INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–1470/2020/23–2–475–483
DOI: 10.17957/IJAB/15.1312
http://www.fspublishers.org

Full Length Article

Key Heat-responsive Genes and Pathways in Tropical and Temperate Maize (*Zea mays*) Germplasms as Revealed by Physiological and Transcriptome Analyses

Qi-Lun Yao[1] and Shi-Zhong Di[2*]
[1]*School of Advanced Agriculture and Bioengineering, Yangtze Normal University, Fuling 408000, P. R. China*
[2]*The Southeast Chongqing Institute of Agricultural Science, Fuling 408000, P. R. China*
[*]For correspondence: yql641@aliyun.com
Received 23 September 2019; Accepted 18 October 2019; Published 16 January 2020

Abstract

Heat stress is becoming a severe threat to maize yield worldwide. Confirmation of the maize germplasm tolerant to heat still remains elusive. In this study, we investigated physiology and transcriptomic profiling in the heat stress response among four different genotypes derived from the tropical and temperate maize germplasms. High temperatures induced pronounced increase in peroxidase (POD) and superoxide dismutase (SOD) activities, and malondialdehyde (MDA), soluble carbohydrate, and protein contents, but there were dramatic reductions in the chlorophyll content and four chlorophyll fluorescence parameters for the four maize genotypes. Heat stress had smaller effects on tropical maize genotypes compared to temperate genotypes. The RNA-seq analysis resulted in 44.5–54.0 million raw reads. A total of 44,241 global expression genes (almost 96.12% of the whole gene-set) were detected and many DEGs associated with specific genotypes were identified. Pairwise comparison between the genotypes revealed that 805 differentially expressed genes (DEGs) were shared. The gene ontology (GO) terms, such as signaling, cellular component organization, cellular component biogenesis, macromolecular complex, molecular transducer activity and structural molecule activity were commonly overrepresented in four genotypes under heat stress. The analysis of DEGs related to stress responses indicated that several crucial genes involved in heat stress were highly up-regulated, and the remaining up-regulated genes were found to be consistently expressed in the four genotypes, showing that the tropical and temperate maize germplasms had similar gene expressions in response to high temperatures. Our findings showed that tropical germplasms conferred potential heat-intolerance.

Keywords: Maize; Heat stress; Physiology; Transcriptome; Responses

Introduction

The high temperature is one of the most important abiotic stress factors limiting crop growth, development, and yield. Heat stress, a rise in the temperature of 10–15°C above the ambient, has become an agricultural problem all over the world (Wahid *et al.* 2007; Yousaf *et al.* 2018). High temperatures accelerate crop growth such as maize, whose phenology is predominantly adjusted by temperature. This has reduced the plant and grain development time, which limits the yield potential attainment. Additionally, pollination can be inhibited and the grain development prevented if heat stress occurs during crop flowering. For plant cell, extreme heat causes the alteration of membrane fluidity related to the membrane function, which plays an important role in carbon assimilation, the onset of oxidative damage resulted from the heat-induced imbalance of physiological activity, generation of reactive oxygen species (ROS), protein turnover, and antioxidant defense (Bita and Gerats 2013). Similar to other abiotic stress factors, the plant deploys morphological, anatomical, physiological, biochemical and molecular mechanisms to respond to heat tolerance including generation of antioxidants, accumulation of compatible metabolite, scavenging of ROS, and the signaling cascade activation resulting in the synthesis of heat-shock proteins (HSPs) as well as late embryogenesis abundant proteins. These molecular chaperones have a key impact on preventing proteins from misfolding and denaturation so as to sustain membrane integrity (Kotak *et al.* 2007). All the responses have been described as underlying regulations of gene expression causing the synthesis of osmoprotectants and transporters, including transcription factors (TFs), phosphatases, and protein kinases (Sakuma *et al.* 2006; Krasensky and Jonak 2012).

The adverse effect of heat stress on plant photosynthesis has been investigated and chlorophyll fluorescence determination considered an efficient and reproducible tool for detecting plant susceptibility to abiotic

To cite this paper: Yao QL, SZ Di (2020). Key heat-responsive genes and pathways in tropical and temperate maize (*Zea mays*) germplasms as revealed by physiological and transcriptome analyses. *Intl J Agric Biol* 23:475–483

stresses (Longenberger *et al.* 2009; Brestic and Zivcak 2013). As a nondestructive diagnostic tool, the chlorophyll fluorescence method determines the susceptibility of the photo system II (PSII) impairment in the photosynthesis electron transport chains (Maxwell and Johnson 2000). Chlorophyll fluorescence parameters such as *Fo*, *Fv/Fm*, and *Qp* can easily be measured without secondary stresses to plant samples (Christen *et al.* 2007).

RNA-seq is a powerful technology for genome-wide expression profiling, especially for studying complex gene regulatory networks and molecular mechanisms of plant thermo-tolerance (McGettigan 2013). In particular, both heat shock transcription factors (HSFs) and heat shock proteins (HSPs) have received much attention (Kotak *et al.* 2007). A positive correlation between expression of HSFs and thermo-tolerance was reported (Katiyar-Agarwal *et al.* 2003; Wang and Luthe 2003; Chauhan *et al.* 2012; Burke and Chen 2015). These heat shock transcription factors (Hsfs) act as crucial regulators of expression of heat shock proteins (Hsps), which prevent proteins from aggregation and unfolding to maintain cellular protein homeostasis under heat stress. Hsfs genes have been identified in various species and thoroughly characterized in *Arabidopsis*, rice, and tomato (Döring *et al.* 2000; Heerklotz *et al.* 2001; Guo *et al.* 2008). Several hsf genes in maize have also been cloned (Gagliardi *et al.* 1995). Transcriptomic profiling of wheat responses to heat stress showed that extreme heat induced a myriad of transcription factors including Zn-finger gene families and members of the HSF (Qin *et al.* 2008). The same gene families have also been found in the transcriptome profile in rice (Zhang *et al.* 2013). Reports indicated that differentially expressed *MYB* genes were up-regulated following heat treatments (El-Kereamy *et al.* 2012; Ambawat *et al.* 2013; Meng *et al.* 2015). There is also evidence from rice studies that over-expression of *MYB* leads to enhanced thermo-tolerance during vegetative growth and reduces the adverse effect of heat stress on grain yield (El-Kereamy *et al.* 2012). Plant thermo-tolerance is characterized by the activity of transcription factors synthesizing Hsps. Hsps are generally sustained in an inert monomer state by associating with molecular chaperones. In response to heat stress, they are converted to active trimmer in aid of oligomerization domains, functioning as sequence specific DNA binding proteins (Bienz and Pelham 1987).

