Pak. J. Bot., 54(1): 65-71, 2022. DOI: http://dx.doi.org/10.30848/PJB2022-1(21)

TRANSCRIPT ABUNDANCE OF HEAT SHOCK PROTEIN GENES CONFER HEAT TOLERANCE IN COTTON (*GOSSYPIUM HIRSUTUM* L.)

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Abstract

The one dark view of industrialization is the emission of greenhouse gases from the burning of fossil fuels which lead towards temperature elevation and global warming. Global warming is a destructive force for agriculture espacially for crop husbandry. Cotton (*Gossypium hirsutum* L.*)* is the most important natural fibre crop grown across the globe and highly vulnerable due to global warming and other climatic changes. It faces biotic and abiotic stresses throughout the world including Pakistan but heat is particularly one of the major abiotic stress that impacting cotton production negatively in Pakistan. Developing heat tolerance in cotton is one of the feasible approaches to overcome the heat problem that needs existence of variability as a pre-requisite. In the present studies, 30 cotton genotypes were assessed for their response to heat stress in randomized complete block design in two replicates at the research area of MNS-University of Agriculture Multan. Relative cell membrane injury was measured to assess heat tolerance. Total RNA was extracted from the one heat tolerant and one heat sensitive genotype. Transcript abundance analysis of HSPs genes (GhHS26 and GhHS97) was performed by RT-PCR to understand the genetic basis of heat tolerance. The CIM-616 was found to be heat tolerant while the SLH-337 heat sensitive genotype.

Key words: Relative cell membrane injury, Differential gene expression, Heat stress, RNA extraction, cDNA synthesis, Quantitative PCR.

Introduction

The emission of greenhouse gases has been increased drastically because of un-judicial burning of fossil fuel to run industry and transportation. The agriculture sector is also to be blamed for methane gas production in livestock farming as well as million hectares cultivation of rice to feed a growing population. This situation is creating global warming and crop farming is highly vulnerable due to heat stress. The high temperature negatively influences the whole phenology of crops and reduces economical yield. The changing climate has significantly reduced the global agricultural productivity by 21% since 1961, and the effect is considerably severe in warmer regions with an overall reduction of 26-34% (Ortiz-Bobea *et al.,* 2021). Cotton is a C_3 plant and is very sensitive to environmental changes. A little change in its optimum growing environment may lead to drastic effect and irreversible growth damage that reduces the economic yield of cotton (Reddy *et al.,* 1992). Optimum temperature for cotton growth ranges from 27-29°C, beyond this range various enzymes get inactivated and decrease the photosynthetic efficiency of cotton plant (Cottee *et al.,* 2010). The temperature above 20°C for 170 days is suitable and plays a positive role on cotton phenology but above 36°C is damaging to growth and development particularly at fruiting (Baloch *et al*., 2000). High temperature affects germination, seedling emergence, plant population per acre, vegetative growth and fruiting of the crops (Rahman *et al.,* 2004). High temperature influence negatively that reduce relative water contents, fresh weight, dry weight of underground and aerial parts of plants (Huang *et al*., 2021). In cotton, elevated temperature results in yield loss due to pollen sterility, flower shedding, boll shedding and reduced fruit setting (Song *et al.,* 2015). The average temperature is continuously increasing due to erratic change in climate and temperature (Rahman *et al.,* 2018). This situation has created alarming conditions for cotton production (Iqbal *et al*., 2016).

It has been projected that there will be an increase in average temperature (seasonal) from 1.52°C to 2.60°C as compared to the seasonal baseline in near-term 2010–2039 (Rahman *et al.,* 2018). High temperature increases the rateof transpiration and photosynthesis process may reduce if water supply is limited that has a negative impact on cotton yield (Hodges *et al*., 1991). Heat stress causes alteration in cell membrane stability that increases the efflux of cytoplasmic molecules. The chemical bonds are altered among the molecules of cell membrane and cellular organelles. Electrolytes such as organic and inorganic solutes leak out from the cytoplasm and cause physiological disorders.Under heat stress the stability of cellular membranes is crucial to perform many physiological processes for plant survival (Wahid *et al*., 2007). A strong negative association of relative cell injury % (RCI %) with yield and fibre traits has been reported in literature (Azhar *et al*., 2009).

