGIOSGA032653* and *BGIOSGA015767*, were validated. *BGIOSGA032653* is involved in phenylpropanoid biosynthesis and *BGIOSGA015676* encodes a heat shock protein (HSP). The qRT-PCR results for these DEGs were all consistent with the RNA-Seq data (Fig.11).

Discussion

Rice plants exposed to high temperature growing conditions during spikelet differentiation inhibited panicle initiation and reduced the spikelet number per panicle (Fig.1 and ~~Fig.~~2). Previous studies have shown that the genes *SP1*, *ASP1*, *TUT1*, *PAA2*, and *OsALMT7* are closely related to branch and spikelet development in rice, but in the current study, we observed no significant difference in expression of these genes between the 40°C treatment and the 32°C control treatment in either rice cultivars. This indicates that these genes ~~did~~ might not respond to the high temperature treatment in young panicles.

In general, the upregulation of HSPs contributes to the heat stress response in plants (Guan *et al*. 2010; Jagadish *et al*. 2010; Jung *et al*. 2013). Moon *et al.* (2014) reported that heterologous overexpression of *OsHSP1* (*BGIOSGA015767*, encoding a heat shock protein) increased heat tolerance in Arabidopsis. However, in the current study, *BGIOSGA15767* expression was upregulated in both HHZ (log2 ~~fold change~~ (HHZ_40/HHZ_32) = 5.7, *P*-value=0) and IR36 (log2 ~~fold change~~ (~~HHZ~~IR_40/~~HHZ~~IR_32) = 5.0, *P*-value=0), and there was no difference in the GO terms between cultivars for HSP. GO enrichment analysis revealed that the DEGs were commonly enriched in response to heat~~,~~ stress and temperature stimuli in the biological process category (Fig.7). These results demonstrate that high temperature ~~growing~~ conditions directly damage young panicle development.

An important factor determining heat tolerance is antioxidant capacity (Lan *et al.* 2016). Buer *et al.* (2010) reported that flavonoids can positively regulate reactive oxygen species, which can affect the transport of plant hormones and influence pollen development. The flavonoid synthesis pathway was overrepresented in the IR36_32 vs IR36_40 group. ~~Specificially~~Specifically, five genes involved in flavonoid synthesis were downregulated at 40°C, which might indicate a reduction in the antioxidant capacity of IR36 under heat stress. In addition, 14 DEGs in the IR36_32 vs IR36_40 group were enriched in the peroxisome pathway. Among these, 10 DEGs were significantly downregulated and four DEGs were significantly upregulated. However, the peroxisome pathway was not significant in the KEGG analysis of HHZ_32 vs HHZ_40 (Fig. 8). BGIOSGA007252 and BGIOSGA011520, which encoded catalase (EC:1.11.1.6), were significantly downregulated at 40°C compared with 32°C in IR36, while no expression differences were observed in HHZ_32 vs HHZ_40. This suggested

that high temperature had ~~s~~greater negative effects on the antioxidant capacity of IR36 than HHZ, which provides primary explanation for the greater heat injury observed in the young IR36 panicles than in HHZ, which provides a primary explanation for the greater heat injury observed in the young IR36 than in the HHZ panicles.

Regulation of endogenous hormones has important effects on the development of young panicles. Wu *et al.* (2017) reported that a lower spikelet number under high temperature growing conditions was associated with cytokinin degradation. In the current study, BGIOSGA001314, which encodes a cytokinin-activity enzyme, did not differ between the 40°C and 32°C treatments in HHZ (log2 (HHZ_40/HHZ_32) = -0.41) or IR36 (log2 (IR36_40/IR36_32) =-0.38). However, ~~T~~the gene BGIOSGA005140, which encodes cytokinin oxidase/dehydrogenase, was significantly upregulated in the IR36_32 vs IR36_40 group (log2 fol~~e~~d change=1.67, *P-value*=0.004), but was not different in the HHZ_32 vs HHZ_40 group (log2 f~~l~~old change=0.86, *P-value*=0.088). These results are consistent with those of Wu et al. (2016) and suggested that spikelet formation is associated with cytokinin degradation, and that more degradation occurred at the high temperature in the heat-susceptible cultivar than in the heat-tolerant cultivar.