Maize, a principal agricultural commodity, is one of the three most widely grown crops worldwide. Like other many plants, maize is often subjected to heat stress. It has been predicted that the expected changes in atmospheric CO_2 and other greenhouse gases will increase global air temperature to be in the range of 2.5–4.5°C until the end of this century. The yield of maize is severely decreased at temperatures above 30°C, which was indicated for US maize germplasm (Schlenker and Roberts 2009). As maize was evolved from an grass species in the tropical lowlands in Mexico and has long been under heat stress, it should be reasonable to predict that there was the potential thermo-tolerance germplasm. In order to reduce heat stress damage, the application of maize varieties tolerant to extreme heat has been considered to be a cost-effective approach. In this study, we investigated physiology and transcriptomic profiling in the heat stress among tropical and temperate maize germplasms. First we assessed the physiological effects of heat stress on maize inbreds from the tropical and temperate germplasms. Next we carried out a transcriptomic prolifing analysis to identify the genes and pathways associate with heat stress. Finally, we confirmed differentially expressed genes in the inbreds.

Materials and Methods

Plant material and treatments

Plant material: This study was based on four advanced stage maize inbred lines, which were bred by Yangtze Normal University, China. The inbreds Suwan-3 and Suwan-10 were sourced from the tropical maize germplasm, and Cim-5 and Cim-16 were derived from the International Maize and Wheat Improvement Center (CIMMYT) temperate populations. Seeds from the elite maize inbreds Suwan-3, Suwan-10, Cim-5, and Cim-16 were germinated in an incubator (25°C) over seven days.

Heat stress treatments: Heat stress treatments were performed in a plant growth chamber. 36 seedlings per genotype (7-day-old seedlings) were planted into 0.8 L pots and covered with fine sand. They were then grown at 30°C/22°C with a light/dark (12/12 h) cycle for 25 days. After the fifth leaf had expanded, plants were separated into two groups: one group (nine plants per genotype) that was heat stressed was grown at 40°C/33°C for seven days and another group (nine plants each) was left at 30°C/22°C as controls. Full-strength Hoagland's nutrient solution was prepared for applying to plants daily.

Physiological characterization

Flag leaves from each maize inbred line (Suwan-3, Suwan-10, Cim-5 and Cim-16) were sampled from heat treatments and controls in three replicates. Following standard methods, the six specific physiological parameters peroxidase (POD) and superoxide dismutase (SOD) activities, chlorophyll, malondialdehyde (MDA), soluble carbohydrate, and protein contents were determined as per protocol of Li (2016). The SPSS statistical package (version 19.0, SPSS Institute Ltd., Armonk, NY, USA) was used to conduct the statistical analysis of physiological data. The level of significance was set at $p < 0.05$ and $p < 0.01$, respectively.

The sixth fully expanded leaf of the plant was analyzed using a portable LI-COR 6400 photosynthetic system (LI-COR Inc., Lincoln, NE, USA) to determine the minimum fluorescence level (*Fo*), the maximum fluorescence level (*Fm*), the steady state fluorescence level (*Fs*), and the light adapted maximum fluorescence level (*Fm*'). The four chlorophyll fluorescence parameters the potential activity

(*Fv/Fo*), the maximum photochemical quantum yield (*Fv/Fm*), the effective photochemical quantum yield (Φ_{PSII}), and the coefficient of photochemical fluorescence quenching (Q_P), were evaluated as follows: *Fv/Fo* = (*Fm* – *Fo*)/*Fo*, *Fv/Fm* = (*Fm* − *Fo*)/*Fm*, Φ_{PSII} = (*Fm*' − *Fs*)/*Fm*', and Q_P = (*Fm*' − *Fs*)/(*Fm*' − *Fo*'), respectively (Kitajima and Butler, 1975; Genty *et al.* 1989; Su *et al.* 2015).

RNA extraction

Eight mRNA libraries were constructed using RNA extracted from Suwan-3, Suwan-10, Cim-5, and Cim-16 that had been subjected to the heat treatments and controls. Briefly, total RNA samples were prepared as follows: equal quantities of RNA isolated from leaves were pooled for each inbred in three replicates following the manufacturer's instructions, using Trizol Reagent (Invitrogene, Nottingham, UK). There were three replicates per inbred.

RNA-seq library construction and transcriptome sequencing

Approximately 5 µg of total RNA was used for the mRNA isolation process. After mRNA was fragmented and converted to cDNA for PCR amplification. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) were subsequently used to qualify each sample library. RNA-seq library construction and sequencing (on the Illumina HiSeq X Ten platform) were performed by Origingene Technology Limited Company for Biology Medicine (Shanghai, China).

Sequencing reads processing and differentially expressed genes (DEGs) identification

The data-processing steps were used to get clean data from trimming the raw data through discarding of adapter sequences and low-quality sequences. Clean reads for each library were mapped to Maize Reference Genome ZmB73_RefGen_v4 (https: //download. maizegdb. org/Zm-B73-REFERENCE-GRAMENE-4.0/Zm00001d. 2. enomic. fa) with GSNAP (Wu and Nacu, 2010). After alignment, normalized gene-level expression values were determined using RSEM version 0.9.3, which were expressed as fragments per kbp of exon model per million fragments mapped (FPKM). After calculating the expression levels of gene, the DEGs were screened by edgeR, with the corrected P-value of < 0.05 between each set of compared samples for each maize genotype under drought stress (Robinson *et al.* 2010). The expression patterns and cluster analyses were then performed using Mev v4.7.4 software with K-Means clustering method and Pearson correlation as distance calculation method (Wu and Nacu 2010). Neighbor-joining cluster analysis was used to analyze the common stress genes.

Gene ontology (Go) annotation of DEGs in maize Inbred lines

Co-modulated DEGs (Common DEGs among the four maize genotypes) were identified using venny graph. GO annotation of co-modulated DEGs was performed. GO categories assigned to DEGs were conducted using the R Bioconductor package GOseq (http: //bioconductor. org/packages/release/bioc/html/goseq. html) (Young *et al.* 2010). For significant GO terms, FDRs were calculated using the Benjamini and Hochberg (1995) to correct the P-values.