Transient reprogramming of gene expression in response to heat shock and elevated temperature is a reaction of biological systems in living organisms (Schoffl *et al*., 1999). Heat stress induces the transcription of many genes that initiate various pathways such as synthesis of hormones and cellular proteins to survive under heat stress conditions. A large set of genes that have potential roles in heat stress responses have been identified using high throughput techniques of biotechnology and genetics. However, transcription of heat shock proteins genes for the synthesis of heat shock proteins (HSPs) is the most important. The heat shock

transcriptional factors play their role to induce transcription of HSPs and protect the plant from heat injury (Vierling, 1991). It has been found that upland cotton had more than 80 heat shock factors which induce the transcription of HSPs genes in response to heat stress. The HSPs protect the plant tissues from heat damage and recover the cell from stresses that help in the survival of plants under high temperature environment (Wang *et al.,* 2014). The expression of HSPs induced by HSPs factors plays a regulatory role stimulated by high temperature and heat stress. Heat stress alters the transcription of many genes at molecular level which modify the whole physiology and phenology of the crop. These proteins have multiple direct and indirect functions that improve the efficiency of physiological mechanisms such as process of photosynthesis, assimilation and partitioning of photosynthates, water uptake, nutrients use efficiency and membrane stability (Camejo *et al.,* 2005).

In view of above, current study was planned to investigate the i) physiological mechanism of heat tolerance and ii) molecular basis ofheat tolerance in cotton.

Materials and Methods

Plant material and growing conditions: Seeds of 30 cotton genotypes were collected from cotton plant material maintained at MNS-University of Agriculture Multan, (MNS-UAM) Pakistan for preliminary heat tolerance assessment assays. All these accessions were indigenous for the Pakistan climate.The plant material was grown in RCBD with three replications at research area of MNS-University of Agriculture Multan located at 30.1575^{o}N, 71.5249^{o}E and 122 m asl. Climate of the experimental site is subtropical which is best suited for cotton cultivation. The temperature ranged 34-36/24-26°C (day/night) during May-June when crop was at seedling stage.

Cell membrane integrity assay: The cell-membrane-thermo-stability was measured following the method proposed by Sullivan (1972). For this purpose, three plants from each genotype were randomly selected for leaf sampling. Leaf samples were taken at 2.00 PM to 4.00 PM on a sunny day from the 12th node position of plant. The samples were washed with d_2H_2O to clean out dust particles and other contaminants. Two leaf discs of 10 mm in diameter were cut from each of two sides of midrib of washed leaves.Leaf discs of each sample were put into separate falcon tubes containing 2 ml distilled water, covered with a lid cotton plugs to avoid evaporation and immediately carried to the laboratory to avoid tissue damage. Set of one falcon tubes containing leaf disc from each side of midrib of samples were incubated at 50°C temperature in water bath for one hour for heat treatment and another one set of falcon tubes was placed at room temperature (25°C) that was used as control. After treatment of heat stress, 10 ml of d_2H_2O was added in both set of falcon tubes containing the leaf samples and were kept at 10°C overnight in a cooling incubator to permit diffusion of maximum electrolytes from the cytoplasm into the water. Next day (24 hours later), both sets of falcon tubes (controlled and treated) were kept at room-temperature for one hour and properly shaken to attain complete mixing of electrolyte in distilled water. Initial electric conductivity-(EC) was measured by EC meter. Then both sets of falcon tubes containing the samples were autoclaved at 0.100 Mpa-pressure and 121°C temperature for 10 minutes to release complete electrolytes from the leaf discs.After autoclaving, falcon tubes containing the samples were placed on working bench to attain room temperature and EC was measured. Relative cell membrane injury percentage was calculated following the formula of Sullivan (1972).

$$RCI\ \% = [1-[\{1-(T_1/T_2)/\ \{1-(C_1/C_2)\}] \times 100$$

where C_1 is the EC of controlled samples before autoclaving and C_2 is after autoclaved. T_1 is the EC of heat-treated samples before autoclaving and T_2 is EC reading obtained after autoclave. This method has also been used by Rahman *et al*., (2004) and Azhar *et al*., (2009) for screening of heat tolerance in cotton.

Measurement of chlorophyll Contents: The plants subjected to heat stress (45°C) at the flowering stage were used to measure chlorophyll contents with SPAD-502 chlorophyll meter (Konica Minolta, Europe) from three randomly selected plants of each genotype.

