Identification and characterization of the ZmHSP20 genes in response to high temperature in maize

Ming Xue  
Agricultural College of Yangzhou University

Yiwen You  
Agricultural College of Yangzhou University

Luyao Zhang  
Agricultural College of Yangzhou University

Jinming Cao  
Agricultural College of Yangzhou University

Saihua Chen (✉ chensaihua@yzu.edu.cn)  
Agricultural College of Yangzhou University

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Abstract

High temperature is an emerging threat to maize productivity due to global warming. The HSP20 gene family has been reported to promote resistance to various abiotic stresses, but whether it is involved in high temperature response in maize is rarely known. Here, we identified 33 HSP20 genes (HSP20s) in maize via a conserved α-crystalline domain (ACD) scanning. The HSP20s can be divided into 14 subfamilies by the phylogenetic analysis. These genes distribute in all chromosomes and ten gene pairs may occur within duplication events. Fourteen members were predicted to have heat stress elements in their promoters, while seven of them locate in previously reported heat-resistance QTL (hrQTL) regions, accounting for 87.5% of the total hrQTL-related ZmHSP20s. Divergent tissue-specific expression profiles of ZmHSP20s were observed under normal conditions. Fourteen ZmHSP20s were co-upregulated in V4 and V5 leaves after high temperature treatment, while seven ZmHSP20s were stimulated concurrently in the kernel at daytime and nighttime high temperatures. These genes also show co-expression interactions with some ZmHSFs that are key factors in the heat-resistance pathway. The dynamic responses of candidate ZmHSP20s were further confirmed by real-time RT-PCR. Our study paves the way for further studies on the roles of ZmHSP20s in heat stress resistance.

Introduction

High temperature is emerging as one type of stress that poses a threat to crop productivity owing to global climate change [1]. The sustained high temperature beyond an optimum crop growth temperature is known as heat stress, which will hinder the development of crops, shorten the field growth period and decrease the yield per unit area [2]. Maize, the most widely planted economic and food crop, may suffer significant damage from heat stress throughout its life, particularly during the flowering and grain filling periods [3, 4].

To date, considerable progress has been made in heat tolerance in plants [5]. Some of these studies revealed the physiological and biochemical effects of high temperature [6–8]. For example, the photosynthetic intensity decreased under high temperature stress in maize leaves [9]. Others have identified heat-resistant genes or loci by quantitative trait locus (QTL) mapping or transcriptome analysis [10]. Among them, heat shock proteins (HSPs) have been proven to participate in heat responses in many plants [11–14]. HSPs in eukaryotes are classified into several families, including HSP100, HSP90, HSP70, HSP60 and small HSPs (sHSP). The sHSP family, also called HSP20, is conserved in different species and consists of small HSPs with a monomer molecular mass ranging from 12 to 42 Kilodaltons (KDa) [15, 16]. All HSP20s are characterized with a highly conserved alpha crystalline domain (ACD) flanked by N- and C-terminal regions [17, 18]. However, not all the ACD-containing proteins belong to the HSP20 family [15]. In angiosperms, HSP20 family members can be divided into several subfamilies based on their subcellular localizations, such as C-I (cytosol I), C-II, C-III, C-IV, C-V, C-VI, ER (endoplasmic reticulum), CP (chloroplasts), M-I (mitochondria I), M-II (mitochondria II), PX (peroxisomal) [16], as well as P (plastidial) [19, 20]. HSP20s can function as ATP-independent molecular chaperones and protect plants by preventing protein aggregation under many stress conditions [19], like heat stress. Many HSP20s have
been shown to play an important role in Arabidopsis heat response. Hsa32, for example, is required for the maintenance but not the induction of acquired thermotolerance [30], whereas HSP21 regulates thermo memory in collaboration with FtsH6 [31]. Exogenous HSP20s also work in transgenic Arabidopsis for heat stress tolerance, such as HSP26 from wheat [20], HSP17.8 from Rosa chinensis [21], and HSP18.2 from rice [22]. Silencing of class I small heat shock proteins decreased seedling thermotolerance in rice, which was increased by overexpression of sHSP17.7 [35, 36]. Up-regulation of 5 sHSPs (HSP26.7, HSP23.2, HSP17.9A, HSP17.4 and HSP16.9A) also plays an important role in heat stress in rice [23]. However, none of the HSP20s has been confirmed to participate in heat tolerance and only a few studies gave hints to the role of HSP20 in conferring heat tolerance in maize. For instance, maize HSF05 and HSFA2 can improve thermotolerance in Arabidopsis [24, 25]. ZmHSP16.9 showed heat tolerance when it was introduced into tobacco [26] and ZmHSP17.2 showed transcriptional accumulation under high temperature in maize [27, 28]. Furthermore, the transcription levels of chloroplast-localized small heat-shock proteins increased with heating duration in C4 species [41]. Accordingly, sHSPs would be critical factors in maize's ability to withstand heat stress.