Real-time RT-PCR analysis

Ten co-modulated DEGs with significant changes were confirmed by qRT-PCR in order to validate of the results obtained from the RNA-Seq assay. Gene-specific primers are designed for qRT-PCR experiments with Primer 5.0 software (Supplementary Table 1S). Independent RNA from leaf samples of Suwan-3 and Cim-16 in both control and heat-stress conditions were isolated for the RNA-Seq assay. The amplification programs were carried out according to the standard protocol of an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) in triplicate as mentioned by Jain (2011). Further, we used the relative quantitative method ($^{2\text{-}\Delta\Delta}$Ct method) to define the expression levels of target genes through their fold change values (Schmittgen and Livak 2008).

Statistical analysis

We used SPSS statistical package (version 19.0, SPSS Institute Ltd., Armonk, NY, USA) to conduct Pearson correlation analysis of RNA-seq versus qRT-PCR data.

Results

Physiological responses to heat stress

There were pronounced increases in POD and SOD activities, and MDA, soluble carbohydrate, and soluble protein contents under drought stress (Table 1). There was no relationship between the four genotypes (inbreds) under the controls. However, there existed significant differences between the tropical Suwan-3 and Suwan-10 genotypes and the temperate Cim-5 and Cim-16 genotypes under high temperature conditions. Increased POD and SOD activities, and higher MDA, soluble carbohydrate, and soluble protein contents were found in Cim-5 and Cim-16 as compared to Suwan-3 and Suwan-10 under heat stress. On the contrary, the chlorophyll content decreased in all four genotypes responsive to heat stress. However, Suwan-3 and Suwan-10 maintained relatively higher values for chlorophyll content than Cim-5 and Cim-16. The results indicated that heat stress had differential physiological impacts on the genotypes derived from the tropical and temperate maize germplasms.

Table 1: Physiological parameters determined in plants responsive to heat stress

Treatment	Genotype	POD (U μg^{-1} FW min^{-1})	SOD (U μg^{-1} FW)	MDA content (nmol g^{-1} FW)	Soluble carbohydrate content (mg g^{-1} FW)	Chlorophyll content (mg g^{-1} FW)	Soluble protein content (mg g^{-1} FW)
Control	Suwan-3	6.08 ± 0.15^{a}	0.27 ± 0.03^{a}	10.52 ± 0.32^{a}	1.47 ± 0.11^{a}	1.63 ± 0.14^{a}	11.96 ± 0.63^{a}
	Suwan-10	6.32 ± 0.13^{a}	0.31 ± 0.03^{a}	9.04 ± 0.30^{a}	1.54 ± 0.10^{a}	1.52 ± 0.13^{a}	12.04 ± 0.58^{a}
	Cim-5	7.10 ± 0.11^{a}	0.30 ± 0.02^{a}	12.17 ± 0.42^{a}	1.40 ± 0.09^{a}	1.57 ± 0.16^{a}	11.33 ± 0.45^{a}
	Cim-16	7.05 ± 0.11^{a}	0.34 ± 0.04^{a}	11.28 ± 0.41^{a}	1.53 ± 0.12^{a}	1.48 ± 0.15^{a}	12.11 ± 0.54^{a}
Heat	Suwan-3	8.35 ± 0.16^{b}	0.52 ± 0.04^{b}	14.05 ± 0.45^{a}	1.78 ± 0.15^{a}	1.39 ± 0.14^{a}	13.49 ± 0.55^{a}
	Suwan-10	8.41 ± 0.14^{b}	0.48 ± 0.05^{b}	15.47 ± 0.51^{a}	1.90 ± 0.17^{a}	1.36 ± 0.15^{a}	13.20 ± 0.66^{a}
	Cim-5	9.72 ± 0.15^{c}	0.81 ± 0.07^{c}	27.28 ± 1.13^{b}	2.64 ± 0.22^{b}	0.80 ± 0.10^{b}	15.24 ± 0.70^{b}
	Cim-16	9.66 ± 0.18^{c}	0.83 ± 0.08^{c}	29.98 ± 1.21^{b}	2.71 ± 0.24^{b}	0.85 ± 0.09^{b}	16.61 ± 0.72^{b}

The same letter after a parameter value represents no significant difference between genotypes at $p<0.05$. Date are expressed as means ± standard error of the mean

To understand the effects of high temperatures on photosynthetic characteristics of maize inbreds, we assessed four chlorophyll fluorescence parameters (*Fv/Fo*, *Fv/Fm*, Φ_{PSII} and Q_P) under control and heat stress conditions. There were no pronounced differences in the four chlorophyll fluorescence parameters among the four maize genotypes under the controls. However, after they were subjected to high temperatures, *Fv/Fo* decreased and the variation range was from 0.82 in Suwan-3 to 0.36 in Cim-16 (Fig. 1A), and the decrease in *Fv/Fm* varied from 3.93 in Suwan-3 to 2.18 in Cim-16 (Fig 1B). The Φ_{PSII} and Q_P parameters were also significantly affected by heat stress. There were significant reductions in the Φ_{PSII} and Q_P of Cim-5 and Cim-16 following heat treatments (Fig 1C and 1D). Furthermore, the lowest Φ_{PSII} and Q_P values were recorded for Cim-16 (0.005 and 0.054). This observation showed that the reductions in *Fv/Fo*, *Fv/Fm*, Φ_{PSII}, and Q_P in Cim-5 and Cim-16 were considerably greater than in Suwan-3 and Suwan-10.

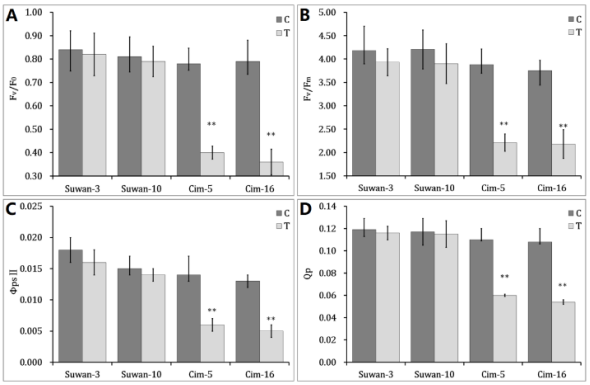

Fig. 1: Changes to F*v/Fo*, *Fv/Fm*, Φ_{PSII}, and Q_P in four maize genotypes subjected to heat stress. * and ** indicate 0.05 and 0.01 significant level, respectively

Transcriptome analysis of maize inbred lines in response to heat stress

A genome-wide analysis of expression genes was carried out using RNA-Seq technology for the four maize inbreds Suwan-3, Suwan-10, Cim-5, and Cim-16. In this work, 'A', 'B', 'C', and 'D' were used instead of Suwan-3, Suwan-10, Cim-5, and Cim-16 during the transcriptome analysis, respectively, and _1 as well as _2 instead of control and heat stress, respectively. On average, the RNA-seq analysis generated between 44.56 and 54.04 million paired-ends reads (Table 2), after the raw data had been filtered. Of those, the clean reads with high quality scores were identified over 97.5% at the Q20 level (Fig. 5).