Seed cotton yield: Seed cotton was picked when all bolls were opened on sunny days. Seed cotton was picked from three guarded plants from each genotypes separately and weighed.

Transcript abundance analysis: Two reported heat shock protein genes, GhHS97 and GhHS26 (Demirel *et al.,* 2014) were selected for transcript abundance analysis in cotton under heat stress condition.Transcript abundance analysis was performed to detect differential response of GhHS97 and GhHS26 in heat tolerant and sensitive cotton genotypes identified in physiological and morphological assays. For this purpose, one heat tolerant genotype (CIM-616) exhibiting relatively high cell membrane thermo-stability and another one heat sensitive genotype (SLH-337) having lowest cell membrane thermal stability were grown in a growth chamber for 14 hours photoperiod, 30/26°C temperature and 80–85% relative humidity. The 20 days old seedlings were subjected to heat treatment at 45°C and leaf samples were collected at 0.75, 1.5, 3, 6, 24, 48, 96 and 192 hours after heat treatment and preserved in liquid nitrogen to extract total RNA. The control cotton general cultivar MNH-886 was maintained at 30°C.

RT-PCR analysis was done to study transcript abundance of HSP genes. Total RNA was extracted from collected leaf samples by RNA purification reagent (catalogue number: 12322-012) and synthesised the first strand of cDNA to be used as template for qPCR analysis. The sequence of the two HSP genes and internal control was retrieved from NCBI and their primers were designed using Primer3 software (Table 1). Efficiencies of three primers was determined by amplification of template serial dilutions and melt curve analysis. The instrumental settings to measure the transcript abundance in cotton genotypes are given in the Table 2. For normalization of qPCR assay ubiquitin gene was used as an internal control.

Table 1. Primer sequences of GhHSP26, GhHSP97and internal control (Ubiquitin) genes.

Gene	Forward 5′	Reverse 5′	Accession Name/Gene Annotation
GhHS97	TGGCAGCCTCTAACGTTGTA	TAACCTCCTCGATCCGCTTC	Gohir.D10G064400.1/IAA-AMINO ACID HYDROLASE ILR1-LIKE 1-RELATED
GhHS26	AGAAAACTCCGCTTTCGTCA	CTCTCTCCGCTGATTTGGAG	Gohir.D03G1 31300.1/K13993-HSP20 FAMILY PROTEIN (HSP20)
Ubiquitin B	ACACGATCGACAACGTGAAG	TCGTCTTGCCGGTTAGAGTC	Gohir.A10G015000.1/K04551-UBIQUITIN B (UBB) GOHIR.

Table 2. The qPCR profile followed in transcript abundance analysis of cotton genotypes.

Step	Temperature.	Time.	Number of cycles.
Denaturation	94.0°C	30s	40
Annealing	50.0°C	30s	
Extension	72.0°C	1minute	
Hold	4.0°C		

Statistical analysis

The mean data were used to make graphs to compare the genotypic performance using MS Excel. The data generated from qPCR analysis was normalized using $2^{-\Delta\Delta Ct}$ method (Wei *et al.,* 2016).

Results

Cell membrane integrity assay: The cell membrane injury was assessed indirectly by EC measurements of ions, leaked out from the leaf discs and calculated as a percentage of total electrolyte leakage. Electrolyte leakage ranged from 19.3 to 89.6% (Fig. 1). Based on electrolyte leakage, thirty cotton genotypes were divided into three groups, 1st) tolerant (<40%), 2nd) moderate tolerant (40-60%), 3rd) sensitive (>60%). Out of thirty genotypes, nine proved to be heat tolerant, nine genotypes showed moderate tolerance and twelve proved to be sensitive for heat stress. The minimum cell membrane injury was observed in CIM-616 (19.3 ± 1.7%) while maximum in SLH-337 (89.6 ± 2.01%).

Chlorophyll contents: Chlorophyll contents were measured with SPAD-502 chlorophyll meter (spade meter) from selected three plants of each cotton genotype. The chlorophyll contents were higher in heat tolerant genotypes and lower in heat sensitive genotypes (Fig. 2). The maximum chlorophyll contents were observed in CIM-616 (59.2 ± 2.63 spade units) and the minimum in SLH-337 (25.0 ± 2.13 spade units).