The DEGs in RHR were enriched in 54 GO terms (Fig.6a). GO term analysis revealed biological processes promoting resistance to heat stress in the heat-tolerant cultivar HHZ. Downregulation of *BGIOSGA022020* in the heterocycle biosynthetic process induces GRAS protein reduction, which promotes BR synthesis to enhance heat tolerance (Vriet *et al*. 2012). In the molecular function category for RHR, 50 DEGs were involved in DNA-binding transcription factor activity. *BGIOSGA006348* encoded an HSF TF and was upregulated in the HHZ_32 vs HHZ_40 group, but there was no difference in the IR36_32 vs IR36_40 group. Wang *et al.* (2009) reported that higher expression of heat shock TFs contributed to high temperature tolerance. WRKY genes encode TFs that play important roles in abiotic stress responses (Chen *et al.* 2010), especially to abscisic acid (ABA) (Zhen *et al.* 2005). In this study, five DEGs were WRKY TFs, namely, *BGIOSGA003134*, *BGIOSGA029574*, *BGIOSGA005924*, *BGIOSGA024948*, and *BGIOSGA033505*, which might promote young panicle development associated with sucrose consumption mediated by ABA under high temperature (Feng *et al.* 2018). However, few studies have reported the relationship between the WRKY family and heat resistance, which should be further studied. *BGIOSGA029574* is a general stress-response gene, which has putative functions in distinct cellular processes, such as transcription regulation, stress response, and sugar metabolism under Fe-excess-induced, dark-induced and drought-induced stress (Ricachenevsky *et al.* 2010). Of the 10 DEGs in the ETH family, five genes were downregulated and the down-regulation of *BGIOSGA017088* reduced the ABA content and promoted gibberellin (GA) signal transduction, which is beneficial for rice plant growth (Yaish *et al.* 2010). Upregulation of *BGIOSGA006285*, *BGIOSGA010867*,

BGIOSGA030019, *BGIOSGA005915*, and *BGIOSGA012535* plays an important role in ethylene response regulation. Cao *et al.* (2006) reported that the upregulation of *BGIOSGA005915* enhanced tolerance to salt, cold, drought, and wounding and the current study revealed that ~~the~~ this gene might also contribute~~d~~ to the improvement of high-temperature stress resistance. *BGIOSGA000303* and *BGIOSGA000304* are genes in the cytokinin receptor family and upregulation of these two genes promotes cytokinin activation (Ito & Kurata 2006). The MADs box gene is related to flower development (Kobayashi *et al*. 2012) and the upregulation of the MAD genes in RHR indicated that the MAD family might enhance heat stress tolerance. The HZ-ZIP TF family might have a similar function.

In the RHR category, the DEGs enriched in the KEGG pathways appear beneficial for heat-stress tolerance, including plant hormone signal transduction and BR biosynthesis. Twenty-one DEGs were involved in plant hormone signal transduction, of which 14 DEGs were upregulated, including the auxin-responsive genes, *BGIOSGA024710*, *BGIOSGA001585*, *BGIOSGA019301* and *BGIOSGA037837*, which facilitate rice plant growth (Hagen & Guilfoyle 2002). In BR biosynthesis, BGIOSGA002945, which encodes D2/CYP90D2 that catalyzes the steps from 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone and from teasterone to 3-dehydroteasterone, was upregulated to promote BR synthesis in the latter pathway (Hong et al. 2003), and BGIOSGA001585 was downregulated to promote BR activity (Sakamoto et al. 2011). These 16 genes might contribute to young panicle development under high temperature. However, BGIOSGA014915, which participates in BR synthesis, was downregulated in RHR. Previous reports have found that BRs can modulate plant metabolic responses to environmental abiotic stresses (Vriet et al. 2012; Wang et al. 2018), but how BR metabolism modulates spikelet development under high temperature needs a further study.

Carbohydrate accumulation is essential for panicle initiation (Tian et al. 2016). KEGG analysis showed that the phenylpropanoid biosynthesis pathway was commonly overrepresented in HHZ_32 vs HHZ_40, IR36_32 vs IR36_40, and IR36_40 vs HHZ_40. The phenylpropanoid biosynthesis pathway is involved in lignin synthesis, which suggests that high temperature inhibits lignin synthesis; however, phenylpropanoid biosynthesis was not associated with the heat tolerance in ~~the different~~our resistant cultivar~~s~~ (Fig. 9).