The HSP20 genes may be involved in the heat response network under many heat shock transcription factors (HSF), as studied recently [17, 29, 30]. For instance, HSFB1 and HSFB2b were reported to be necessary for the expression of 10 HSP20s under heat stress conditions, which is necessary for acquired thermotolerance in Arabidopsis [31]. In wheat, HSFA6f can directly regulate HSP16.8, HSP17, HSP17.3 and lead to a positive impact on thermotolerance [32]. Likewise, HSFA6e was manifested to be involved in the regulation of HSP17 in wheat [33].

Our preliminary gene prediction conducted by ACD domain scanning found that there are numerous members in maize, suggesting that there may be an HSP20 superfamily. However, whether these ACD-containing proteins function as HSP20s and whether they are involved in heat response are still unclear. In this study, we conducted a comprehensive analysis of ZmHSP20 genes in maize, which would help us better understand their roles in adapting to heat stress.

Materials And Methods

Genome-wide identification of ZmHSP20 family genes in maize

Three approaches were adapted to identify ZmHSP20 family genes in maize step by step. First, the Hidden Markov Model (HMM) was used to obtain ACD-containing proteins in maize according to HSP20 family protein (PF00011) profile (P < 0.001). Second, the protein sequences retrieved from known HSP20 family members of Arabidopsis (Siddique et al., 2008) and rice (Sarkar et al., 2009) were used as queries in a blast search for the maize genome database, with the results filtered with an e-value ≤ 1e⁻³. Third, taking “HSP20” and “small heat shock protein” as keywords, an overall scan was also employed to search against the maize genome database. After removing the redundant sequences, unique sequences were submitted to CDD (https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) and Pfam (http://pfam.xfam.org/) to confirm the existence of the conserved HSP20 domain. Their isoelectric point
(pI) and molecular weight [34] were then calculated through the ExPASy tool (https://web.expasy.org/compute_pi/). These identified proteins were named according to their molecular weight [35, 36] and their chromosomal locations were determined by sequence alignment within the maize genome database (https://www.maizegdb.org/).

**Multiple sequence alignment and phylogenetic analysis**

The amino acid sequences of all the ZmHSP20s were aligned using the MEGA6 software's Muscle tool for the phylogenetic tree construction. Neighbor joining analysis was performed with the pairwise deletion option with Poisson correction. Bootstrap analysis was conducted with 1000 replicates by the MEGA program. The rice and Arabidopsis HSP20 amino acid sequences were obtained from the Ensemble Plants database (http://plants.ensembl.org/index.html). ZmHSP20s were classified into different subfamilies by their distances to these known rice and Arabidopsis HSP20s in the phylogenetic tree. The gene duplication was confirmed according to the method used in potato HSP20 and ZmHSF identification [37, 38]. For all the candidate HSP20s, the similarity should be > 80% and the coverage should be > 70% when their amino acid sequences were aligned to known HSP20s.

**Cis-acting regulatory element prediction in promoter regions of ZmHSP20**

About 1.5 kilobases of promoters upstream of the initiation codon (ATG) of 33 ZmHSP20s were downloaded from the MaizeGDB database. The PlantCARE database was used to anticipate conserved cis-acting regulatory elements in these promoters [46].

**Expression analysis**

Based on the RNA-seq data sets from the NCBI database (Supply Table 1), we compared the expression levels of these ZmHSP20s in various tissues under normal conditions as well as the induced pattern under heat-stress conditions. The expression patterns of ZmHSP20s were studied in fifteen different tissues of B73, including shoot, root, root hair, leaf, ovule, shoot apical meristem (SAM), 10-day ear, 10-day embryo, 10-day endosperm, seed, cob, tassel, anther, silk and pollen. We also summarized the expression changes of these ZmHSP20 genes in response to heat stress in leaf [39] and kernel tissues [40], using the maize V4 genome as a reference. The threshold of a false discovery rate (FDR) corrected by the P value < 0.05 and a fold change ≥ 2 was used to identify significant differential expression between two samples.

**Plant material and growth conditions**

Maize (*Zea mays* L.) inbred line W22 was grown in the chamber under normal conditions (32°C/22°C, day/night). At V4 stage, half of the seedlings were kept in the normal condition (32°C, daytime), while the other half were moved into a higher temperature circumstance (42°C, daytime) for heat stress treatment. From that moment on, leaves in each condition were collected every three hours and snap-frozen immediately in liquid nitrogen and stored at 80°C.