After data-processing steps through trimming the raw data, the RNA-seq analysis generated between 44.56 and 54.04 million paired-end reads per sample (Table 2). Of those, the clean reads with high quality scores were identified over 97.5% at the Q20 level (a base quality greater than 20 and an error probability of 0.01). A high proportion of clean reads (80.62, 81.47, 83.45, and 79.96% for each genotype, respectively) were readily mapped to maize reference genome sequence for each heat stress library (from A to D), as well as control sample library (81.61, 80.27, 83.27, and 80.00% for each genotype, respectively). A total of 44,241 global expression genes assigned to 96.12% of the whole gene-set were identified and many DEGs associated with specific genotypes were determined. Subsequently, GSNAP software was used to quantify the abundance of global expression genes through reported as fragments per kilobase of exon model per million mapped reads (FPKM) (Supplementary Table 2S). Additionally, through hierarchical clustering of normalized expression levels for all globally expressed genes, distinct gene expression profiles in the four maize genotypes responses to heat stress are illustrated in Fig. 2.

DEGs under heat stress in maize inbred lines

Transcriptional changes related to heat stress were determined by comparing the four different maize genotype leaf transcriptomes in stress conditions with their corresponding controls. The DEGs were identified using edgeR software. Pair-wise comparisons of the Suwan-3, Suwan-10, Cim-5 and Cim-16 samples under heat stress treatment with their corresponding control samples were performed. The results showed that most of the DEGs were up-regulated in the four maize genotypes responsive to heat stress (Supplementary Table 3S). Furthermore, a pair wise comparison between the genotypes revealed that 805 DEGs were shared, with 1444, 1281, 2509, and 1732 DEGs being detected in comparisons of the control with Suwan-3, Suwan-10, Cim-5 and Cim-16, respectively (Fig. 3).

Table 2: The sequence data from Illumina sequencing analysis

Sample	Raw reads	Clean reads	Mapped reads	Unique match	Mapping ratio (%)
A_1	50343898	50122754	40907182	37113126	81.61
A_2	47288288	46988200	37881174	34743622	80.62
B_1	44561894	44322380	35576618	32155690	80.27
B_2	45213754	45029636	36685366	33922382	81.47
C_1	45955384	45699412	38055954	35739332	83.27
C_2	54038184	53836898	44927520	42564740	83.45
D_1	49973722	49834474	39867196	37063692	80.00
D_2	50962344	50765020	40589546	37112116	79.96

Further, a sample of 10 DEGs was chosen for qRT-PCR analysis to validate the accuracy of RNA-seq results. These DEGs include Zm00001d034027, Zm00001d035139, Zm00001d002801, Zm00001d030557, Zm00001d046357, Zm00001d022445, Zm00001d043205, Zm00001d042848, Zm00001d053975, and Zm00001d042148, which encode ethylene-responsive protein, MA3 domain-containing protein, GT-2 like 1 protein, alanine aminotransferase 2 mitochondrial, beta-galactosidase, alpha-galactosidase 3, ethylene-responsive transcription factor 4, laccase-7, beta-amylase 8, and cytokinin oxidase 2, respectively. Of 10 genes, 8 showed up-regulation and 2 down-regulation in the heat treatment condition (Fig. 4), when comparing the inbred lines Suwan-10 and Cim-5. The correlations between the qRT-PCR and RNAseq data demonstrated that the qRT-PCR data was highly consistent with their RNASeq expression profiles.

DEGs annotation and KEGG pathways

The Gene Ontology (GO) term analysis was conducted to identify the function of the detected DEGs (Fig. 6; Supplementary Table 4S). The GO terms "signaling", "cellular component organization", "cellular component biogenesis", "macromolecular complex", "molecular transducer activity", "structural molecule activity" were commonly overrepresented in four maize genotypes in response to heat stress. The results above suggest that these DEGs with the identified biological functions may be the crucial contributors to responses of the four genotypes to heat stress.

Discussion

Heat stress often causes adverse changes in plant growth, physiological processes, and development. It was demonstrated that modifications of physiological processes by gene expression alterations led to the tolerance or adaptation of high temperatures (Moreno and Orellana, 2011). Identification and confirmation of the maize germplasm tolerant to heat stress still remain largely elusive, although some studies have used the RNA-seq technology to identify heat-responsive genes in various crop species (Wahid *et al.* 2007; Zenda *et al.* 2019).

Physiological impairment occurs in plants under high temperature conditions (Halliwell 2006; Mahmood *et al.*

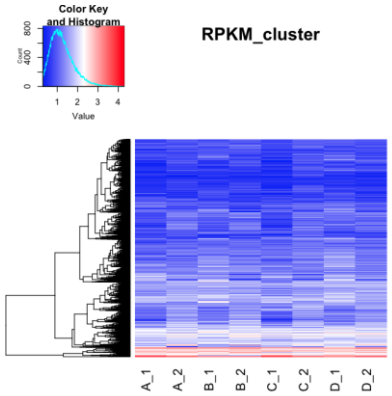

Fig. 2: Clusters of DEGs related to heat stress in different maize genotypes

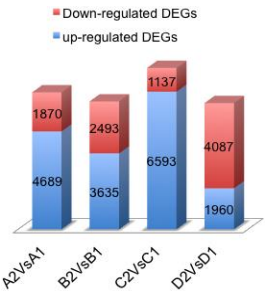

Fig. 3: Transcriptional changes related to heat stress were determined by comparing leaf transcriptomes of four different maize genotypes with each responding control sample

2010). High temperature can induce oxidative stress by the membrane lipid peroxidation and disruption of cell membrane stability (Camejo *et al.* 2006). Oxidative stress induced by high temperature leads to enzyme deactivation, protein degradation, and membrane damage, which has been described in a variety of crops such as rice (Hurkman *et al.* 2009); soybean (Djanaguiraman *et al.* 2011), sorghum (Djanaguiraman *et al.* 2014) and wheat (Ristic *et al.* 2007). It has been reported that oxidative stress may alter metabolic pathways that result in the accumulation of harmful ROS such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) in plant cells (Asada, 2006). The extent of membrane impairment can be reflected by alterations in MDA content, since POD catalyzes lipid peroxide to generate MDA. SOD functions as catalyzing the dismutation of O^{-2} to O_2 and H_2O_2, showing the level of

