Int.J.Curr.Microbiol.App.Sci (2019) *8*(6): 347-358

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 06 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.806.039

Expression Profiling of Heat Shock Protein Genes in Two Contrasting Maize Inbred Lines

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Keywords

Heat shock proteins, Maize, *In-silico* analysis, Real-time PCR, Heat tolerance

Article Info

Accepted:
04 May 2019
Available Online:
10 June 2019

A B S T R A C T

High temperature stress is one of the most detrimental abiotic stresses which adversely affect productivity of maize (*Zea mays* L.) in tropics and subtropics. Plants respond to high temperature stress by regulating expression of an array of genes, heat shock proteins (HSPs) being one of them. Owing to highly differential expression of HSPs in various crop species under high temperature stress, these could be considered as key stress responsive genes. Since HSPs gene family contain various members, identification of specific gene(s) playing crucial role in heat stress tolerance could be beneficial for developing stress resilient genotypes. Here we report *in-silico* characterization of five HSP genes and their expression analysis in two contrasting maize inbred lines i.e. LM17 (heat tolerant) and HKI1015WG8 (heat susceptible) subjected to high temperature stress at seedling stage. The five maize specific HSP genes, *viz.*, *ZmHsp26, ZmHsp60, ZmHsp70, ZmHsp82* and *ZmHsp101* exhibited distinctive expression pattern in response to heat stress. Higher up-regulation of Zm*Hsp70* was found throughout the stress exposure in the heat tolerant line as compared to the susceptible line. Sharp up-regulation and rapid decline in expression of *ZmHsp82* in LM17 than HKI1015WG8 after 12 hours heat stress exposure suggested its possible role in plant acclimatization to heat-stress conditions. Further, higher up-regulation of *ZmHsp101* even after removal of stress (recovery for 24 hrs) indicated its possible role in recovering plant from adverse effects of heat stress. The study opens up scope for investigation through transgenic (RNAi and/or over-expression) approach to further characterize and elucidate precise role of Zm*Hsp101*, Zm*Hsp82* and Zm*Hsp70* in heat stress tolerance in maize.

Introduction

A plethora of environmental factors referred to as abiotic stresses, *viz*., drought, heat, cold, flooding, salinity, *etc*. exert a negative impact on growth and development of crop plants, leading to significant reduction in grain yield (Tuteja and Gill, 2013). With the ever-changing climatic conditions, the impact of these abiotic stresses is expected to enhance in near future. The constantly rising ambient temperature (heat stress) is one of the most important abiotic stresses that severely affect the plant growth, development, metabolism,

grain quality and yield in major cereal/food crops, hence becomes most remarkable global concern (Wilhelm *et al.,* 1999; Gooding *et al.,* 2003; Jagadish *et al.,* 2007; Shi *et al.,* 2017). In general, a transient increase in temperature, usually 10-15°C above the optimum temperature, is considered as heat stress (Wahid *et al.,* 2007). The annual mean air temperature of nearly 23% of the land on the earth is estimated above 40°C (Leone *et al.,* 2003). It is predicted that the global temperature will increase by 1.7–3.8°C by the end of twenty-first century (Wigley and Raper, 1992; IPCC, 2014). The climate modeling studies have anticipated the increase in day and night temperature in the future and hence expected significant reduction in the global food production (Lobell *et al.,* 2011; Cairns *et al.,* 2012). For instance, in 1980 and 1988, US heat waves resulted in reduction in agricultural production with estimated loss of about 55 and 71 billion dollars, respectively (Mittler *et al.,* 2012). Over the past three decades (1980–2008), heat stress has caused a decrease of 3.8% and 5.5% in the global yields of maize and wheat, respectively (Lobell *et al.,* 2011). Therefore, sustaining high yield under heat stress is an utmost challenge in front of scientific community.