**RNA extraction and RT-PCR analysis**
Total RNA was extracted using the Trizol reagent (Vazyme, Nanjing). For RT-PCR analysis, 500 ng total RNA were reverse-transcribed into cDNA by the PrimeScript™ Reverse Transcriptase Kit (TaKaRa, Japan). Quantitative RT-qPCR assays were performed with a SYBR Green RT-PCR Kit (Taq Universal SYBR Green Supermix, Vazyme) according to the manufacturer's instructions. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized by the 0 h treatment under each condition. The maize GAPDH gene (Zm00001d049641) was employed as an internal control. Three biological replicates were used for gene expression analysis. The primers used in this study are listed in Supplemental Table 2.

**Results**

**Genome-wide scanning of ZmHSP20s**

A total of 55 ACD-containing proteins were found by BLAST and these proteins were taken as candidate ZmHSP20 proteins. We downloaded these 55 protein sequences from MaizeGDB (https://www.maizegdb.org/). For genes with multiple transcripts, all of the protein sequences corresponding to the various transcripts were downloaded and compared; the most intact one was kept at the end. Three proteins were excluded after the Pfam program's conserved domain analysis because they lacked significant HSP20 domains. And then, a maximum likelihood phylogenetic tree was calculated by MEGA6 based on these 52 proteins together with other ACD-containing proteins retrieved from 17 other higher plants [17]. Consequently, 33 nonredundant ZmHSP20s, including 7 reported ones [26], were established by their close distance to known HSP20s within the clusters of the phylogenetic tree (Supplemental Fig. 1). The predicted molecular weights [34] of these 33 ZmHSP20s were between 14.0 kDa (ZmHSP14) and 27.4 kDa (ZmHSP27.4), along with their amino acid numbers in a range from 123 to 252. Their predicted isoelectric (PI) points varied from 4.95 (ZmHSP21.9) to 8.66 (ZmHSP17.4B). All the information about gene name, sequence ID, protein size, molecular weight and PI was listed in Table 1.

**Gene organization, location and duplication**

The structure analysis of the ZmHSP20 genes revealed that 60.61% (20/33) of those genes are intron-less. Other genes contain one intron except for ZmHSP17.4B which contains two introns (Supplemental Fig. 2). Their schematic structures clearly showed that they shared a great deal in common with their corresponding members in other species [36, 41].

The amino acid sequences of 33 ZmHSP20s, 23 OsHSP20s [36] and 19 AtHSP20s [41] were used to reconstruct an unrooted phylogenetic tree (Fig. 1). The ZmHSP20s were divided into 14 different subfamilies according to the classifications of HSP20s in rice and Arabidopsis. More than half of the ZmHSP20s belong to the C, C and C subfamilies, of which 8 are in the CI subfamily, 5 in the C subfamily, and 4 in the C subfamily. In the CIX, CVIII, CX, MII, CXI, CVI, and PX subfamilies, only one ZmHSP20 gene can be found, respectively. The remaining 9 ZmHSP20s were divided into 4 different subfamilies: 3 in the ER subfamily, 2 in the C subfamily, 2 in the MI subfamily and 2 in the P subfamily.
The 33 ZmHSP20s were distributed across 10 chromosomes in the maize genome. However, the numbers on each chromosome varied widely, from six ZmHSP20 genes on both chromosome 3 and 9 to only one ZmHSP20 on chromosome 10 (Fig. 2). When compared with the previously reported heat resistance QTLs, we found that ten members locate within the hrQTL regions, indicating a potential linkage between these ZmHSP20s and heat resistance.

It has been reported that the maize genome experienced a round of genome duplication approximately 12 million years ago [42]. Consistent with the genome duplication event, 7 duplicated ZmHSP20 gene pairs were identified within a chromosome (Fig. 2 and supplemental table 3). There is a pair of homologs on chromosome 3, which are ZmHSP17.2B and ZmHSP14. Also, three homologs, ZmHSP17A, ZmHSP17B and ZmHSP17.4C, were located on chromosome 9 and the amino acid sequences of ZmHSP17A and ZmHSP17B are completely identical. Likewise, among the three ZmHSP20 genes on chromosome 1 (ZmHSP17.8B, ZmHSP17.8A and ZmHSP17.9B), there was only one amino acid variation between ZmHSP17.8B and ZmHSP17.8A. These results suggested that additional duplication events may have occurred on the two sites. Apart from those homologs within a chromosome, the remaining 16 pairs of duplicated genes were all distributed among various chromosomes (Fig. 2). For instance, ZmHSP17.1 on chromosome 3 was paired with ZmHSP17.3 on chromosome 8. Meanwhile, the counterpart of ZmHSP21.9, which is located on chromosome 6, is on chromosome 8. ZmHSP18 on chromosome 9 matches with ZmHSP17.9B, ZmHSP17.8B and ZmHSP17.8A on chromosome 1.