In the SHR category, seven DEGs were enriched in the starch and sucrose metabolism pathway (Fig. ~~9~~10b). This pathway was also highly represented in CHR (Fig. ~~9c~~10c). Such genes are involved in the downregulation of genes encoding beta-fructofuranosidase, fructokinase, beta-glucosidase, trehalose-6-phosphate phosphatase, alpha-trehalase, and others. Sucrose degrades into uridine 5'-diphosphoglucose and fructose, which are major forms of carbon that are utilized as energy supplements. A

reduction in the activities of enzymes involved in sucrose hydrolysis inhibits sucrose utilization, which impairs panicle development. Trehalose-6-phosphate synthase, trehalose-6-phosphate phosphatase, and alpha-trehalase are involved in trehalose synthesis. Trehalose plays an important role in abiotic stress resistance, and trehalose-6-phosphate, an intermediate product of trehalose synthesis participates in sucrose signal transduction (Lunn *et al*. 2006; Ruan 2014). Nunes *et al*. (2013) reported that trehalose-6-phosphate served as a sugar signal that could induce the expression of genes associated with the alleviation of abiotic stress injury. In this study, some DEGs in CHR were also upregulated to promote trehalose-6-phosphate synthesis, and the upregulation of *BGIOSGA026976*, *BGIOSGA009181*, and *BGIOSGA030796* promoted trehalose-6-phosphate synthesis in SHR. The gene encoding trehalose-6-phosphate phosphatase, *BGIOSGA000509*, was significantly downregulated in IR36 at 40°C compared with 32°C, which might have reduced trehalose content and in turn disrupted the carbohydrate distribution. ~~The~~ Our results suggest that trehalose-6-phosphate metabolism was disordered under the high temperature growing condition and that the heat-susceptible cultivar experienced greater inhibition than the heat-tolerant cultivar.

~~In SHR, seven DEGs were associated with starch and sucrose metabolism.~~ Among these seven DEGs, the genes encoding sucrose synthesis, namely, *BGIOSGA010570* and *BGIOSGA026140*, were significantly downregulated in the IR36_32 vs IR36_40 group, while no difference in expression was observed in the HHZ_32 vs HHZ_40 group. Impairment of sucrose synthase activity reportedly reduced resistance to environmental stress, and *OsSUS3* inhibition reduced the heat tolerance of rice at the grain filling stage (Hirose *et al*. 2008; Takehara *et al*. 2018). The results of the present study suggested that sucrose impairment in the heat-susceptible cultivar aggravated spikelet reduction.

There is a close relationship between endogenous hormones and carbohydrate accumulation, which may suggest that the regulation of endogenous hormones in heat-tolerant varieties promoted the utilization of carbohydrates. Molecular marker-assisted selection can be carried out according to DEGs associated with hormone metabolism in the study of RHR.

Conclusions

In summary, heat stress-responsive DEGs in young panicles were identified by a transcriptome analysis of a heat-tolerant rice cultivar and a heat-susceptible rice cultivar grown at high temperature (40°C) and control temperature (32°C). Statistical analysis of a total of 5533 DEGs revealed three categories of genes (RHR, SHR, and CHR) containing a total of 4070 DEGs. We highlighted differential expression of a group of DNA-binding TF that was significantly enriched in the RHR category and differential

expression of genes involved in the starch and sucrose metabolism pathway that were overrepresented in the SHR category. Overall, the up-regulation of DEGs related to plant hormones and signal transduction were specifically beneficial for young panicle development grown at high temperature. Heat-tolerant cultivars increase endogenous hormones and maintain a stable carbohydrate metabolism pathway under high temperatures. ~~In addition~~However, certain metabolic pathways, including starch and sucrose metabolism, were much more~~specifically~~ damaged in heat susceptible cultivars under high temperatures, thus aggravating the inhibition of panicle development. The identification of DEGs in this study improves our understanding of the molecular mechanisms of heat resistance in young panicles~~; .~~ ~~heat-tolerant cultivars increase endogenous hormones and maintain a stable carbohydrate metabolism pathway under high temperature growing conditions.~~

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