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Radhwan Abd Baqer
Ministry of Education,
Directorate of Education,
Dhi Qar, Iraq

Hassan AbdulKareem Qaseim
Ministry of Education,
Directorate of Education,
Basra, Iraq

Weam Munadhil Hussain
Department of Biology, College
of Education of Pure Sciences,
Basra, Iraq

Corresponding Author:
Radhwan Abd Baqer
Ministry of Education,
Directorate of Education,
Dhi Qar, Iraq

Gene expression profile of the heat shock protein Hsp 23.6 in wheat leaves (*Triticum aestivum* L.) subjected to NaCl stress

Radhwan Abd Baqer, Hassan AbdulKareem Qaseim and Weam Munadhil Hussain

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Abstract

This study was conducted with the aim of detecting and studying the gene expression pattern of a small molecular weight protein located in the mitochondria called the mitochondrial small heat shock protein with a molecular weight of 23.6 (M-sHSP 23.6) in the leaves of the local bread wheat plant (Ebba 99 Cultivar) growing under conditions of NaCl salinity at three levels 50, 100 and 150 mM in addition to the control treatment for four weeks. RNA was extracted from salt-stressed plant leaves for gene expression analyzing during different levels of salt stress. The gene expression of HSP 23.6 was determined by semi- quantitative-PCR. The results showed that increased salt stress intensity led to increased gene expression of the protein under study, indicating its vital role in salinity adaptation. These observations suggest that heat shock protein (HSP 23.6) may be a potential biomarker for determining salinity tolerance, thus guiding improvement programs aimed at enhancing plants' ability to withstand environmental risks.

Keywords: Heat Shock Proteins (HSPs), gene expression, salt stress, wheat plant, semi-quantitative PCR

Introduction

Approximately 20% of soils worldwide suffer from salinity problems, which are more pronounced in arid and semi-arid regions, in addition to other human and climatic factors (Lopez *et al.*, 2002; Munns & Tester, 2008; Ding *et al.* 2021) [16, 18, 5]. In response to the rapid growth of the world's population, agricultural production must increase by 70% by 2050 (Hassan *et al.*, 2020) [14]. Abiotic stressors result in a 50% decrease in agricultural yield, posing a significant danger to world food security (Seleiman *et al.*, 2019) [21]. Plant response to high-salt environments has become a major topics of interest to researchers in the field of agriculture and crop production due to its close connection to the source to the human food supply (El Sayed, 2011) [7]. Wheat is the world's leading grain producer and is a major food source for about 36% of the world's population, containing 20% of calories and 55% of carbohydrates (El Sayed, 2011; Hasanuzzaman *et al.*, 2017) [13, 7].

Many stressors exist that directly harm plants, such as drought, salinity, extreme temperatures, and exposure to chemicals, causing secondary damage such as oxidative stress and salt stress. Plant exposure to these factors is inevitable. Plants have developed a range of mechanisms that enable them to resist these stresses, whether morphological or physiological. Exposure to environmental stress stimulates the formation of a group of heat shock proteins, a phenomenon common in many living organisms. Heat shock proteins are a widespread protein group that forms as a rapid response to oxidative and salt stress, especially at high temperatures. These proteins are divided into five types depending to their molecular weights, including those with low molecular weights, which are then called low heat shock proteins (Al-Whaibi, 2011; Park and Seo, 2015) [3, 20].

Low molecular weight protein(LMWPs) including heat shock proteins (HSPs) perform many vital roles, such as maintaining cellular homeostasis and as molecular companion proteins that enable the plant to adapt to different stresses and prevent and maintain protein structure variation (Huttner & Strasser, 2012; Swindell *et al.*, 2007) [15, 25]. Minimal heat shock proteins