Heat stress mainly results in improper folding of protein which in turn leads to protein dysfunction and aggregation (Singh and Shono, 2005). The misfolding of proteins/enzymes adversely affects plant overall growth and development. To cope up with heat stress, crop plants alter their metabolism in many ways such as, by activating signalling cascades and regulatory proteins like heat shock transcriptional factors (HSFs), activating/modifying antioxidant defence system to maintain cellular homeostasis, synthesizing and accumulating compatible solutes (polyamines, sugars, proline, betains, etc) which assist in osmotic adjustment (Wahid *et al.,* 2007; Bokszczanin and Fragkostefanakis, 2013; Hasanuzzaman *et al.,* 2013). At the molecular level, heat stress causes alterations in expression of an array of genes encoding for osmoprotectants, ion transporters, detoxifying enzymes, transcription factors and heat shock proteins (HSPs) (Wahid *et al.,* 2007; Qin *et al.,* 2008; Sarkar *et al.,* 2014; Dutra *et al.,* 2015; Frey *et al.,* 2015, Yadava *et al.,* 2015). These adaptive changes in plants in response to heat stress in turn help in minimizing the adverse effect of stress on plants by maintaining the near-optimal conditions for plant growth and development (Yadava *et al.,* 2016). Among the heat stress responsive genes, HSPs are the most frequently and quantitatively observed genes under high temperature stress condition in various crop species (reviewed by Kotak *et al.,* 2007; Reddy *et al.,* 2016; Mishra *et al.,* 2018). HSPs are molecular chaperones which are involved in protein quality control, mainly by assisting proper re-folding of misfolded proteins during stress condition which in turn prevents protein aggregation hence play a crucial role in conferring heat and other abiotic stress tolerance in crops (Reddy *et al.,* 2016; Singh *et al.,* 2016; Mishra *et al.,* 2018). Based on their molecular weight, HSPs have been classified into five sub-classes: HSP100, HSP90, HSP70, HSP60 and small sHSPs or low molecular weight HSPs (Wang *et al.,* 2004, Singh and Shono, 2005). In addition to stress tolerance, members of HSP families also have their role in normal growth and development in plants.

Maize (*Zea mays* L.), is the second most widely grown crop in the world. In comparison to other grain crops, demand for maize would rapidly increase because of its myriad uses in various industrial products and processes and requirement for animal feed. By 2030, global maize production has to increase significantly from the current levels and that too with limited resources, shrinking arable land and a changing climate which

anticipate increasing temperature. Maize crop is highly sensitive to drought and high temperature stress, particularly at reproductive phase, *viz.,* flowering and early grain filling stages (Dass *et al.,* 2010; Cairns *et al.,* 2012). Most of the tropical maize cultivating areas in South Asia is prone to heat stress (Prasanna, 2011). The consequences of heat stress in maize are tassel blast, leaf firing, enhanced leaf senescence and reduced photosynthesis (Crafts-Brander and Salvucci, 2002; Hussain *et al.,* 2006; Chen *et al.,* 2010. Further, high temperature during reproductive phase reduces pollen viability (Schoper *et al.,* 1987; Singh and Shono, 2003), silk receptivity and leads to reduced number of kernel per ear which in turn results in poor seed set and reduced grain yield (Johnson, 2000, Singh *et al.,* 2017). It has been shown that each degree day spent above 30°C reduced the final maize yield by 1% and 1.7 % under favorable growing and drought stress conditions respectively (Lobell *et al.,* 2011).

In order to curtail the yield losses caused by high temperature stress in maize and to develop thermo tolerant genotypes, a better understanding of heat stress responsive key genes and master regulators such as transcription factors, playing pivotal role in tolerance mechanism is needed.

Owing to their highly altered expression during heat stress, HSPs are considered as potential candidates to address the issue of heat stress. However, not much information is available regarding the transcript profiling of HSP genes in tropical maize under high temperature stress. Therefore, in the present study, expression analysis of five HSP genes in two contrasting maize inbred lines i.e. LM17 (heat tolerant) and HKI1015WG8 (heat susceptible) subjected to high temperature stress during seedling stage was performed. The expression profiling revealed distinctive expression patterns for HSPs in response to heat stress.

