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EXPRESSION PROFILING OF HEAT SHOCK PROTEIN GENES AS KEY EARLY RESPONDERS TO HEAT STRESS IN INDIGENOUS LETTUCE (LACTUCA SATIVA L)

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Abstract

Global warming-induced high temperatures can negatively impact lettuce's productivity as a cool-season crop. Plants' heat shock protein (HSPs) serves as molecular chaperones, facilitating stress tolerance and protein stability, are critical to plant survival in the context of heat stress (HS) aggravated by global warming. In this study, we determine the expression pattern of HSP genes i.e. LsHSP70A, LsHSP70B, LsHSP83A and LsHSP83B in indigenous lettuce (lactuca sativa L.) by quantitative real-time PCR (qRT-PCR) following temperature stress. The four HSP genes exhibited distinctive expression patterns in response to HS exposure at 37°C at 1, 4, 16 and 24 hours. The findings showed that the expression levels of the two HSP genes, LsHSP83A and LsHSP83B were significantly upregulated at all time points after heat stress, whereas LsHSP70A, LsHSP70B revealed variable expression patterns exhibiting both up- and downregulation at various time points, suggesting diverse regulatory mechanisms. Our study showed putative early heat-responsive HSP genes in lettuce, which could be possible candidates for breeding efforts to create heat-tolerant lettuce cultivars.

INTRODUCTION

Lettuce (*Lactuca sativa* L.), the "king of salad crops" is recognized as world's most economically valuable leafy vegetables, commonly found in healthy food (Lee *et al.*, 2015). Lettuce is belongs to family Asteraceae, estimated to be the largest plant family with an estimated 23,000-30,000 species (Noumedem et al., 2017). Lettuce is an annual cool season crop in temperate regions (Lal *et al.*, 2024). It grows rapidly in temperature between 17°C and 28°C, with flowers initiation appearing between 21°C and 27°C (Wallace et al., 2012). Heat stress refers to environmental temperatures beyond the optimum range for certain plant and animal species, which negatively impacts growth and development (Hussain et al., 2023). Physiological conditions such as tip burn, rib discoloration, bloated heads, and premature bolting are frequently caused by heat stress in lettuce, which drastically lowers its nutritional value and marketability (Lee et al., 2015; Kang et al., 2021).

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Climate change has a significant impact on the world's food production, with crop yields being threatened by rising temperatures and more frequent droughts. National Oceanic and Atmospheric Administration (NOAA) reported that ocean surface and global land temperatures elevated by a mean of +0.98°C in 2020, making it the 2nd warmest record year in the previous 141 years (NOAA, 2020). Crop growth and yield productivity have been significantly affected by climate change, which not only contributes to a persistent increase in average temperatures through the greenhouse effect but also induces short-severe heat events (Mearns et al., 1984). Peng et al. (2004) observed that in dry season, crop yield declined by arround 10% for each degree Celsius as temperature elevated. However, plants rely on complicated physiological and cellular processes for stress-tolerance and adaptability because plants cannot migrate to evade persistent and constant environmental pressures (Wu et al., 2007).

Heat shock proteins belong to highly conserved stress-related proteins that are expressed in hard environment (Saeed et al., 2021). Based on sequence homology and approximate molecular weight, HSPs are divided into six key subfamilies including *Hsp40*, *Hsp60*, *Hsp70*, *Hsp90*, *Hsp100/110* and small Hsps (Wang et al., 2004), have particular roles in protection mechanisms and responses to stressful circumstances. *HSP70* has been considered the evolutionary conserved and widely distributed chaperones across all HSP families. It mainly exists in endoplasmic reticulum, mitochondria and cytoplasm and plays an important protective role in intracellular protein transport and folding, degradation of proteins aggregates, and regulating growth and development in plants under several stress circumstances (Wang et al., 2024). *HSP83* also referred to as cytoplasmic *HSP90*, belongs to the *HSP90* protein family and is detected in plants and other organisms. Phylogenetic analysis showed that *HSP83s* and *-90s* diverged from a common ancestor separately, however *HSP90s* evolved earlier (Zhang et al., 2014). With the exception of the Archaea, all species have been identified to comprise HSP90 with a molecular weight that varies from 82-90 kDa across different organisms (Kim et al., 1998). *HSP83*, a cytoplasmic *HSP90* protein, a molecular chaperone, is essential for aiding in refolding of denatured proteins and prevent aggregation, structural integrity and cytosolic proteins regulation under stress conditions (Sun et al., 2015).