Have molecular weights ranging from 15–42 kDa and have a conserved region at the carboxyl end (Sun *et al.*, 2002) [24]. Structural alignments, cross-reactivity and cellular location are employed to classify LMWPs. This classification consists of six classes. Three of these classes are resides within the nucleus or cytoplasm, and the remaining three exist in the endoplasmic reticulum, mitochondria, and chloroplasts (Vierling, 1991; Sun *et al.*, 2001) [26, 23]. These proteins (LMW-HSPs) in various environmental stresses was protein intermediates-saving. At very early stages of the unfolding, thereby averting their irreversible aggregation. Organellar (LMW-HSPs) represent a distinctive characteristic of plant cells (Escobar *et al.*, 2021) [8]. These proteins serve as molecular chaperones, performing essential functions throughout plant growth stages, development processes, and stress response (Wang *et al.*, 2024) [28]. In addition to elevated temperatures, low molecular weight proteins can be induced by many stressors, including water stress, salinity, and cold exposed. They may also be generated during other developmental phases, such as germination of seeds, embryogenesis, and the development of the fruit (Sun *et al.*, 2002) [24]. Plant-stress responses and the elimination of reactive oxygen species (ROS) generated by the electron transport chain depend heavily on mitochondria. A variety of abiotic stimuli cause the up-regulation of genes expression that encoding proteins related stress, such as mitochondrial small HSPs (M-sHSPs) (Escobar *et al.*, 2021) [8].

Nuclear genes that encode mitochondria-localized low molecular weight proteins have been found and examined in numerous plant species. Two mitochondrial small heat shock protein genes (TaHSP23.5 and TaHSP23.6) were identified as being expressed in wheat during heat shock conditions (Basha *et al.*, 1999) [4]. Showed increased expression of the genes for the low molecular weight proteins HSP17.4, HSP17.7, and HSP19.1 in wheat grown under influence salt stress (Muthusamy *et al.*, 2017) [19]. A recent work indicated that the HSP23.9 protein is crucial for the wheat plant's response to salinity and heat stress. Moreover, Wang *et al.* (2020) [29] documented a significant rise in HSP23.9 expression in response to these stressors. Another study conducted on durum wheat grown under the influence of salinity also indicated an increase in the concentrations of a number of LMWPs proteins known as HSPs with different molecular weights (17.8, 26.3, 70 and 101 kDa) (Al Khateeb *et al.*, 2020) [2].

The development of LMW-HSPs has been recorded in many plants are stressed by various types of stressors such as elevated temperatures, drought, and salinity (Ahn & Zimmerman, 2006; Volkov *et al.*, 2006; Zahur *et al.*, 2009) [1, 27, 30]. These works demonstrate that LMWPs (HSPs) can significantly contribute to a plant's empowered to endure environmental constraints. The aimed of our study was to study the gene expression profile of the HSP 23.6 protein in the leaves of a local wheat variety grown under influence NaCl salinity stress.

Materials and Methods

Plant Growth Conditions and NaCl Treatment

The current study utilized the bread wheat (*Triticum aestivum* L.) cultivar Ebba 99. The seeds were surface sterilization with 70% ethanol. They were rinsed three times with sterile distilled water, then surface sterilized using sodium hypochlorite 5% for 30 minutes. Subsequently, they were rinsed three times with sterile distilled water, each

rinse lasting 30 seconds. Following that, the seeds were cultured in five aseptic Petri dishes containing twenty seeds to evaluate seed viability and determine the germination percentage. Seeds were incubated in a growth chamber at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, under to a 16/8 hour light/dark photoperiod. Seeds were planted in plastic containers supplied with a 2:1 mixture of river sand and peat moss. For the initial 10 days, all of the pots were watered with distilled water. The pots were classified into four categories: the first was administered distilled water (control), the second was irrigated with 50 mM NaCl, the third with 100 mM NaCl, and the fourth with 150 mM NaCl. The plants were grown in a controlled growth chamber, where the temperature was maintained at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and the photoperiod consisted of 16 hours of light and 8 hours of darkness.

Gene expression analysis

The effect of sodium chloride treatment on the gene expression pattern of the nuclear gene encoding a small mitochondrial protein with a molecular weight of 23.6 kDa was studied using a semi-quantitative method. Four-week-old plant leaves, grown under controlled conditions, were used and then exposed to different levels of sodium chloride (50, 100, and 150 mM). Subsequently, RNA was extracted, and semi-quantitative polymerase chain reaction (PCR) analysis was performed to determine gene expression levels.

Total RNA isolation from leaves

Using an RNA extraction kit (Z3100; Bromage, USA), RNA was isolated from plant leaves. RNA quality was assessed using an ethidium bromide-stained agarose gel, and its concentration was measured using a Nano drop spectrophotometer.