Materials and Methods

Plant material and growth conditions

Maize inbred lines, HKI1015WG8 and LM17 which have been identified as heat susceptible and heat tolerant, respectively, were used in the present study (Debnath *et al.,* 2016, Singh *et al.,* 2017). The two inbred lines were grown under controlled condition in greenhouse at ICAR-IIMR, Pusa Campus, New Delhi. The seedlings were raised in small thermocol cups (7 cm top diameter) filled with a mixture of vermiculite, coco peat and soil (1:1:2). One set of two weeks old seedlings were exposed to heat stress (42°C) for different intervals of time (3, 6, 9 and 12 hours) while other set was kept at 25°C in plant growth chambers. The leaf samples from both the sets were collected at each time-point (3, 6, 9, 12 hours) and after recovery for 24 hrs (24 hrs recovery by growing at 25°C after 12 hrs heat exposure). The collected leaf samples were immediately frozen in liquid nitrogen and stored at -80 °C until used for total RNA extraction.

RNA isolation

Total RNA was isolated from the leaf samples using Ambion Pure Link™ Plant RNA kit (Invitrogen) according to the manufacturer's protocol. The quality and concentration of the isolated RNA was assessed by Nano Drop spectrophotometer (Nano 200) and the integrity of the RNA was also verified on gel electrophoresis. The RNA was stored at -80 ^{o}C.

Quantitative real-time PCR (qRT-PCR) analysis

First strand cDNA was synthesized using 1 µg of total RNA using Affinity Script qRT-PCR

cDNA synthesis kit (Agilent Technologies, USA) according to the manufacturer's instructions. Maize Hsp gene sequences were obtained from NCBI and gene specific qRT-PCR primers (Table 1) were designed using Primer Quest software (http://eu.idtdna.com).

The qRT-PCR was performed in triplicate using the Brilliant-III Ultra-fast SYBR Green master mix in AriaMx real-time PCR (Agilent Technologies, USA) detection system. The *Actin* gene was used as reference gene to normalize the expression values. The expression level in leaf tissue from un-stressed/control plants was selected as calibrator.

The fold change value ($\log_2$ scale) for mRNA expression level compared/relative to expression in control plants (grown at 25°C) was calculated using comparative $\Delta\Delta C_t$ method (Livak *et al.*, 2001). In this method the fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_{t\ (\text{gene of interest})} - C_{t\ (\text{actin})})_{\text{test}} - (C_{t\ (\text{gene of interest})} - C_{t\ (\text{actin})})_{\text{control/calibrator}}$.

In-silico analysis of *Hsp* genes

The theoretical pI (isoelectric point) and Mw (molecular weight) of HSP proteins were predicted by Expasy–Computer pI/Mw tool (http://www.expasy.org). The WoLF PSORT program (https://wolfpsort.hgc.jp/) was used to predict the sub-cellular localization of *Zm*HSPs.

The amino acid sequences were further used for predicting the domain architecture using Inter Pro (http://www.ebi.ac.uk/interpro) and Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Further, signature sequence unique to any protein family was identified using PROSITE tool (https://prosite.expasy.org/cgi-bin/prosite/PSScan.cgi).

Results and Discussion

Identification and *in-silico* characterization of *ZmHsp* genes

Five heat shock protein encoding genes belonging to different families were retrieved from the maize genome database (https://www.maizegdb.org/gene_center/gene) and their respective amino acid sequences were retrieved from NCBI. The amino acid sequences were analyzed by different bioinformatics software used to predict molecular weight, isoelectric point (pI) and sub-cellular localization, enlisted in Table 2. On the basis of molecular weight, these *Hsps* were grouped into different families (Table 2).

The unique signature sequence prediction by PROSITE tool confirmed the respective family of these five *Hsp* genes. Protein domain analysis predicted the domain architecture of five HSP proteins as enlisted in Table 3. The low complexity regions (LCRs), repetitive sequences or sequences enriched in one/few aminoacids, were predicted in all five HSPs (Figure 1 and Table 3). These LCRs have been reported in extreme abundance in eukaryotic proteins (Golding 1999; Marcotte *et al.*, 1999). The LCRs have shown to contribute to variability/diversity across protein families and involved in protein–protein and protein–nucleic acid interactions modulation (Xiao and Jeang 1998; Shen *et al.*, 2004). In *ZmHsp82* and *ZmHsp101*, adenosine triphosphate (ATP) binding domain which binds to and hydrolyzes ATP, *viz.*, HATPase_c and AAA, respectively were predicted (Figure 1 and Table 3). In general, HSPs derive energy from ATP hydrolysis for molecular chaperone activities (remodeling or disaggregation of protein aggregates) (Burton and Baker, 2005; reviewed by Sable and Agarwal, 2018).