Some quantitative trait loci (QTLs) have been identified which are related to heat stress tolerance [10, 51–53]. The QTL intervals were mapped to the genome to examine the positional relationship between the QTLs and the ZmHSP20 genes. The results showed that 5 QTL intervals related to 7 different heat-related traits were found to be co-located with 11 ZmHSP20 genes (Fig. 2 and Table 2). Among all the duplicated genes, at least seven members are associated with the QTL intervals. These ZmHSP20 genes may be copied and then have new functions in protecting plants from heat stress.

**Cis-elements analysis in the ZmHSP20 promoters**

*Cis*-elements in the promoter regions are responsible for gene regulation, which plays an important role in responses to any exogenous environment, like biotic or abiotic stresses. The examination of each ZmHSP20’s stress sensitive elements will therefore clarify the functions of each protein in the stress response. Since many phytohormones have been confirmed in the balance between growth and stress response, we mainly focused on two groups of cis-elements, including phytohormone responsiveness and stress responsiveness (Fig. 3 and Supplemental Table 4). In total, six kinds of phytohormone responsiveness elements were found. They were abscisic acid responsiveness (ABRE), MeJA-responsiveness (CGTCA-motif, TGACG-motif), ethylene-responsiveness (ERE), auxin responsiveness (AuxRR-core and TGA-element), gibberellin-responsiveness (GARE-motif, P-box), salicylic acid responsiveness (TCA-element). It is noteworthy that the majority of them are MeJA and abscisic acid responsiveness elements, which have been proved in stress responses ([43, 44]).
The stress-responsive cis-regulatory element, ARE, DRE (DRE core), GC-motif, LTR, MBS, STRE, TC-rich repeats, W box, WRE3, WUN-motif, box S, and HSE and were found in 15, 25, 10, 12, 18, 30, 5, 11, 19, 4, and 14 promoters of ZmHSP20s, respectively (Fig. 3). They were primarily responsible for anaerobic stress, wound responsiveness, heat stress, and other abiotic stress. These results suggest that complex regulatory networks might be involved in the transcriptional regulation of ZmHSP20 genes. For the HSE-containing promoters, seven of them are located in the hrQTL regions, which accounts for 87.5% of all the hrQTL-conjugated ZmHSP20s.

**Expression patterns of ZmHSP20 genes under normal growth conditions**

To further investigate the possible functions of each ZmHSP20 gene, the expression levels of 33 ZmHSP20 genes derived from RNA-Seq data of different tissues were obtained and a heatmap was constructed using FPKM values and normalized by \( \log_2^{\text{FPKM}} \) (Fig. 4 and Supplemental Fig. 3).

ZmHSP20 genes within four clusters showed significantly divergent expression patterns (Fig. 4). ZmHSP20 genes in cluster I were found to be constitutively expressed in almost all tissues as ZmHSP17.8A and ZmHSP17.8B. Those in cluster showed preference in several tissues, like higher expression of ZmHSP19.9 and ZmHSP17.2B in root and silk, respectively. On the contrary, ZmHSP20 genes in cluster showed specific-tissue expression patterns in some tissues, while genes in cluster are relatively silent under normal conditions, which indicates that they may respond to some stimuli. Using FKPM > 100 as a criterion, some ZmHSP20 genes showed absolute higher expression levels (FKPM > 100) in specific tissues, such as ZmHSP17.9A in ear, ovule and silk; ZmHSP17.2B in silk; ZmHSP17.4A in leaf and shoot; ZmHSP19.9 in root and shoot; ZmHSP23.8 in endosperm and roothair; ZmHSP17.9B in roothair and silk; ZmHSP17.8C in endosperm and silk; ZmHSP18.3 in ovule; ZmHSP17A and ZmHSP17B in silk; ZmHSP22.3 in another; ZmHSP18 in roothair. Whereas, ZmHSP17.4B and ZmHSP17.5 were barely expressed in any tissue and they were classified into the Cluster III subfamily. The results indicated that the ZmHSP20 genes showed great variation in expression levels at different tissues or developmental stages.