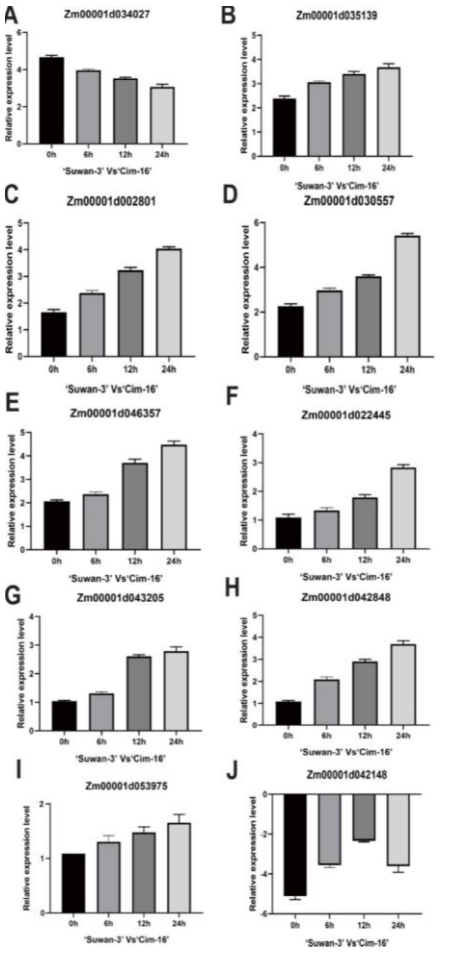

Fig. 5: Relative expression levels (q-RT PCR) of 10 selected genes for Suwan-3 vs Cim-16. X-axes are time course (from 0 to 24 h) and Y axes represent the scales of relative expression levels. All genes with negative values of expression level indicate that they were down-regulated under heat stress

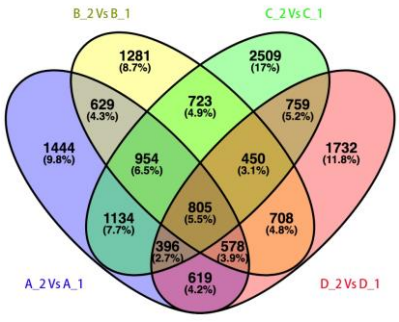

Fig. 4: Venny graph for heat-stress specific DEGs

H_2O_2 accumulation. A decrease in chlorophyll content was also a result of lipid peroxidation of chloroplast and thylakoid membranes due to heat stress (Mohammed and Tarpley 2010). High temperatures impose negative impacts on carbohydrate and protein syntheses. The carbohydrate and

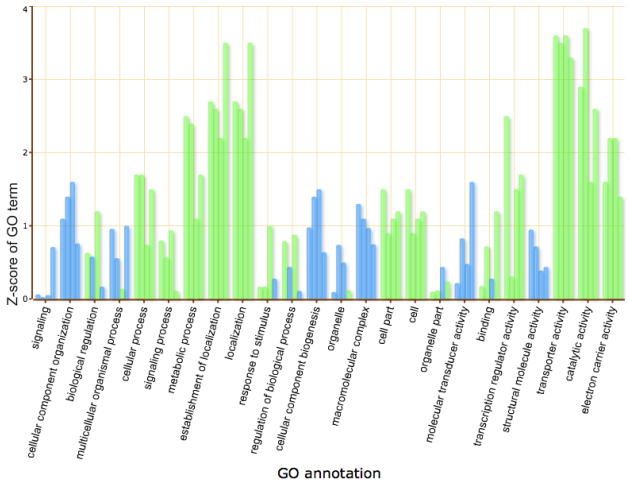

Fig. 6: GO annotation of DEGs in four different maize genotypes responsive to heat stress. The Y-axis denotes the abundance of GO term. The X-axis defines GO terms

protein contents have been used as indicators of plant physiochemical metabolisms induced by heat stress (Sumesh *et al.* 2008). Taken together, the changes of these specific physiological components in crops implicated their participation in heat stress. In this study, maize inbreds generally displayed physiological accommodation enhancement under heat stress. Furthermore, greater effects of heat stress on the temperate maize lines as compared to the tropical maize lines were evidenced by pronounced changes of six specific physiological parameters. Interestingly, the content of soluble carbohydrate and protein increased in all four genotypes under heat stress as compared to controls. This was expected since water deficit causes loss of plant water content and a corresponding enhancement in the relative contents of soluble carbohydrate and protein.

Photosynthesis, a heat sensitive physiological process in plants, is markedly affected by high temperatures (Crafts-Brandner and Salvucci 2002; Mabapa *et al.* 2018). A high temperature greatly affects carbon metabolism and photochemical reactions in chloroplast. Reduction in photosystem II (PSII) photochemistry ratio is the reason for damaged photosynthesis that hampers chloroplast functions (Suwa *et al.* 2010). Chlorophyll fluorescence has been an index of detailed analysis on functional and structural changes of PSII under abiotic stresses (Kalaji *et al.* 2011; Mathur and Jajoo 2014). In this study, four chlorophyll fluorescence parameters (*Fv/Fo*, *Fv/Fm*, Φ_{PSII} and Q_P) were selected to distinguish the changes in PSII performance induced by heat stress. Under heat stress, *Fv/Fo*, *Fv/Fm*, Φ_{PSII}, and Q_P declined consistently and were significant higher in the inbreds from the tropical germplasm than those from the temperate. Smaller *Fv/Fo* decreases in the inbreds from tropical germplasm indicate that their chloroplasts play an essential role in defending photosystems from severe damages to maintain a larger photosynthetic activity of PSII. Both *Fv/Fm* and Φ_{PSII} are commonly employed to evaluate

the photochemical quantum yield of PSII. Reductions in *Fv/Fm* and Φ_{PSII} implicate that heat stress have adverse impact on the PSII photochemical efficiency. Larger decreases in *Fv/Fm* and Φ_{PSII} show more damages of photosystems in utilizing the absorbed light energy for photochemistry in the temperate inbreds than in the tropical inbreds responsive to heat stress. Another chlorophyll fluorescence parameter Q_P often increases when the light intensity of growth environments is higher than the ability for photosynthesis in plants (Ebbert *et al.* 2001). Previously, the reduction in Q_P was thought to result from alterations of thylakoid membrane and monomerization of light harvesting complex of PSII under abiotic stresses (Essemine *et al.* 2012; Yoshioka-Nishimura *et al.* 2014; Chen *et al.* 2018). The correlation of significant Q_P decreases with high temperatures further revealed more severe impairments to photosystems in the temperate inbreds compared to the tropical inbreds.