Seedcotton yield: The seedcotton yield ranged from 102 to 278 g/plant (Fig. 3). The cotton genotype CIM-616 produced highest seedcotton of 278 g/plant while SLH-337 produced 102 g/plant. Eleven genotypes produced above average yield of 163 g/plant.

Transcript abundance analysis: Transcript abundance analysis revealed that the expression of heat shock proteins genes was inductive. When cotton plants were subjected to heat stress the differential gene expression was observed in cotton genotypes (Figs. 3 & 4). At 45ºC, the genes namely GhHS97, GhHS26 and Ubiquitin (housekeeping internal control) amplified their respective gene fragments in CIM-616and MNH-886, while only ubiquitin amplified in SLH-337 (Table 3). The maximum gene expression of heat shock proteins genes was observed in CIM-616 and control cultivar MNH-886. Cotton genotype CIM-616 and check variety MNH-886 appeared asheat tolerant while SLH-337 as heat sensitive.

Table 3. Amplification ofGhHS97, GhHS26 and Ubiquitin B (internal control) in heat tolerant (CIM-616), heat sensitive (SLH-337) and check(MNH-886) genotypes/varieties of cotton.

Gene	MNH-886	CIM-616	SLH-337
GhHS97	+	+	-
GhHS26	+	+	-
Ubiquitin B	+	+	+

+, Amplified; -, Not amplified

Discussion

Heat is one of the major abiotic stresses which causes reduction in the seed cotton yield. Heat shock proteins (HSPs) genes play a crucial role in conferring heat tolerance. In response to high temperature, heat shock proteins inducing factors are formed which induces the transcription of heat shock proteins. The HSPs seems to be involved in mechanisms maintaining the membrane stability of heat tolerant genotypes. It's also evident by the current findings as well.

Temperature influences the cellular membranes structure and proteins by thermo-dynamic effect. Commonly these alterations are fast and high temperature can affect every cellular molecule. It has been reported that alteration in environmental heat stress can be received by cells of plant in view of alteration in rigidity of membrane (Vigh *et al.,* 2007). Heat stress is significantly responsible for the damages pre /post-harvest leading to sun burns and scorching of leaves, stems, branches, senescence and abscission of leaf, root and shoot growth retarded. Heat stress causes damage to chloroplast and fruits and decline in yield of fruit crops (Vollenweider & Gunthardt, 2005). Several plant characters have been correlated with tolerance of stresses like stability of cell membrane, water contents of leaf by Saleem *et al.,* (2015), osmotic potential (Ball & Oosterhuis, 2005), leaf age (Xue *et al.,* (2010) and leaf

fluorescence (Burke, 2001). However, relative CM injury percentage has been reported as a satisfactory and proven parameter for screening the cotton genotypes for temperature stress tolerance in many crops. The findings of current research showed that cell membrane was damaged by heat and consequently membrane leakage was increased (Fig. 1). The electrolyte leaked out from the plasma membrane present in the cytoplasm is commonly measured by EC determination procedure. Low leakage of electrolytes shows thermo-stability of the membrane in heat tolerant genotypes and vice versa in sensitive genotypes. In present studies, heat tolerant genotypes CIM-616, MNH-886, CIM-446, CIM-600, FH-142, CRS-124, CIM-482, BS-15 and MNH-992 showed low electrolyte leakage i.e., RCI below 40%, high spade value (above 40; Fig. 2) and high seed cotton yield (above 150 g/plant; Fig. 3). Rahman *et al*., (2004), Azhar & Khan, (2005) and Azhar *et al*., (2009) earlier reported that heat tolerant cotton accession produced more seed cotton. Differential responses same genotypes/varieties in current studies and previous studies (Azhar *et al.*, 2009) might be due to poor purity maintenance at the source (breeder's end), aging and natural genetic alterations.

In heat tolerant cotton genotypes, chlorophyll contents (spade units) were higher than sensitive one. The highest chlorophyll content was observed in CIM-616 and minimum in SLH-337. In heat sensitive genotypes chlorophyll contents were degraded due to heat stress while remained stable in heat tolerant. Chlorophyll contents are an important parameter for screening of cotton genotypes for heat tolerance (Vacha *et al.*, 2007). These contents are directly proportional to heat tolerance and vice versa.In other crops e.g., in wheat higher total chlorophyll contents have been reported in heat tolerant genotypes and lower in sensitive one (Rehman *et al*., 2016).