Expression analysis of *ZmHsp* genes at seedling stage

The qRT-PCR based expression analysis of identified *ZmHsp* genes was performed in contrasting maize inbred lines at different time-points after heat stress exposure (3, 6, 9 and 12 hours) and after recovery. The increased expression / up-regulation of all five *Hsps* were observed at various time intervals after heat stress treatment in both the lines with respect to their respective control (non-stressed) plants, which suggested that heat stress induced the expression of all 5 *Hsp* genes investigated in this study (Figure 2). However, the level of up-regulation varied at different time-points in the contrasting lines. Out of five *Hsps*, up-regulation of two *Hsps* (*ZmHsp26* and *ZmHsp60*) was higher in susceptible genotype compared to the tolerant one. The expression of *ZmHsp26* increased rapidly in susceptible genotype after 6 hours of heat exposure but lacked any specific pattern. Expression of *ZmHsp60* was higher in susceptible genotype at all the time-points than in the tolerant one. The greater up-regulation in susceptible line suggested that these two *Hsps* genes might be playing role in normal cellular growth/development/ maintenance and not be crucial for imparting heat stress tolerance in tropical maize. The level of up-regulation for remaining three *Hsps* (*ZmHsp70, ZmHsp82* and *ZmHsp101*) was significantly higher in tolerant line compared to the susceptible line (Figure 2). Previously, it has been shown that *Hsp100* and *Hsp90* work in association with *Hsp70* and constitute chaperone complexes, which in turn evaded protein aggregation under stress condition (Reddy *et al.,* 2016; Mishra *et al.*, 2018). Further, *Hsp90* and *Hsp70* and their co-chaperones (sHSPs) had shown to interact with various components of signalling molecules like hormone receptors, tyrosine/ threonine/ serine-kinase receptors and resulted into acquired tolerance (Wang *et al.*, 2004). Therefore, these three *Hsps* (*ZmHsp70, ZmHsp82* and *ZmHsp101*) might be crucial for imparting thermotolerance and sufficient up-regulation of them required for the same. In our study, higher up-regulation of these three *Hsps* was observed in tolerant genotype than in the susceptible genotype.

The higher up-regulation of *ZmHsp82* (HSP90 family member) and *ZmHsp101* (HSP100 family member) was detected in LM17 (heat tolerant) than HKI1015WG8 (heat susceptible) after 12 hours stress treatment and after recovery, respectively. In case of *ZmHsp82*, rapid and very sharp up-regulation was observed after 12 hours of heat exposure while very less transcript level was found after recovery. The up-regulation in tolerant line was almost twice than up-regulation in susceptible line after 12 hours of heat stress treatment. This transient induction in expression suggested that higher expression of *ZmHsp82* was required at much later time-point during heat stress exposure to acclimatize plants to heat stress and basal level or very minimal expression is required under normal conditions. In *Arabidopsis*, HSP90 has been shown to regulate the heat shock response that is responsible for heat acclimation (Yamada *et al.,* 2007). HSP90 in association with HSP70, constituted a major part of chaperone complexes and helped in protein folding. Similarly, several other studies had also shown up-regulation of *Hsp90* under high temperature stress (Majoul *et al.*, 2004; Hu *et al.*, 2009; Li *et al.*, 2013).