It is well known that the plant has evolved adaptive mechanisms to abiotic stress response (Zenda *et al.* 2019). We conducted comparative transcriptomic analyses of four genotypes to detect key regulatory genes and elements related to heat stress (Supplementary Table 3S). Transcription factors (TFs) such as WRKY, MYB, DREB, and NAC have been designated as important regulators in plant responsive to abiotic stress. In this study, a heat shock transcription factor (HSF) (Zm00001d029270, heat stress transcription factor B-4d), belonging to type B2 HSF, was identified to be highly up-regulated in the tropical maize genotype Suwan-3 under heat. The heat shock transcription factor (HSFA3) in *Arabidopsis* is modulated by the AP2-type transcription factors DREB2A and DREB2B (Larkindale and Vierling 2008), implying that other transcription factors can activate HSFs, although DREB transcription factors were typically associated with cold and dehydration responses. Highly up-regulated DEGs in Suwan-3 also included a transcription factor (Zm00001d035139). Under high temperature conditions, a gene (Zm00001d030557) in the tropical maize genotype Suwan-10, encoding alanine aminotransferase 2 mitochondrial, was highly up-regulated, and reported to be crucial for the regulation of energy availability in wheat in response to abiotic stresses (Kendziorek *et al.* 2012). Another high heat-induced gene (Zm00001d002801) in the temperate inbred Cim-5 encodes GT-2 like 1 protein, which was reported to regulate plant stress responses (Zheng *et al.* 2016). For the rest up-regulated genes involved in heat stress, most were detected as consistently expressed in the tropical genotypes compared to the temperate genotypes, showing the similarity of gene expression in the tropical and temperate maize germplasms when responding to high temperatures.

Metabolic pathways relevant for phytohormones have been attributed to plant thermo-tolerance (Kotak *et al.* 2007). For example, ROS accumulation and lipid peroxidation can be decreased by means of treating ABA, SA, as well as AVG (aminoethoxy vinyl glycine) application (Koprivova *et al.* 2008). More so, the enzymatic antioxidant defenses against heat stress were also modified by phytohormones (Lubovská *et al.* 2014). It was shown that ethylene levels were the crucial signals for stress and senescence induction (Morgan and Drew 1997). Two up-regulated genes (Zm00001d034027 and Zm00001d043205) were affected by heat in our dataset (Supplementary Table 3S). Another phytohormone cytokinin was described as affecting the leaf water potential and stomatal conductance, which led to an enhanced heat and drought tolerance in tobacco (Macková *et al.* 2013). Expression of a cytokinin glycosyltransferase improves adaptation of Arabidopsis for drought stress (Li *et al.* 2015). In this study, a gene (Zm00001d042148) encoding cytokinin oxidase 2 was detected to be down-regulated in four genotypes under heat stress (Supplementary Table 3S), suggesting that the differentially expression of this gene is crucially associated with responding to heat stress in maize inbreds.

Bio-molecular metabolism has been described as corresponding regulation in plants adapting abiotic stresses. Our GO term enrichment analyses showed that macromolecular complex, cellular component organization, and cellular component biogenesis were commonly enriched terms in all the four genotypes (Supplementary Table 5S). These three GO terms comprised of 62 genes which were overlapping. Among 62 genes, a gene (Zm00001d047077) was associated with carbohydrate process. The rest DEGs primarily involved in the process of protein, amino acid, and nucleotide regulation. Furthermore, bio-molecular activity related DEGs, including molecular transducer and structural molecule activity, were also co-modulated in four genotypes responsive to heat stress (Supplementary Table 5S). A total of 43 DEGs participated in these pathways (Supplementary Table 4S), such as Zm00001d035139 (MA3 domain-containing protein), Zm00001d048424 (evolutionarily conserved C-terminal region 2), and Zm00001d037620 (60S ribosomal protein L13).

Plant photosynthesis involves electron carrier system and a reduction in the electron carrier activity may decrease photosynthetic ability (Song *et al.* 2014). Photosynthetic energy is primarily captured through linear electron flow related to light-stimulated electron transfer between PSI and PSII (Livingston *et al.* 2010). Our results indicated that 13 DEGs were identified for electron carrier activity. Of 13 DEGs, eight were implicated in cytochrome P450s with more DEGs being down-regulated than up-regulated, especially in the temperate maize genotypes (Supplementary Table 4S). Cytochrome P450s has been reported to play an important role in protecting organisms from oxidative damage, and their induction is relative to biotic and abiotic stresses (Guo *et al.* 2007). Consistent with chlorophyll fluorescence analysis, it is presumed that decline in photosynthetic capacity is attributed to oxidative damage to electron carrier system by heat stress.

Conclusion

Differential physiological responses and transcriptomic profiles in maize genotypes derived from tropical and temperate maize germplasm are responsive to high temperatures. The tropical maize genotypes showed better thermo-tolerance than the temperate genotypes. This study contributes to our understanding of the hypothesis that the heat stress induces DEGs which, in turn, can trigger pathways related to hormones, antioxidants, and photochemical reactions that activate physiological heat responses. This revealed that the molecular mechanisms underlying heat responses may accelerate the development of thermo-tolerance maize varieties.

Acknowledgements

This project was supported by National Natural Science Foundation of China (No. 31371633) and University Outstanding Achievements Transfer Aid Project of Chongqing Municipal Education Commission (CGZH1633).