Transcription of HSPs-genes produces HSPs that play a primary role and provide general homeostasis to cells for heat stress (Vabulas *et al.*, 2001). Heat stress induces the transcription of HSPs genes and their expression varies with temperature as well as duration of heat stress. The transcription of HSPs gene may start as early as response to heat stress but expression terminates with the increase of stress duration in heat sensitive genotype (Jing *et al*., 2018). Two reported heat shock protein genes, GhHS97 and GhHS26 (Demirel *et al.*, 2014) were selected for transcript abundance analysis in cotton under heat stress condition. Two contrasting genotypes displaying differential RCI, spade value and seed cotton yield response to heat stress along with general cultivar were used to study molecular basis of heat tolerance through transcript abundance analysis. In transcript abundance analysis, CIM-616 and MNH-886 showed gradual temporal increase in transcript abundance of both HSPs genes while didn't express in SLH-337 when subjected to high temperature (45^0C) (Figs. 4 & 5). When temperature was increased up to 45^0C and heat interval was increased, CIM-616 and MNH-886 showed stability in its transcript abundance and all three genes expressed, while SLH-337 showed only stable expression of Ubiquitin B gene. The transcript abundance of HSPs genes in SLH-337 was too low and negligible when subjected to high temperature.Transcription of HSPs genes was poor and cannot meet the requirement for homeostasis in heat stress. Although the transcript abundance of both HSPs genes was similar but varying phenotypic responses showed that the mechanism of heat tolerance in both heat tolerant genotypes was different. It has also been reported earlier as well (Lee *et al.*, 1995; Priya *et al*., 2019).