In case of *ZmHsp101* transcript level started increasing with the onset of high temperature stress in both the lines. However the up-regulation was significantly higher (more than 2.5 fold) in the tolerant line than the susceptible line after 24 hours of recovery. The study suggested that higher expression of *ZmHsp101*which sustained even after stress is removed might play a major role for heat

stress acclimation of the maize plant. Previous studies have shown that disaggregating chaperone, HSP100, promoted protein disaggregation under heat stress condition hence required for both basal and acquired thermotolerance (Parsell *et al.,* 1994; Glover and Lindquist, 1998; Quietsch *et al.,* 2000: reviewed by Mittler *et al.,* 2012). It has been reported essential for acquisition of high temperature tolerance in yeast (known as *Hsp104*), and plants (known as *Hsp101*) such as soybean, *Arabidopsis*, tobacco and wheat (Sanchez and Lindquist, 1990; Lee *et al.,* 1994; Schirmer *et al.,* 1994; Wells *et al.,* 1998; Hong and Vierling, 2000). Further, over expression of *Hsp101* gene in *Arabidopsis* (Quietsch *et al.,* 2000) and rice (Katiyar-Agarwal *et al.,* 2003) exhibited high temperature tolerance in transgenic plants. Our studies also suggested higher expression of Zm*Hsp101* even after stress removal could be responsible for conferring thermotolerance in maize.

The expression level of *ZmHsp70*, was higher in tolerant line than susceptible one subjected to heat stress for 3 to 12 hours. Further, shifting the plants to normal temperature conditions for 24 hours after 12 hours of heat treatment resulted into significant reduction in its expression in the tolerant line only. *Hsp70*, has been reported to promote refolding of denatured proteins once released from the protein aggregates (reviewed by Parsell and Lindquist, 1993; Miernyk, 1999). Over expression of *Hsp70* in *Arabidopsis*, tobacco and rice has been proven useful in imparting thermotolerance by suppressing programmed cell death and preventing fragmentation and degradation of genomic DNA during heat stress (Cho and Choi, 2009: Montero-Barrientos *et al.,* 2010; Qi *et al.,* 2011). Recent studies in rice (Sarkar *et al.,* 2013) and tea plant (Chen *et al.,* 2018) have also shown induced expression of *Hsp70* under heat stress. Higher expression of *Hsp70* in tolerant line in our study showed strong correlation between transcript level and thermotolerance. The three highly expressed *Hsps* (*ZmHsp70, ZmHsp82* and *ZmHsp101)* in LM 17, a heat tolerant maize inbred line, could play a crucial role in conferring heat tolerance by re-folding of misfolded proteins during stress and need to be further investigated more comprehensively.

Table.1 List of primers used for qRT-PCR analysis

S. No.	Gene name	Primer Sequence (5'->3')	Tm [°C]
1	*Hsp101*	F- ACCGCAAGTACGTGGAGAAG	59.4
		R- GTACCTCGCGCATAGCTGTG	61.4
2	*Hsp26*	F- CGACGTACAGGTTAGCCAGA	59.4
		R- GTCCATCGTGTCCAGCATCT	59.4
3	*Hsp82*	F- ACGCTGTCCATCATCGACTC	59.4
		R- GTGGTGACCATGACCCTGTC	61.4
4	*Hsp60*	F- CCTTACCGGAGGAGAGGTAATA	60.3
		R- CTCCAGCGCCATCAAGAATA	57.3
5	*Hsp70*	F- AAGTAAGGAGGAGATCGAGAAGA	58.9
		R- CTGATGGTGTTGCGCATATTG	57.9
6	*Actin*	F- CAATGGCACTGGAATGGT	53.7
		R- ATCTTCAGGCGAAACACG	53.7

Table.2 Characteristics of the five *Zm*HSP proteins in maize

Gene Name	Accession Number	Molecular weight (Dalton)	Isolectric Point (pI)	Family name	*Subcellular Localization
ZmHsp26	NP_001105583.1	26377.94	7.86	sHSP	chlo: 13, nucl: 1
ZmHsp60	NP_001105690.1	60935.09	5.67	HSP60	mito: 12, chlo: 2
ZmHsp70	NP_001148198.1	71138.34	5.05	HSP70	cyto: 9, cysk: 4, chlo: 1
ZmHsp82	NP_001135416.3	81802.65	5.03	HSP90	cyto: 7, E.R.: 3, nucl: 1, plas: 1, vacu: 1, golg: 1
ZmHsp101	NP_001104935.2	101118.68	5.84	HSP100	cyto: 4, nucl: 2, vacu: 2, E.R.: 2, pero: 2, mito: 1, plas: 1