The development of heat-tolerant lettuce cultivars is vital since lettuce that exhibits symptoms commonly associated with heat stress has an exceedingly low acceptance among consumer's level. There are limited transcriptomic studies on lettuce. Knowing the genes that respond to heat stress and gaining insights on their expression patterns during heat stress is essential. This information can assist the development of the next generation of lettuce cultivars with enhanced heat tolerance by utilizing cutting-edge molecular breeding approaches for crop resilience.

Material and Methods

Plant materials, growth conditions and treatments

Seeds of native lettuce (*Lactuca sativa* L.) cultivars were obtained from a greenhouse in Quetta city. A total of 200 seeds were obtained and sown on appropriate organic media (peat & perlite) with a broadcast method. Irrigation was provided on moisture need basis with sprinklers. 50 cell trays with seeds of indigenous lettuce were planted, and the plants were raised in the green house, which was kept at a constant 22/20°C (day/night) temperatures. Seedlings were moved inside a temperature-controlled LED growth chamber eighteen days after the germinations. White and blue light spectrum were used in an alternative pattern to grow the seedlings, and a constant temperature and humidity range of 60–65% were maintained. Then at 25-day-old plants were subjected to 37°C heat stress treatments at various intervals, including 1 h, 8 h, 16 h and 24 h. A sample of the lettuce's aerial foliage section was taken with sterilized sharp razor instantly frozen in liquid nitrogen, and kept at ultra-centrifugation temperatures *i.e.* -80°C. Plants were returned to the ideal temperature for phenotypic change observation after a 24-hour heat treatment.

Genomic RNA isolation and cDNA synthesis

Total RNA was isolated from the frozen leaf samples using TriZol reagents (Thermo Scientific, USA) following the manufacturer's instructions. The

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cDNA was synthesized from the DNase I treated RNA using the RevertAid first-strand synthesis kit as per recommended instructions (Thermo Scientific, USA). Isolated RNA and cDNA quantification was performed using NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, USA) and stored at -86°C until further use.

Differentially expressed genes

The study conducted to determine various HSP genes expression pattern to compare samples grown at high temperature (37°C day/night, experimental group) with those grown at optimal temperature conditions (20°C day/night, control group). For each pair of comparisons, fold chain (FC) was computed for statistical analysis. Genes with significant variations in expression were identified based on a p-value threshold of <0.05.

Quantitative real time PCR (qRT-PCR) analysis

The quantitative real time PCR analysis was performed in ABI 7500 real-time PCR system (Applied Biosystem, USA) utilizing the synthesized cDNA to assess the mRNA expression pattern of selected four HSP genes (*LsHSP70A*, *LsHSP70B*, *LsHSP83A* and *LsHSP83B*) at different experimental time point during heat stress in indigenous lettuce (Lactuca Sativa L.) (Table 1). A total of 20 µl was used as final volume to prepare the PCR reaction, including 1 µl cDNA, 12 µl Power-Track SYBR-green Master-Mix (Thermo Scientific, USA), 1 µl each forward and reverse primers (8 µM) and 5 µl of DEPC water. The thermal cycling conditions included an initial denaturation at 95C for 5 min, followed by 40-cycles of 95°C for 10 sec, 60°C for 1 min. The relative transcriptional levels of the selected HSP genes were normalized by using the reference gene, UBQ21 (Borowski et al. 2014) as an internal control using the $2^{-\Delta\Delta Ct}$ method.

Table 1: Primers used for the amplification of studied HSP70A, HSP70B, HSP83A and HSP83B genes

GENE NAME	FORWARD PRIMER 5'	REVERSE PRIMER 3'	TEMP °C
Reference gene/Housekeeping gene UBQ 21	TCTTAGATCACCGTCCCATCGT	TCTGAGATTGTCCGAGGATATGAG	60
LsHSP70A	AGCTGAGGATAAAGTTGGTGG	CTCATCTTCCGCCTTATACC	60
LsHSP70B	TCTAAGCGGTGAAGGCAACC	TCGGAATGGTGGTGTTTCGT	60
LsHSP83A	CTGTGCAAGACGATCAAGGA	ACTCCCCAGTCACCAAACAG	60
LsHSP83B	AAAGTGGTGGTTTCCGACAG	CGCCTTCATTATCCTCTCCA	60

Statistical Analysis

Statistical Package for Social Sciences (SPSS V 22.0) was used for statistical analysis with statistical significance was assigned at $p < 0.05$.