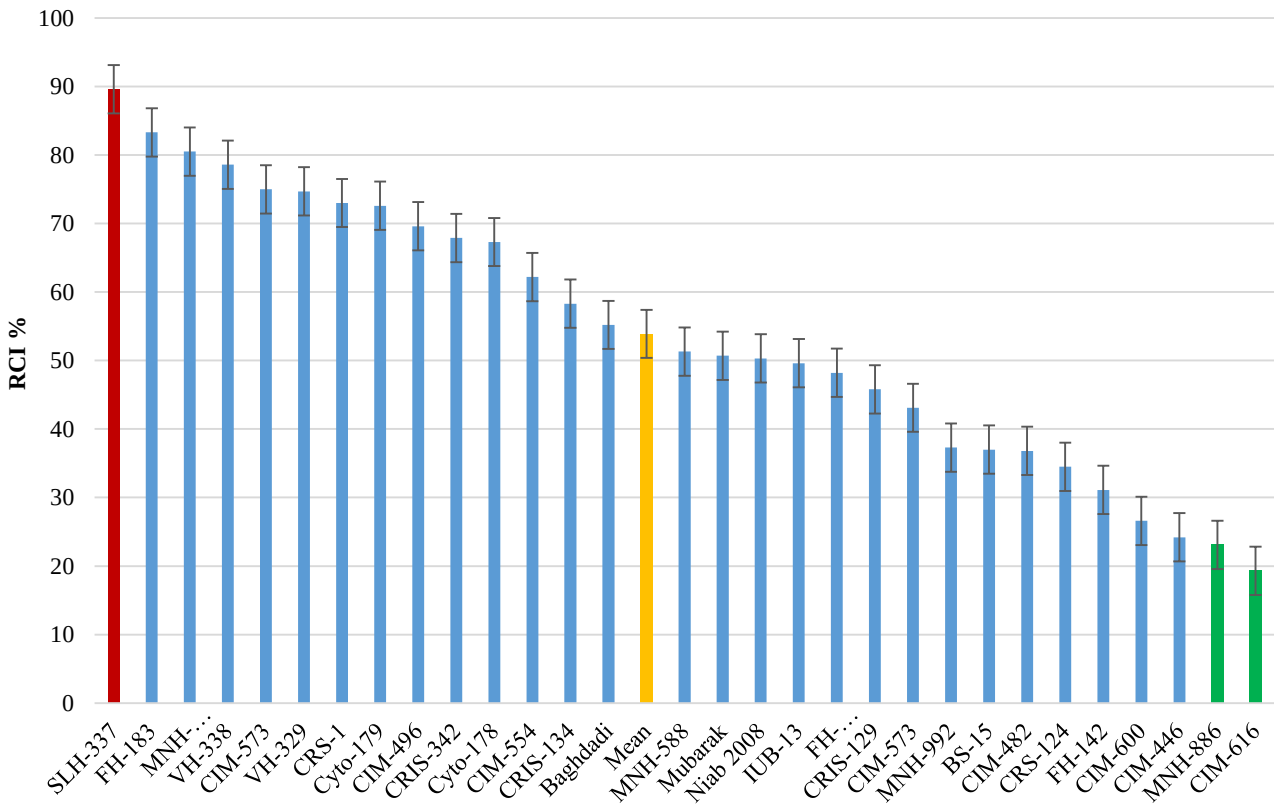

Fig. 1. Relative cell membrane injury of 30 cotton genotypes.

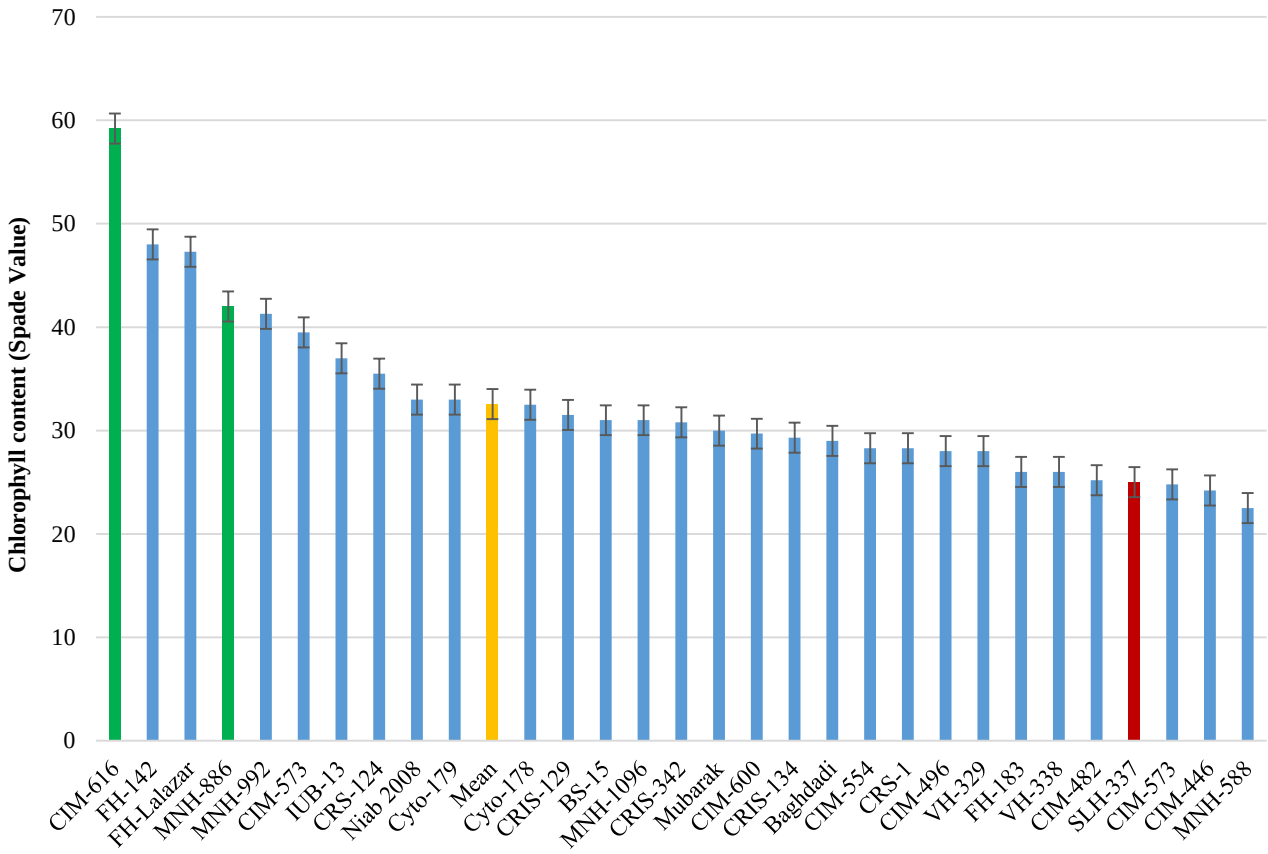

Fig. 2. Chlorophyll contents (spade units) measured in 30 cotton genotypes.