After normalization by row scales, the relative abundance among genes in each tissue was also visualized by a heatmap, according to which ZmHSP20 genes were classified into three clades, clade , , and (Supplemental Fig. 3). In general, genes in clade may play major roles in silk; genes in clade are more predominant in root hair, followed by silk. The bulk of the clade genes are abundant in other tissues, such as ZmHSP19.9 and ZmHSP21.2 in the root; ZmHSP17.2A, ZmHSP17.4A, ZmHSP21.9, ZmHSP17.5 and ZmHSP16.7 in the shoot; ZmHSP16.7, ZmHSP17.4B, ZmHSP22.3 and ZmHSP16.6 in the anther.

**Expression profiles of ZmHSP20 genes in response to heat stress in leaves**

There is a close relationship between gene induction and function. In order to explore the possible regulation mechanism of ZmHSP20 genes in the heat stress response, the transcript patterns of ZmHSP20 genes with continuously increased heat-stress treatments (31°C/21°C, 33°C/23°C,
35°C/25°C and 37°C/27°C, Day/night) in V4 or V5 leaves from W22 were compared. Apart from three barely detected ZmHSP20 genes [39], ZmHSP17.4B, ZmHSP22.8A, ZmHSP17.4C, the remaining 30 ZmHSP20 genes were investigated. On a whole, a great majority of members showed up-regulated expression patterns as the temperature increased from 31°C to 35°C, whereas a slight reduction was found when the temperature altered from 35°C to 37°C. In addition, an overwhelming portion of ZmHSP20 genes were expressed higher in V5 leaves than in V4 leaves (Fig. 5A).

The optimal temperature range for maize growth is 25–33°C during the day and 17–23°C at night [45]. Since little difference was found between 31°C and 33°C in V4 leaves, we mainly focused on the changes of V4-35°C vs. V4-31°C, V4-37°C vs. V4-31°C, V5-35°C vs. V5-31°C and V5-37°C vs. V5-31°C. Fourteen ZmHSP20 genes were upregulated consistently in the four treatments (Fig. 5B). In addition, some genes were induced in V5-35°C leaves but not in V4-35°C leaves, such as ZmHSP17.3, ZmHSP22.8B, ZmHSP16.6 and ZmHSP17.5. Unexpectedly, the gene, ZmHSP19.9, was down-regulated at V5-35°C compared with V5-31°C.

Intriguingly, four CI subfamily genes (ZmHSP18, ZmHSP17.1, ZmHSP17.9B and ZmHSP14), one CII subfamily gene (ZmHSP17.8C), one P subfamily gene (ZmHSP26.4), and two ER subfamily genes (ZmHSP23.4, ZmHSP20.2) were drastically enhanced (fold change ≥ 50) under high temperature conditions in V5 leaves at 35°C.

Expression profiles of ZmHSP20 genes in response to heat stress in kernels

To further explore ZmHSP20 genes responding to high-temperature stress in kernels, the transcriptomes were studied at 10 days and 25 days after pollination in the waxy maize kernels [40]. Compared with the gene expression levels in B73 seed under normal condition (Fig. 4), a similar expression pattern was found in normal day/night (NN) kernels of waxy maize (Fig. 5C). The number and pattern of changed gene expression were basically constant in hot day/normal night (DH) and hot day/hot night (DNH). In brief, 7 ZmHSP20 genes were highly induced in the two treatments (Fig. 5D). Among them, three genes (ZmHSP17.5, ZmHSP17.1 and ZmHSP14) were extremely induced (fold change ≥ 50). However, few gene were induced in normal day/hot night (NH).

The differential expression patterns in leaf and kernel indicated that there were different response and regulatory mechanisms of the ZmHSP20 family under heat stress conditions. only one P subfamily gene (ZmHSP26.4), one ER subfamily gene (ZmHSP20.2) and three CI subfamily genes (ZmHSP17.2B, ZmHSP17.1 and ZmHSP14) were significant induced in both leaf and kernel heat stress.

Heat stress response of ZmHSP20 genes verified by real-time qRT-PCR

To further verify heat stress-responsive ZmHSP20 genes, real-time RT-PCR analysis was used to assay the expression levels of ten selected genes under both normal temperature and heat stress conditions in V4 leaves (Fig. 6). The dynamic response of these selected genes to the high temperature varied. After heat treatment, HSP16.7 and HSP17.4A showed the same expression pattern as those under normal
conditions, which indicates that they were barely induced by heat (Fig. 6A and 6B). In contrast, eight HSE-containing genes, \( HSP26.4 \), \( HSP23.4 \), \( HSP17.1 \), \( HSP18 \), \( HSP17A \), \( HSP23.8 \), \( HSP14 \) and \( HSP15.8 \) enriched significantly at 3 hours after heat treatment, which suggested that these genes had a robust reaction to the heat (Fig. 6C-J). Besides the transient induction during the first 3 hours, another obvious fluctuation of \( HSP17A \) and \( HSP23.8 \) was observed at 12 hours after heat treatment, which indicates that their underlying mechanism involved in heat response may varied from the others. Although no HSE was predicted in \( HSP14 \) and \( HSP15.8 \), they also raised in the first beginning, which suggests that they may be induced indirectly and function in the cascades of heat signals.