*Chlo: chloroplast, cyto: cytoplasm, ER: endoplasmic reticulum, golg: golgi apparatus, mito: mitochondria, nucl: nucleus, pero: peroxide, plas: plasma membrane, vacu: vacuole, cysk: cytoskeleton

Table.3 Unique signature sequence and domain architecture of the five *Zm*HSP proteins in maize

Gene Name	Predicted unique signature sequence	Amino acid positions of predicted sequence	Protein family to which signature belongs	*Predicted domain
ZmHsp26	sHSP domain	124 - 240	sHSP family	low complexity
ZmHsp60	AAVEEGIVpGGG	438 - 449	Chaperonins cpn60 (HSP60) family	low complexity, coiled coil
ZmHsp70	IDLGTTyS, IFDLGGGTfdvSLL & VvLvGGsTRIPrVqQ	12 – 19, 203 – 216 & 340 - 354	HSP70 family	low complexity, coiled coil
ZmHsp82	YsNKEIFLRE	35 - 44	HSP90 family	HATPase_c, coiled coil, low complexity
ZmHsp101	DAANLFKPmLarG & RIDmSEYmEQhSvA-RLiGA	297 – 309 & 633 - 651	Chaperonins clpA/B (HSP 100) family	low complexity, AAA, coiled coil, ClpB_D2-small

***** HATPase_C: Histidine kinase-like ATPases, AAA: ATPases associated with a variety of cellular activities, ClpB_D2-small: C-terminal, D2-small domain, of ClpB protein

Fig.1 Distribution of protein domains in selected *ZmHSPs*. HATPase_C: Histidine kinase-like ATPases, AAA: ATPases associated with a variety of cellular activities, ClpB_D2-small: C-terminal, D2-small domain, of ClpB protein. Low complexity region and Coiled-coil region represented by pink and green color respectively