Results

This study explores the effects of heat stress on lettuce's early vegetation growth. To determine their response, the lettuce plants were exposed to high temperatures (37°C) for 24 hours. We investigated the expression pattern of particular HSPs including *LsHSP70A*, *LsHSP70B*, *LsHSP83A* and *LsHSP83B* in lettuce plants under heat stress. The quantitative real time PCR (qRT-PCR) was used to determine the amount of transcript at different intervals during heat treatment (1, 8, 16 and 24 hours). Their patterns of periodic expression provided valuable insight in response to heat stress.

The HSP genes exhibited differential upregulated and downregulated expression patterns at different points over the heat stress treatment period compared to control. The *LsHSP70A* gene of lettuce showed a dynamic expression pattern when exposed to heat stress. At 1-hour, it was observed to be upregulated (1.35-fold), peaked at 8-hours (5.06-fold) and maintained at 16-hours (1.47-fold). Its expression, however, sharply downregulated (0.12-fold) at 24 hours compared to the control group, suggesting a potential regulatory mechanism upon prolonged heat exposure. The *LsHSP70B* gene

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showed a dynamic expression pattern to heat stress in lettuce. At 1 hour, it was observed significantly elevated (6.17-fold), suggesting an early-stress response. However, *LsHSP70A* gene expression sharply decreased at hours (0.6-fold), further declined at 16 hours (0.4-fold) and 24 hours (0.14-fold) compared to control lettuce plant. Moreover, the *LsHSP83A* and *LsHSP83B* genes, in contrast to *LsHSP70A* and *LsHSP70B* genes showed persistent expression pattern throughout the period of the experiment, demonstrating their sustained involvement in the heat stress response and possible contribution to long-term thermotolerance mechanisms. The *LsHSP83A* gene exhibited upregulation throughout the experimental period to heat stress, at 1 hour (1.32-fold), 8 hours (24.42-fold), 16 hours (22.28-fold) and 24 hours (5.91-fold) compared to control suggesting a continuing demand for upgraded protection and repair process by cells under heat stress. *LsHSP83B* exhibited a similar pattern of gene expression in lettuce in response to heat stress, showing upregulation at 1 hour (1.13-fold), sharply elevated at 8 hours (10.56-fold), reduced at 16 hours (5.06-fold) and further decreased at 24 hours (1.63-fold) compared to control group.

A one-way analysis of variance (ANOVA) was performed to determine the mean Ct values of studied differentially expressed HSP genes at different time points under experimental heat stress conditions at 37°C. The findings showed a statistically significant difference ($p = 0.038$) among the genes was observed, suggesting a significant difference in expression patterns in response to heat stress (Table xxx).

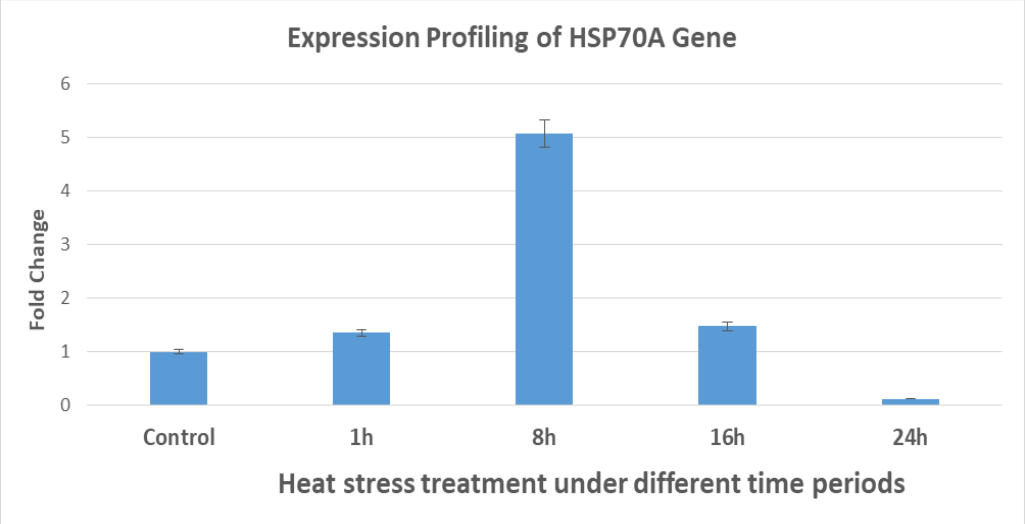

Figure 1: Differential expression of *HSP70A* gene at various time intervals during heat stress