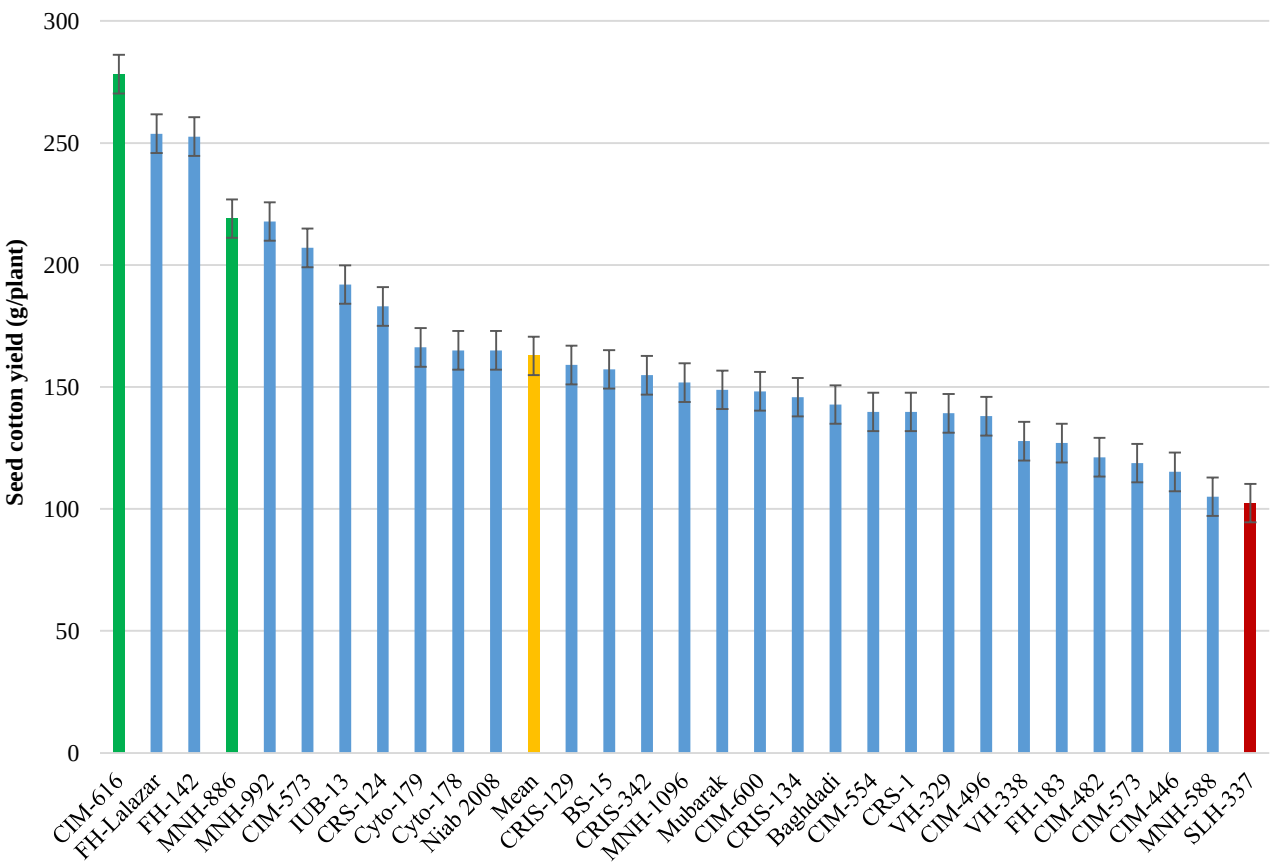

Fig. 3. Seedcotton yield (g/plant) produced by 30 cotton genotypes.