Co-expression interaction networks between \( ZmHSP20s \) and \( ZmHSFs \) under high temperature

The heat-responsive \( ZmHSP20s \) genes can be directly or indirectly triggered by upstream \( HSFs \) \([46, 47]\), which have been proved as key factors in heat resistance pathway. Up to date, 25 \( ZmHSF \) genes have been identified \([29, 38]\), thus we analyzed their association patterns with \( ZmHSP20s \) in different samples and identified highly coordinated genes by the Weighted Gene Co-Expression Network Analysis. A total of five modules containing different \( ZmHSP20s \) and \( ZmHSFs \) genes were identified (Fig. 7). For the largest module, there is a strong co-expression relationship between \( ZmHSF03 \) and 11 \( ZmHSP20s \), including \( ZmHSP18, 14, 26.4, 16.7, 17.9A, 18.3, 20.2, 23.4, 17.2B, 17.8B \) and \( 23.8, \) six of which (\( ZmHSP18, 26.4, 17.9A, 23.4, 17.8B \) and \( 23.8) \) have HSE in their promoters and five of which (\( ZmHSP18, 14, 26.4, 23.4, \) and \( 23.8) \) been verified in heat response by real-time qRT-PCR (Fig. 7A). Besides, other 4 small modules were found in the co-expression network, including \( ZmHSP18, 26.4, 17.2B \) and \( ZmHSP15.8 \) with \( ZmHSF01; ZmHSP21.9, ZmHSP19.9 \) and \( ZmHSP16.6 \) with \( ZmHSF17 \) and \( ZmHSF08 \), \( ZmHSP22.8A \) and \( ZmHSP17.1 \) with \( ZmHSF10 \) and \( ZmHSF19 \), \( ZmHSP22.8B \) and \( ZmHSP17A \) with \( ZmHSF21, ZmHSF07 \) and \( ZmHSF09 \). Among all the \( ZmHSP20s \), only \( ZmHSP17.8B \) were repeatedly identified in two modules.

Discussion

With the rise in average global temperature, maize is experiencing an increase in the frequency of high-temperature events. Lots of studies have revealed that \( HSP20s \) inhibit the irreversible aggregation of denaturing proteins as molecular chaperones, thus enhancing the stress tolerance of many plants \([16, 17]\). In the current study, we used expression pattern analysis to find possible \( ZmHSP20 \) genes in relation to heat stress responses.

In this work, 33 putative \( ZmHSP20s \), 19 \( AtHSP20s \), and 23 \( OsHSP20s \) were grouped into 14 subfamilies, and \( ZmHSP20s \) distributed in each family. Likewise, the schematic structures of \( ZmHSP20s \) share similar exon–intron structures with other species. These results indicate that the evolution of \( HSP20s \) in gramineous species was conserved. However, the numbers of \( HSP20s \) differ among species. There are 13 \( HSP20s \) in barley \([48]\), 19 in Arabidopsis \([41]\), 23 in rice \([36]\), 35 in pepper \([49]\), 42 in tomato \([50]\), 44 in watermelon \([51]\), 63 in switchgrass \([52]\), 45 in cucumber \([51]\), 47 in soybean \([53]\), 48 in berry \([54]\), 48 in potato \([37]\), 94 in cotton \([55]\) and 109 in wheat \([56]\). The tremendous diversification of \( HSP20s \) in plants may reflect in plant adaptations to stresses \([17]\) and may be due to the chromosome ploidy and genome
size. As it is well known, maize genomes have undergone duplication and ZmHSP20 gene family may also be expanded and diversified along with the genome duplication, which may contribute to widely functional divergence [57]. In our study, 20 ZmHSP20 genes (61%) were demonstrated to be involved in gene duplication, which suggests that duplications contributed significantly to the amplification of the ZmHSP20 gene family. Notably, genes doubled inside chromosomes, presumably denoting their importance and acting as a selective advantage for plants under stress [15] or being preferentially maintained [65].

Although the HSP20 subfamilies are various among the species, we found that most HSP20 genes clustered into the CI, CII and CIII subfamilies. However, the functions of the CI and CII subfamily genes are inconsistent in different species. CI HSP20s, for example, have been reported to be thermo-protective, whereas CII HSP20s do not [66]. Our research also discovered that the ZmHSP20s in the CI subfamily, but not those in the CII subfamily, are highly expressed under heat stress. The CI subfamily HSP20s may be more efficient in maintaining their oligomeric structure at higher temperatures than CI proteins [58].