Complementary DNA (cDNA) synthesis by reverse transcription

After total RNA extraction, complementary DNA (cDNA) was generated by reverse transcription using the GoScript™ Reverse Transcription System (A5000; Promega, USA) according to the manufacturer's protocol, with 1 μL of total RNA and the Oligo(dT)15 primer. Incubation conditions included 5 minutes at 25°C , 60 minutes at 40°C , and 15 minutes at 70°C . The cDNA was amplified using specific primers for the target gene and a primer for the α -tubulin gene as a reference gene (housekeeping gene) Table 1. PCR was conducted with the following program using Master Mix (Geneaid) Table 2. 1 cycle per denaturation step at 95°C for 5 min; 30 cycles of denaturation/annealing/elongation at 95°C for 30 s/ 60°C for 30 s/ 72°C for 1 min followed by one cycle final extension at 72°C for 7 min. resulting PCR amplicons were analyzed, and the reactions were loaded on ethidium bromide stained 1% agarose gels for visualization. The quantification of the band was conducted utilizing scanning density measurement with BIO-RAD (Image Lab 6.0.1). The measurement yielded band intensity as the unit of expression. The equation established the relative expression of target genes (RE) (Martiansyah *et al.*, 2018) [17].

Equation RE = expression level of target gene/expression level of reference gene.

Data analysis

Use analysis of variance (ANOVA) to data analyzed, and mean differences were compared by the Least Significant Difference (LSD0.05) test by GenStat program.

Table 1: Specific primers employed in Semi quantitative PCR

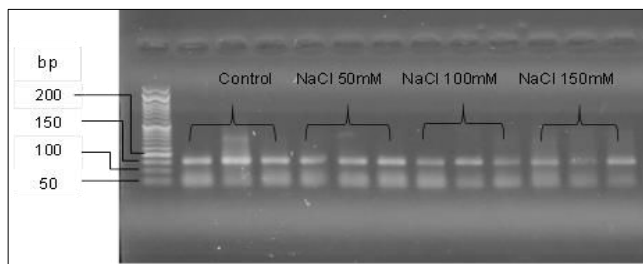
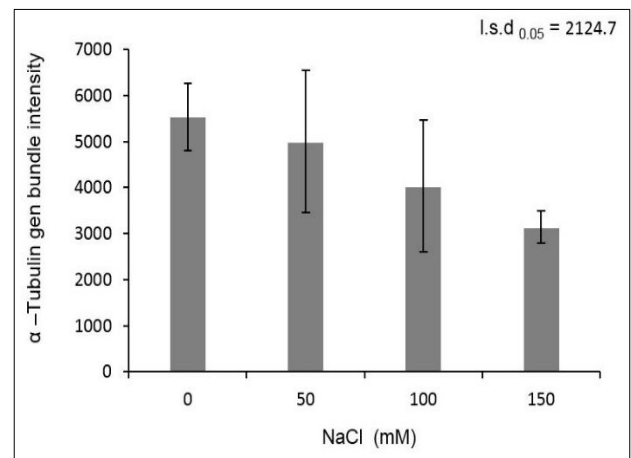
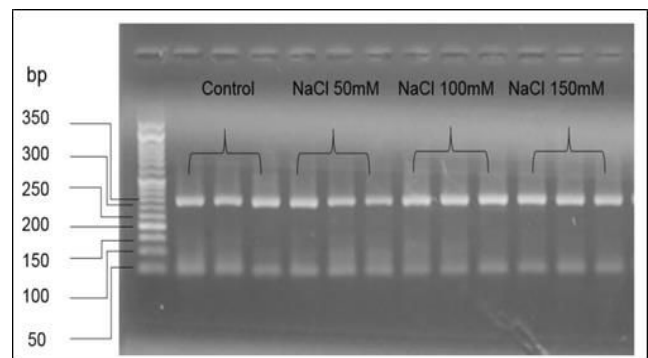
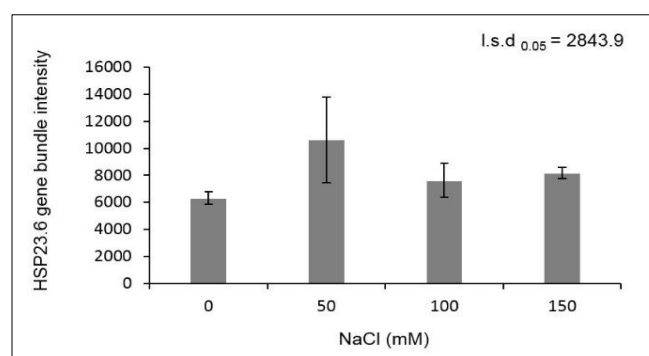
Gene		Primer Sequences	Product size	Accession number
<i>sHSP 23.6</i>	F	CAAGGTCATCCTCTTCATCG	339 bp	AF097363
	R	TTCTCGACGTACTTGCGGTA		
<i>α-Tubulin</i>	F	GTACATGGCCTGCTGTCTCA	150 bp	U76558
	R	CCTGGTGGCTGGTAGTTGAT		