The expression of these HSPs genes may provide heat tolerance by the synthesis of heat shock proteins (Lee *et al*., 1995). However, the transcript of HSPs gene in CIM-616, was higher and more expressed. HSPs have gained special attraction in plant sciences due to their important role in innate immunity in plants (Lee *et al*., 1995). Molecular chaperons such as small HSPs, having molecular weight between 15-42 kDa are ubiquitously distributed throughout the living organism like bacteria to humans and provide buffering capacity under heat stress. Although chaperons/HSPs proteins are different from other classes of HSPs due to their molecular weight, binding with large oligomers, ATP independence and structure conserved then higher molecular weight proteins HSPs excluding alpha crystallin domain. These small HSPs have capacity to exert their protective role under versatile heat stress conditions in different ways that have not been found in remaining members of the protein family (Adam *et al*., 2001). Additionally, these proteins also boost up the catalytic activity of 26S proteasome, thus stimulating the breakdown of ubiquitinated proteins in heat stresses conditions (Wang *et al.,* 2003). These proteins have versatile function and have been detected in mitochondria where they inhibit the degradation of NADH and protect them from heat stress (Zhang *et al.,* 2002). Heat stress is a great concern for the plant scientists, plant breeders, farmers, planners, textile industry and national economy. In future perspective, use of functional genomics and expression of specific molecular markers associated with heat tolerance would be helpful to develop heat tolerant genotypes. The use of high throughput technology integrated with conventional breeding techniques would assist to cope with heat stress through conferring heat tolerance in cotton for sustainable productivity under climate change and global warming scenario.

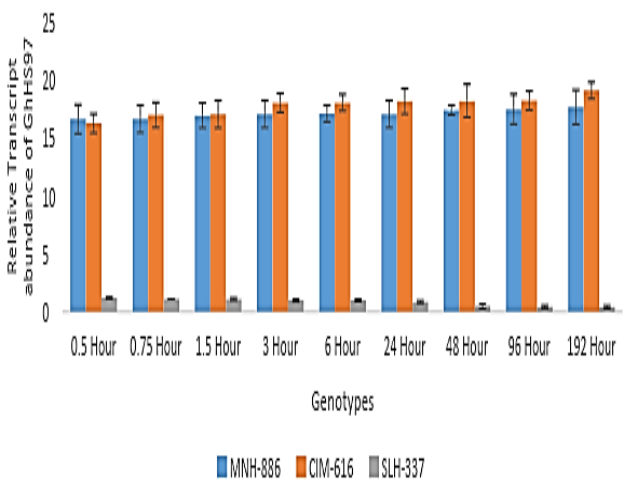

Fig. 4. Relative transcript abundance of GhHS97 gene in heat tolerant genotype (CIM-616), heat sensitive genotype (SLH-337) and general cultivar (MNH-886) of cotton.

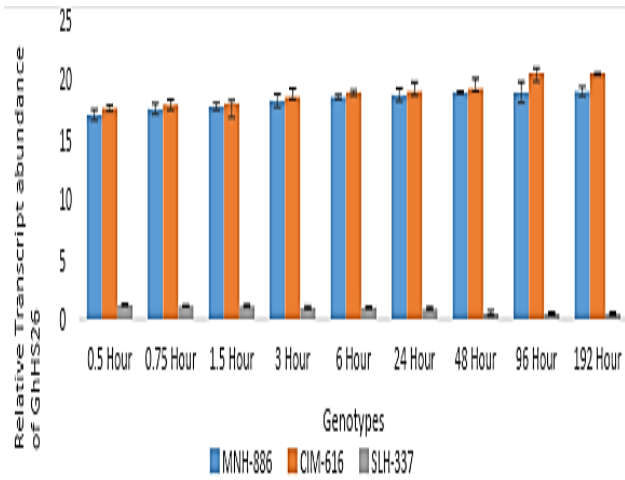

Fig. 5. Relative transcript abundance of GhHS26 gene in heat tolerant genotype (CIM-616), heat sensitive genotype (SLH-337) and general cultivar (MNH-886) of cotton.

Conclusion

Cotton genotypes CIM-616, MNH-886, CIM-446, CIM-600, FH-142, CRS-124, CIM-482, BS-15 and MNH-992 are heat tolerant having low electrolyte leakage, high spade value and high seed cotton yield. GhHS97 and GhHS26 expressed in heat tolerant cotton genotypes CIM-616 and MNH-886 while didn't express in sensitive genotype SLH-337.

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(Received for publication 2 March 2020)