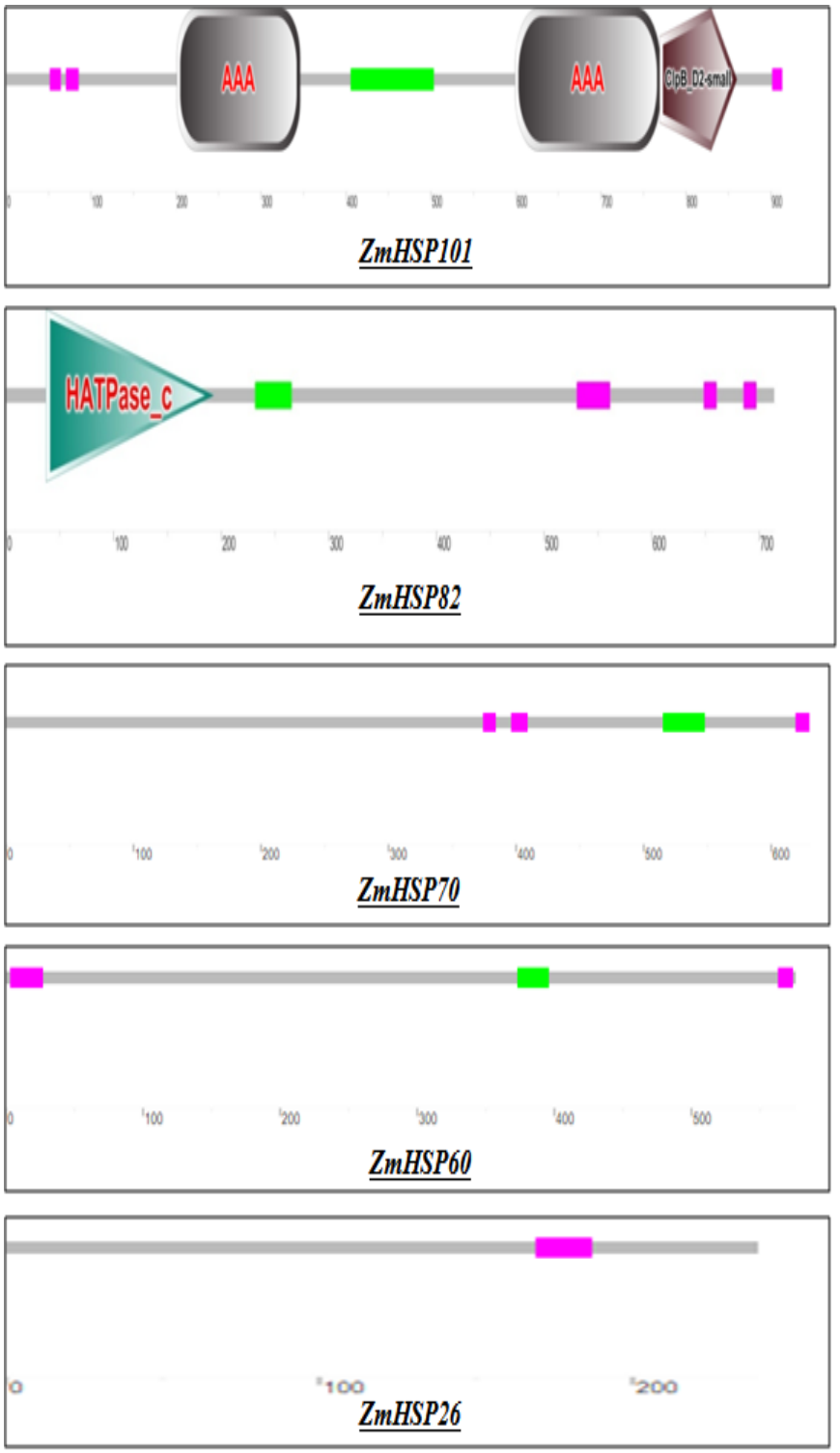
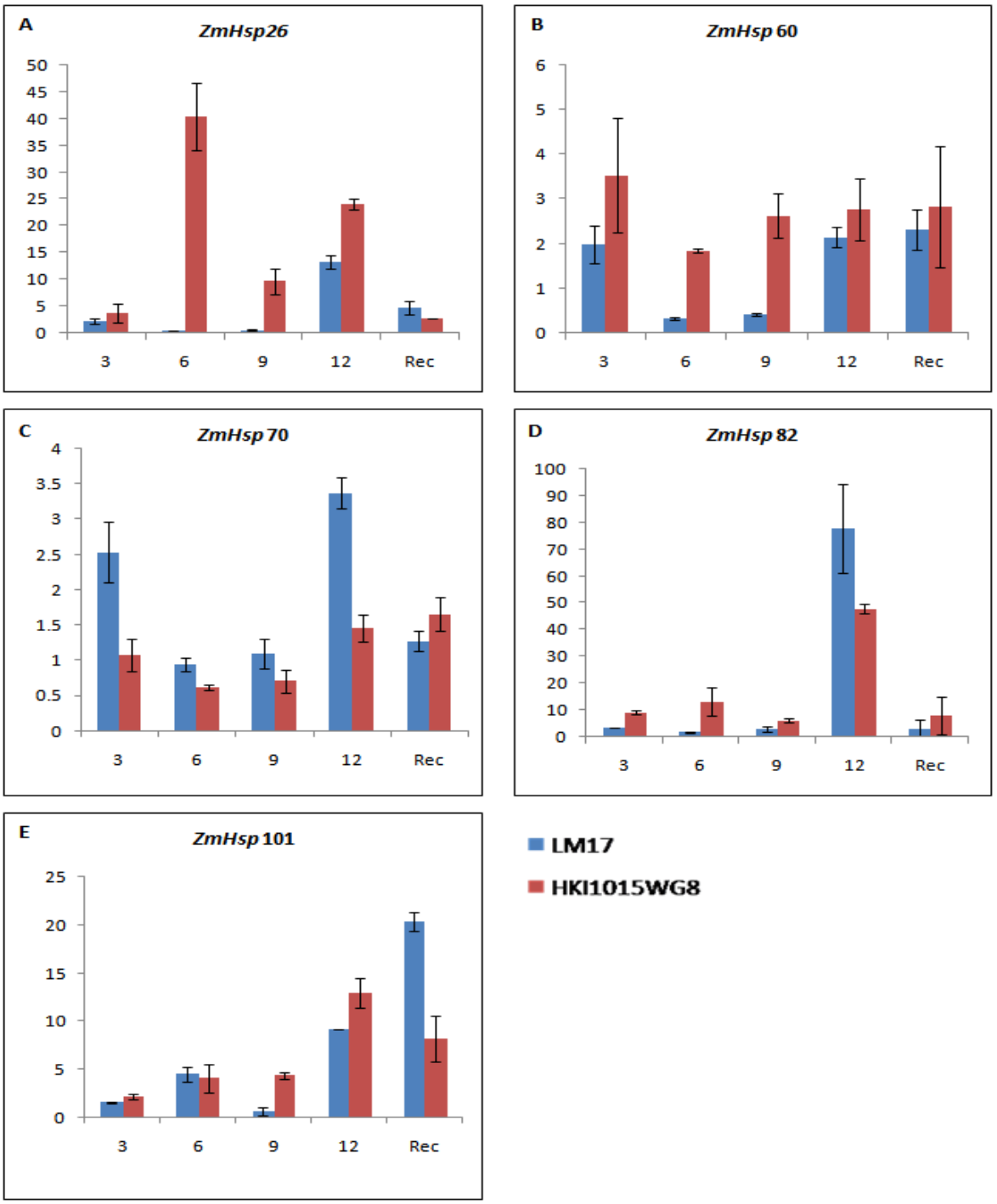