Through the expression analysis of these ZmHSP20s, we found that these genes were differentially expressed in various tissues and there was no single expression pattern for the ZmHSP20 genes based on RNA-seq analysis, even the members of the same ZmHSP20 subfamily or the genes encoding for similar HSP20s. The spatio-temporal expression analysis of the identified ZmHSP20s revealed differential expression with potential roles in maize growth and development (Fig. 4). Two genes, ZmHSP17.8A and ZmHSP17.8B, belonging to the CI subfamily were expressed constitutively in almost all the tissues, implying that these genes might have specific housekeeping function under normal growth conditions in maize. Twelve ZmHSP20 genes showed selectively high expression level in some of the tissue which indicates their special functions in these tissues. Furthermore, the responses of each gene to heat stress varied across tissues. For example, only ZmHSP17.8C showed high expression after transient heat stress during the tetrad stage of pollen development [59]. In contrast, our data shows that more than half of the ZmHSP20 genes are upregulated in response to high temperatures in the leaf or kernel, suggesting that these genes may be crucial for maize resistance to heat stress in these two tissues. Many ZmHSP20 genes are also induced by 33°C, day/23°C, night treatment at V5 leaves, which indicates that heat stress impacts may occur after a long-term treatment even at 33°C. It is evident that seed set could be reduced when corn plants were exposed to high night temperature during flowering [60]. But the effects are less in the NH treatment than in the DH and DNH treatments [40], which was somehow proved by the results showing that only two ZmHSP20 genes were highly expressed during stress imposed at night. According to the previous results, 5 ZmHSP20 genes were induced both in leaf and kernel under heat stress. ZmHSP26.4, a P subfamily gene, was up-regulated and reached a peak when it responded to a slow rise in temperature to 37°C in maize [39] and differential upregulation proteomic data also demonstrated its crucial role (Supplemental Fig. 4). ZmHSP17.2B, one of the CI subfamily genes, accumulates during environmentally high temperatures [27] and confers heat tolerance in transgenic tobacco [26]. Moreover, their homologs, three CI subfamily genes (HSP17.4CI, HSP17.6ACI and HSP18.1CI), have been examined to function in acquired thermotolerance by phenotype analysis of single gene knockouts in Arabidopsis [61]. It is worth believing that the 5 ZmHSP20 genes have non-redundant
functions in heat stress response. In our RT-PCR results, eight ZmHSP20 genes (ZmHSP26.4, 23.4, 17A, 23.8, 14, 15.8, 18 and 17.1) were found to be involved in heat response in V4 leaves, suggesting their potential roles in heat resistance in maize.

By constructing a co-expression network, five modules containing different ZmHSP20 and ZmHSF genes were identified, which indicates different interactions between ZmHSP20 and ZmHSF family in maize. We found that most of ZmHSP20 genes are enhanced, which can avoid or reduce heat damage to plants [19], but there were only a few HSF genes with medium or up-regulated expression levels in the leaves of seedlings in maize at V4 and V5 stage leaves and in the kernel, respectively (Supply Fig. 3). As mentioned previously, an increasing number of articles report that HSFs can regulate the expression of a group of HSP20s by recognizing heat stress elements (HSE, 5’-GAANNTTC-3’) in their promoters upstream of the TATA box [16, 62]. A total of 531 cis elements were identified and they were divided into phytohormone responsiveness and stress responsiveness. We are delighted that HSEs were found in almost half of the ZmHSP20 gene promoters, suggesting their potential heat-stress response under high temperature conditions. Although ZmHSP17.2B has been demonstrated to give heat tolerance in transgenic tobacco, no HSE was found in it, implying that some ZmHSP20s may function indirectly downstream of HSFs.

HSP20s play important roles in plant immunity [77] and other stress responses [16, 56, 63, 78]. The elevation of ZmHSP20 gene expression with heat treatment indicates their functions in heat stress tolerance. Nevertheless, there is still a shortage of data from null mutants or other genetic materials in maize. Furthermore, alternative splicing (AS) of HSPs has been linked to heat stress in vegetative tissues [72–76], which needs to be confirmed in maize. There are still many unsolved issues about the function of ZmHSP20s and more studies are required to uncover their roles in heat response.