References

Ambawat S, P Sharma, NR Yadav, RC Yadav (2013). *MYB* transcription factor genes as regulators for plant responses: an overview. *Physiol Mol Biol Plants* 19:307–312

Asada K, (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol*, 141:391–396

Benjamini Y, Y Hochberg (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B. (Methodological)* 57:289–300

Bienz M, HR Pelham (1987). Mechanisms of heat-shock gene activation in higher eukaryotes. *Adv Genet* 24:31–72

Bita CA, T Gerats (2013). Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Front Plant Sci* 4:273

Brestic M, M Zivcak (2013). PSII fluorescence techniques for measurement of drought and high temperature stress signal in crop plants: protocols and applications. *In: Molecular Stress Physiology of Plants*, pp: 87–131. Springer, Dordrecht, The Netherlands

Burke JJ, J Chen (2015). Enhancement of reproductive heat tolerance in plants. *PLoS One*, 10:e0122933

Camejo D, A Jiménez, JJ Alarcón, W Torres, JM Gómez, F Sevilla (2006). Changes in photosynthetic parameters and antioxidant activities following heat–shock treatment in tomato plants. *Funct Plant Biol* 33:177–187

Chauhan H, N Khurana, A Nijhavan, JP Khurana, P Khurana (2012). The wheat chloroplastic small heat shock protein (sHSP26) is involved in seed maturation and germination and imparts tolerance to heat stress. *Plant Cell Environ* 35:1912–1931

Chen JP, JJ Burke, ZG Xin (2018). Chlorophyll fluorescence analysis revealed essential roles of FtsH11 protease in regulation of the adaptive responses of photosynthetic systems to high temperature. *BMC Plant Biol* 18:11–24

Christen D, S Schönmann, M Jermini, RJ Strasser, G Défago (2007). Characterization and early detection of grapevine (*Vitis vinifera*) stress responses to esca disease by *in situ* chlorophyll fluorescence and comparison with drought stress. *Environ Exp Bot* 60:504–514

Crafts-Brandner SJ, ME Salvucci (2002). Sensitivity of photosynthesis in a C_4 plant, maize, to heat stress. *Plant Physiol* 129:1773–1780

Djanaguiraman M, PVV Prasad, DL Boyle, WT Schapaugh (2011). High-temperature stress and soybean leaves: Leaf anatomy and photosynthesis. *Crop Sci* 51:2125–2131

Djanaguiraman M, PVV Prasad, M Murugan, R Perumal, UK Reddy (2014). Physiological differences among sorghum (Sorghum bicolor L. Moench) genotypes under high temperature stress. *Environ Exp Bot* 100:43–54

Döring P, E Treuter, C Kistner, R Lyck, A Chen, L Nover (2000). The role of AHA motifs in the activator function of tomato heat stress transcription factors HsfA1 and HsfA2. *Plant Cell* 12:265–278

Ebbert V, B Demmig-Adams, WW Adams, KE Mueh, LA Staehelin (2001). Correlation between persistent forms of zeaxanthin-dependent energy dissipation and thylakoid protein phosphorylation. *Photosynth Res* 67:63–78

El-Kereamy A, YM Bi, K Ranathunge, PH Beatty, AG Good, SJ Rothstein (2012). The rice R2R3-MYB transcription factor *OsMYB55* is involved in the tolerance to high temperature and modulates amino acid metabolism. *PLoS One* 7:e52030

Essemine J, S Govindachary, D Joly, S Ammar, S Bouzidm, R Carpentier (2012). Effect of moderate and high light on photosystem II function in Arabidopsis Thaliana depleted in digalactosyl-diacylglycerol. *Biochim. Biophys. Acta Bioenergetics* 3:1367–1373

Gagliardi D, C Breton, A Chaboud, P Vergne, C Dumas (1995). Expression of heat shock factor and heat shock protein 70 genes during maize pollen development. *Plant Mol Biol* 29:841–856

Genty B, JM Briantais, NR Baker (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta (BBA)-Gen Subj* 1:87–92

Guo J, J Wu, Q Ji, C Wang, L Luo, Y Yuan, Y Wang, J Wang (2008). Genome-wide analysis of heat shock transcription factor families in rice and Arabidopsis. *J Genet Genomics* 35:105–118

Guo P, G Bai, B Carver, R Li, A Bernardo, M Baum (2007). Transcriptional analysis between two wheat near-isogenic lines contrasting in aluminum tolerance under aluminum stress. *Mol Genet Genomics* 277:1–12

Halliwell B (2006). Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 97:1634–1658

Hanif A, A Wahid (2018). Seed yield loss in mungbean is associated to heat stress induced oxidative damage and loss of photosynthetic capacity in proximal trifoliate leaf. *Pak J Agric Sci* 55:777–786

Heerklotz D, P Döring, F Bonzelius, S Winkelhaus, L Nover (2001). The balance of nuclear import and export determines the intracellular distribution and function of tomato heat stress transcription factor HsfA2. *Mol Cell Biol* 21:1759–1768

Hurkman WJ, WH Vensel, CK Tanaka, L Whitehand, SB Altenbach (2009). Effect of high temperature on albumin and globulin accumulation in the endosperm proteome of the developing wheat grain. *J Cereal Sci* 49:12–23

Jain M (2011). Next-generation sequencing technologies for gene expression profiling in plants. *Brief Funct Genomics* 11:63–70

Kalaji HM, K Bosa, J Koscielniak, Z Hossain (2011). Chlorophyll a fluorescence–a useful tool for the early detection of temperature stress in spring barley (*Hordeum Vulgare* L). *Omics* 15:925–934

Katiyar-Agarwal S, M Agarwal, A Grover (2003). Heat-tolerant basmati rice engineered by over-expression of *hsp101*. *Plant Mol Biol* 51:677–86

Kendziorek M, A Paszkowski, B Zagdańska (2012). Differential regulation of alanine aminotransferase homologues by abiotic stresses in wheat (*Triticum aestivum* L.) seedlings. *Plant Cell Rep* 31:1105–1117

Kitajima M, W Butler (1975). Quenching of chlorophyll luorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochim Biophys Acta Biomembr* 376:105–115

Koprivova A, KA North, S Kopriva (2008). Complex signaling network in regulation of adenosine 5'-phosphosulfate reductase by salt stress in Arabidopsis roots. *Plant Physiol* 146:1408–1420

Kotak S, J Larkindale, U Lee, P Koskull-Doring, E Vierling, KD Scharf (2007). Complexity of the heat stress response in plants. *Curr Opin Plant Biol* 10:310–316

Krasensky J, C Jonak (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J Exp Bot* 63:1593

Larkindale J, E Vierling (2008). Core genome responses involved in acclimation to high temperature. *Plant Physiol* 146:748–761

Li XF (2016). *Plant Physiology Experiment Manual (2)*, pp: 141–158. High Educational Press, Beijing, China

Li YJ, B Wang, RR Dong, BK Hou (2015). AtUGT76C2 an Arabidopsis cytokinin glycosyltransferase is involved in drought stress adaptation. *Plant Sci* 236:157–167

Livingston AK, JA Cruz, K Kohzuma, A Dhingr, DM Kramera (2010). An Arabidopsis mutant with high cyclic electron flow around photosystem I (*hcef*) involving the NADPH dehydrogenase complex. *Plant Cell* 22:221–233