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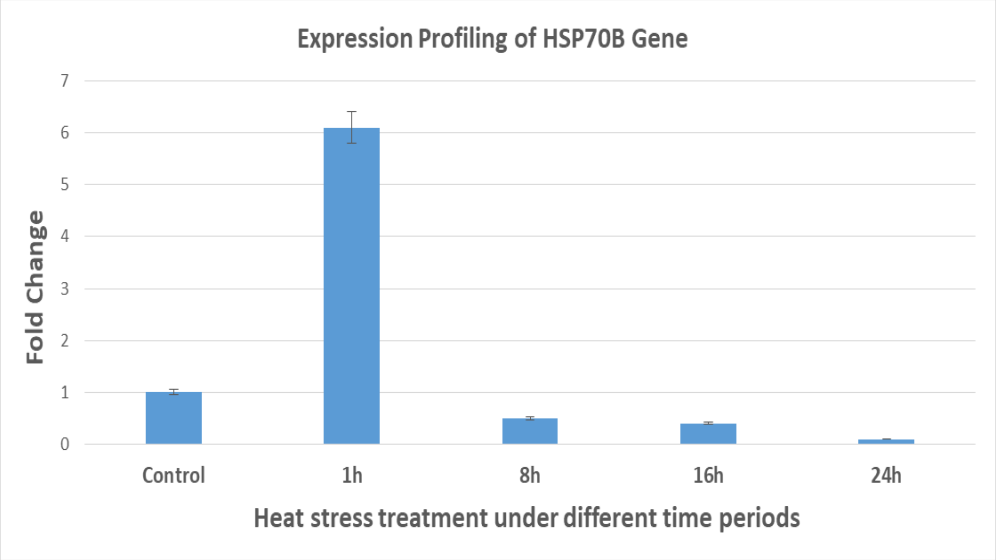

Figure 2: Differential expression of *HSP70B* gene at various time intervals during heat stress

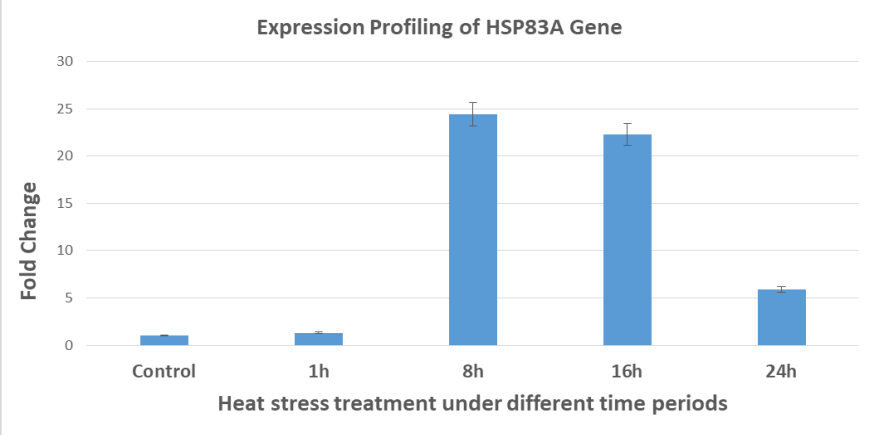

Figure 3: Differential expression of *HSP83A* gene at various time intervals during heat stress.

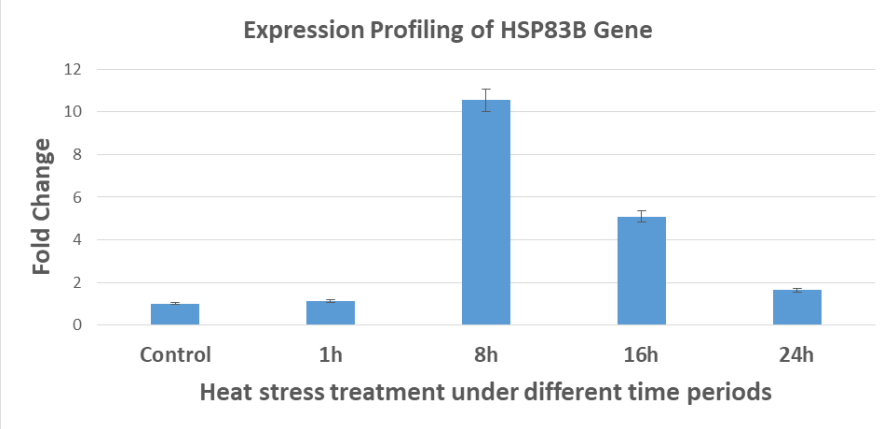

Figure 4: Differential expression of *HSP83B* gene at various time intervals during heat stress.