Fig.2 (A-E) Expression analysis of *ZmHsp* genes in LM17 (represented by green colour) and HKI1015WG8 (represented by red colour) maize inbreds in response to heat stress treatments. Values on *X*-axis represents heat stress treatment in hours while rec denotes 24 hrs recovery by growing at 25°C after 12 hrs heat exposure and *Y*-axis represents the log2 fold change in expression level in in response to heat stress treatment (42°C) compared to respective control (25°C). Error bars show standard deviation



In conclusion, identifying key heat stress responsive gene(s), playing crucial role in stress adaptation to plants, is important to engineer plants for heat stress tolerance which in turn would result into sustainable yield in the era of climate change and global warming. Thus, it is essential to understand the mechanisms by which plants react and adapt to heat stress. An array of genes like HSPs is known to be induced in plants under heat stress and play a fundamental role in cellular homeostasis during stress conditions. In this study, *in-silico* analysis of five heat responsive HSP genes were performed and expression of these genes in two contrasting tropical maize inbred lines i.e. LM17 (heat tolerant) and HKI1015WG8 (heat susceptible) subjected to high temperature stress were carried out at seedling stage under controlled conditions. Out of five, three highly expressed *Hsps* (*ZmHsp70*, *ZmHsp82* and *ZmHsp101*) in LM 17, a heat tolerant maize inbred line, were identified which might be playing a crucial role in conferring heat tolerance. However, role of these *Hsps* in heat stress adaptation needs to be further investigated more comprehensively through over-expression and/or RNAi strategies.

Acknowledgement

The authors are thankful to the Director, ICAR-IIMR for providing necessary facilities to carry out this work under in-house project "Physiological and molecular basis of heat tolerance in maize". The research was supported in part by funds from the National Agricultural Science Fund.

Author contribution

IS and PY conceived and planned the experiments, which were carried out by CA, IT and AKJ. KK supervised the bioinformatic and molecular experiments, analyzed the collected data and wrote the primary draft of the manuscript. SR, IS and PY provided specific comments and improved the draft. All the authors read, commented and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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How to cite this article:

Krishan Kumar, Ishwar Singh, Chetana Aggarwal, Ishita Tewari, Abhishek Kumar Jha, Pranjal Yadava and Sujay Rakshit. 2019. Expression Profiling of Heat Shock Protein Genes in Two Contrasting Maize Inbred Lines. *Int.J.Curr.Microbiol.App.Sci.* 8(06): 347-358.
doi: https://doi.org/10.20546/ijcmas.2019.806.039