Longenberger PS, C Smith, S Duke, B McMichael (2009). Evaluation of chlorophyll fluorescence as a tool for the identification of drought tolerance in upland cotton. *Euphytica* 1:25–33

Lubovská Z, J Dobrá, H Storchová, N Wilhelmová, R Vanková (2014). Cytokinin oxidase/dehydrogenase overexpression modifies antioxidant defense against heat drought and their combination in Nicotiana tabacum plants. *J Plant Physiol* 171:1625–1633

Mabapa PM, KK Ayisi, IK Mariga (2018). Comparison of gas exchange in *Moringa oleifera* and other drought tolerant tree species for climate change mitigation under semi-arid condition of Northern South Africa. *Intl J Agric Biol* 20:2669–2676

Macková H, M Hronková, J Dobrá, V Turečková, O Novák, Z Lubovská, V Motyka, D Haisel, T Hájek, IT Prášil, A Gaudinová, H Štorchová, E Ge, T Werner, T Schmülling, R Vanková (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. *J Exp Bot* 64:2805–2815

Mahmood S, A Wahid, F Javed, SMA Basra (2010). Heat stress effects on forage quality characteristics of maize (*Zea mays*) cultivars. *Intl J Agric Biol* 12:701–706

Mathur S, A Jajoo (2014). Alterations in photochemical efficiency of photosystem II in wheat plant on hot summer day. *Physiol Mol Biol Plants* 20:527–531

Maxwell K, GN Johnson (2000). Chlorophyll fluorescence-a practical guide. *J Exp Bot* 51:659–668

McGettigan PA (2013). Transcriptomics in the RNA-seq era. *Curr Opin Chem Biol* 17:4–11

Meng X, JR Wang, GD Wang, XQ Liang, XD Li, QW Meng (2015). An *R2R3-MYB* gene *LeAN2* positively regulated the thermotolerance in transgenic tomato. *J Plant Physiol* 175:1–8

Mohammed AR, L Tarpley (2010). Effects of high night temperature and spikelet position on yield-related parameters of rice (*Oryza sativa* L.) plants. *Eur J Agron* 33:117–123

Moreno AA, A Orellana (2011). The physiological role of the unfolded protein response in plants. *Biol Res* 44:75–80

Morgan PW, MC Drew (1997). Ethylene and plant responses to stress. *Physiol Plantarum* 100:620–630

Qin D, H Wu, H Peng, Y Yao, Z Ni, Z Li, C Zhou, Q Sun (2008). Heat stress responsive transcriptome analysis in heat susceptible and tolerant wheat (*Triticum aestivum* L.) by using Wheat Genome Array. *BMC Genomics* 9:432

Ristic Z, I Momcilovic, JM Fu, E Callegaric, BP DeRidder (2007). Chloroplast protein synthesis elongation factor, EF-Tu, reduces thermal aggregation of Rubisco activase. *J Plant Physiol* 164:1564–1571

Robinson MD, DJ McCarthy, GK Smyth (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140

Sakuma Y, K Maruyama, F Qin, Y Osakabe, K Shinozaki, K Yamaguchi-Shinozaki (2006). Dual function of an Arabidopsis transcription factor DREB2A in water-stress-responsive and heat-stress responsive gene expression. *Proc Natl Acad Sci USA* 103:18822–18827

Schlenker W, M Roberts (2009). Nonlinear temperature effects indicate severe damages to US crop yields under climate change. *Proc Natl Acad Sci USA* 106:15594–15602

Schmittgen TD and KJ Livak (2008). Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3:1101–1108

Song YP, QQ Chen, D Ci, XN Shao, DQ Zhang (2014). Effects of high temperature on photosynthesis and related gene expression in poplar. *BMC Plant Biol* 14:111

Su LY, ZW Dai, SH Li, HP Xin (2015). A novel system for evaluating drought–cold tolerance of grapevines using chlorophyll fluorescence. *BMC Plant Biol* 15:82–94

Sumesh KV, P Sharma-Natu, MC Ghildiyal (2008). Starch synthase activity and heat shock protein in relation to thermal tolerance of developing wheat grains. *Biol Plantarum* 52:749–753

Suwa R, H Hakata, H Hara, HA El-Shemy, JJ Adu-Gyamfi, NT Nguyen, S Kanai, DA Lightfoot, PK Mohapatra, K Fujita (2010). High temperature effects on photosynthetic partitioning and sugar metabolism during ear expansion in maize (*Zea mays* L.) genotypes. *Plant Physiol Biochem* 48:124–130

Wahid A, S Gelani, M Ashraf, MR Foolad (2007). Heat tolerance in plants: An overview. *Environ Exp Bot* 61:199–223

Wang D, DS Luthe (2003). Heat sensitivity in a bentgrass variant. Failure to accumulate a chloroplast heat shock protein isoform implicated in heat tolerance. *Plant Physiol* 133:319–327

Wu TD, S Nacu (2010). Fast and SNP-tolerant detection of complex variants and splicing in short reads. *Bioinformatics* 26:873–881

Yoshioka-Nishimura M, D Nanba, T Takaki, C Ohba, N Tsumura, N Morita, H Sakamoto, K Murata, Y Yamamoto (2014). Quality control of photosystem II: direct imaging of the changes in the thylakoid structure and distribution of FtsH proteases in spinach chloroplasts under light stress. *Plant Cell Physiol* 55:1255–1265

Yousaf MI, K Hussain, S Hussain, A Ghani, M Arshad, A Mumtaz, RA Hameed (2018). Characterization of indigenous and exotic maize hybrids for grain yield and quality traits under heat stress. *Intl J Agric Biol* 20:333–337

Zenda TS, ST Liu, X Wang, G Liu, HY Jin, AY Dong, YT Yang, HJ Duan (2019). Key maize drought-responsive genes and pathways revealed by comparative transcriptome and physiological analyses of contrasting inbred lines. *Intl J Mol Sci* 20:1268

Zhang X, W Rerksiri, A Liu, X Zhou, H Xiong, J Xiang, X Chen, X Xiong (2013). Transcriptome profile reveals heat response mechanism at molecular and metabolic levels in rice flag leaf. *Genes* 530:185–192

Zheng X, HP Liu, HT Ji, YN Wang, BD Dong, YZ Qiao, MY Liu, X Li (2016). The wheat GT factor *TaGT2L1D* negatively regulates drought tolerance and plant development. *Sci Rep* 6:27042