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Table 2: Comparison between the Ct values of the selected HSP genes

SOV	Sum of Squares	df	Mean Square	F-cal	Sig.
Between the Groups	93.317	3	31.106	3.571	0.038
Within the Groups	139.375	16	8.711		
Total	232.692	19			

Discussion

Heat stress is a serious issue that limits the growth of plants and metabolism, which reduces the potential yield of several crops cultivated across the globe (Fahad et al. 2017). The detrimental effects of heat stress on crops are getting exacerbated due to the rising frequency of extreme climate changes. This poses an imminent threat to agricultural sustainability and global food security and necessitates immediate adaptation measures and innovative approaches. Every plant species grows effectively at a specific spectrum of temperatures, including the lowest, maximum and optimum ranges required for optimal growth and development (Hatfield and Prueger, 2015). As a cool-season crop, lettuce thrives in temperature ranging from 17°C to 20°C (Holmes et al. 2019). In order to ensure robust growth, high yield and general crop quality, this optimal range needs to be maintained.

Heat stress triggers a variety of defense mechanisms, such as the activation of particular genes that produce stress-associated molecular chaperones known as HSPs. These proteins play a vital role in defending cells by limiting protein denaturation, misfolding, and aggregation under stress (Haq et al. 2019). Moreover, several HSPs assist plants grow resilient to biotic and abiotic stresses (Timperio et al. 2008), facilitate survival and recovery once optimal growth conditions are restored, maintains plants continued to develop.

The expression levels of *HSP70A*, *HSP70B*, *HSP83A*, and *HSP83B* were examined in lettuce samples harvested at particular time periods under high temperatures to determine the early responses of lettuce HSP genes to heat stress. The analysis showed that the HSB83A and HSP83B exhibited persistent expression levels throughout the experimental time points whilst HSP70A and HSP70B showed varied expression levels detected at different experimental periods. The specific functions of *HSP70A* and *HSP70B* perform at various phases of stress exposure or recovery are possibly shown by the differential expression patterns of these genes, leading to a dynamic and precisely regulated response to heat stress. This observation is consistent with studies involving other plant species that show differential regulation of *HSP70* family members under stress. Thus, a comprehensive study of radish (*Raphanus sativus*) discovered 34 *RsHSP* genes with distinct expression patterns under heat stress, suggesting functional diversity within the HSP70 family (Pan et al. 2024). The intricacy of HSP70-mediated stress responses was further highlighted by the observation of 61 *GmHSP70* genes in soybean (*Glycin max*) exhibited stress-inducible expression patterns under both stress and drought stresses (Zhang et al. 2015).

HSP83 is homologous to *HSP90*, comprises a histidine kinase/HSP90-like ATPase domain, together with HSP70 and other co-chaperones facilitate protein folding from the initial phases of protein synthesis (Szabo et al. 1994; Li et al. 2020; Wang et al. 2022). The findings of this study show that *HSP83A* and *HSP83B* exhibited stable expression patterns throughout the experimental periods, suggesting an intrinsic impact on the plant's response to heat stress and in retaining cellular homeostasis despite variations in the ambient temperature. Our findings are consistent with previous observations by Conner et al. (1990) that heat shock has been reported to significantly elevated *HSP83* expression dynamic in Arabidopsis thaliana, suggesting a potential contribution in the heat stress response. Kang et al. (2021) reported elevated expression levels of *LsHSP83* under heat stress in *Lactuca sativa* L. *LsHSP70* and *LsHSP83* are therefore considered to be crucial for shielding lettuce cells from heat stress by enhancing cellular stability and protection.

Conclusion

In the present study, we observed differential expression dynamics of the studied genes in *Lactuca sativa* L. under heat stress. Fluctuating expression

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patterns of *LsHSP70A* and *LsHSP70B* genes suggest more intricate regulatory mechanisms, while the persistent expression dynamic of *LsHSP83A* and *LsHSP83B* genes demonstrated their vital role in heat stress response. Our findings highlight the need for further studies into the functional significance of these HSPs by identifying potential temporal association between their expression profiles.

Acknowledgment

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Conflict of Interest

The authors declared that there is no conflict of interest.

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