Conclusion

In this study, 33 ZmHSP20 genes have been identified and divided into 14 subfamilies. Ten ZmHSP20 gene pairs may occur as a result of the genome duplication. Moreover, fourteen ZmHSP20 genes were predicted to have HSE in their promoters. Based on tissue-specific expression patterns, the ZmHSP20 genes show diversity, indicating their multiple roles in plant growth, development or stress resistance. By analyzing the expression of ZmHSPs in response to heat stress in leaves and kernels, candidate heat-responsive genes were found, some of which were further proved by qRT-PCR. Finally, the co-expression networks of ZmHSP20 and HSF genes were analyzed and five modules were identified. Our results lay the foundation for understanding the complex mechanisms of the ZmHSP20 genes involved in heat stress.

Declarations

Ethics approval and consent to participate

The genomic data were obtained from MaizeGDB databases (https://www.maizegdb.org/). All the procedures were followed in accordance with the relevant guidelines under the Ethics approval and
consent to participate heading. The Maize (*Zea mays*) W22 were obtained from our lab.

**Consent for publication**

All the participants confirmed the consent for publication.

**Availability of data and materials**

The accession numbers of data analysed during the current study are included in the “supplemental table 1”.

**Conflict of Interests**

There are no competing interests in the manuscript.

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**Author contributions**

M.X. and S.H.C. performed most of data analysis. Y.W.Y., L.Y.Z. and J.M.C. participated in the experiments. M.X. and S.H.C. wrote the main manuscript text. All authors reviewed the manuscript.

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**References**

1. Uncategorized, References.


Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures
Figure 1

Phylogenetic analysis of Hsp20 proteins. The unrooted neighbor-joining phylogenetic tree was conducted with maize (Zm), rice (Os) and *Arabidopsis* (At) Hsp20 proteins. Bootstrap supports from 1000 replicates are indicated at each branch. Displaying of different background colors to distinguish the clade.
Figure 2

Chromosomal location, gene duplication of ZmHsp20 families and co-located QTL. The ring represents chromosome ideograms. The approximate distribution of each ZmHsp20 gene is marked with a short black line on the circle. Possible duplicated genes were marked with same color and are connected by different lines. Genes with black color were not involved in duplication. The ring marked with red color represents QTL intervals.
Figure 3.

Investigation of cis-acting element numbers in ZmHsp20 genes. (A) The different colors and numbers of the grid indicated the numbers of different promoter elements in these ZmHsp20 genes. (B) The number of different types of cis-acting. Blue represents for non-heat stress cis-regulation elements. Orange represents for heat stress cis-regulation elements.
Figure 4

Analysis of expression profile of maize Hsp20 genes. The data are normalized by “Row scale”. The color scale signal values are shown on the right. The different tissues and/or organs are noted on the bottom of each lane. Cluster dendrograms are shown on the left and bottom, respectively.
Expression profiles of ZmHsp20 genes in response to heat stress. (A) Heatmap of ZmHsp20 expression in V4 or V5 leaves from W22 with continuous heat stress treatment (31°C/21°C, 33°C/23°C, 35°C/25°C, and 37°C/27°C). (B) Heatmap of ZmHsp20 expression in kernels waxy maize 10 day and 25 day after pollination with heat stress treatment. These treatments were applied from 1 DAP to 15 DAP (NN, normal day/normal night, 28/20°C; DH, hot day/normal night, 35/20°C; NH, normal...
day/hot night, 28/27°C; and DNH, hot day/hot night, 35/27°C). Cluster dendrograms are shown in the center. The color scale signal values are shown on the right.

**Figure 6.**

### Figure 6

RT-qPCR analysis of gene expression in W22 plants at room temperature (RT) and high temperature (HT). RNA was extracted from ten days of seedlings after treated 0, 3, 6, 9 and 12 hours. Gene expression patterns were assessed for \( \text{HSP16.7} (\text{Zm00001d017813}), \text{HSP17.4A} (\text{Zm00001d039941}), \text{HSP26.4} (\text{Zm00001d028408}), \text{HSP23.4} (\text{Zm00001d025508}), \text{HSP17.1} (\text{Zm00001d039933}), \text{HSP18A} (\text{Zm00001d047841}), \text{HSP17A} (\text{Zm00001d047548}), \text{HSP23.8} (\text{Zm00001d052194}), \text{HSP14} (\text{Zm00001d039935}) \) and \( \text{HSP15.8} (\text{Zm00001d044728}). \) Transcript levels were normalized to \( \text{ZmGAPDH} (\text{Zm00001d049641}). \) Relative levels to seedlings treated 0 hours are presented. Data are mean ± SD.
Figure 7.

Co-expression interaction networks between ZmHsp20s and ZmHsfs

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• Tables.xlsx
• supplementaltable.xlsx
• supplyfigures.pptx