Table 2: Components of the PCR reaction

Components	Volume
Master mix	5μl
cDNA	5μl
Forward primer	1μl
Reverse primer	1μl
Nuclease free water	8μl
Total volume	20μl

Results and Discussion

The statistical analysis results in Figures 1 and 2 showed a significant decrease in α -tubulin gene bundle intensity with an increase in NaCl concentrations, specifically at 100 and 150 mM concentrations, compared to the control treatment. The results shown in Figures 3 and 4 illustrated that elevated NaCl concentrations caused a significant enhancement in gene bundle intensity of HSP 23.6 at concentrations of 100 and 150 mM, relative to the control treatment. In comparison, the 50 mM treatment did not cause any significant increase compared to the control.

**Fig 1:** Products amplified by PCR of α -tubulin, utilised as a housekeeping gene**Fig 2:** The impact of treatment using varying concentrations of NaCl on α -Tubulin gene bundle intensity**Fig 3:** PCR amplification products of HSP23.6 (target gene)**Fig 4:** The impact of treatment using varying concentrations of NaCl on HSP 23.6 gene bundle intensity

The analysis of variance presented in Figure 5 revealed an up regulation in the expression of the HSP23.6 gene in wheat leaves subjected to NaCl stress compared to the control treatment of the examined cultivar. An increase in the relative expression rate of the HSP23.6 gene was noted. A significant difference was detected at 100 and 150 mM

concentrations, resulting in values of 3.05 and 3.28, respectively, in contrast to the control treatment, which exhibited the minimal gene expression level, recorded at 1.15. Furthermore, the 50 mM concentration showed the least gene expression level in relation to the 100 and 150 mM concentrations.

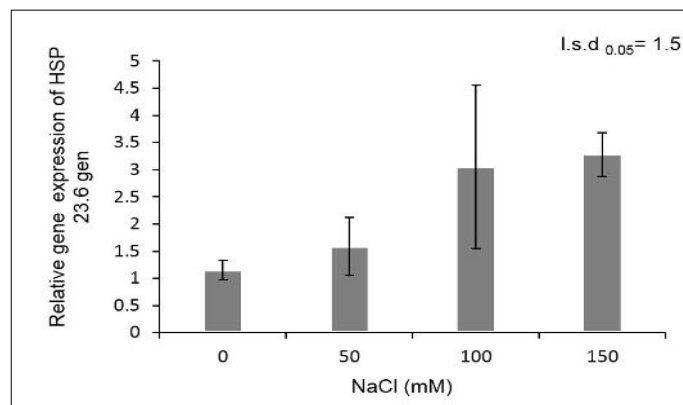


Fig 5: Effect of treatment with various concentrations of NaCl on relative gene expression in HSP 23.6 gene'

Gene expression of stress-related proteins, including M-sHSPs, is changed by many abiotic stimuli, and mitochondria are key components of the plant's stress response and free radical detoxification, as they generate reactive oxygen species during electron transport. (Escobar *et al.*, 2021) [8]. The study showed that treatment with 100 and 150 mM concentrations of sodium chloride increased gene expression of the 23.6HSP protein compared with the control group. Gene expression of sHSP 23.6 was observed in all treatments, whether in sodium chloride-treated or untreated wheat plants. When plants are subjected to salt stress, many transcription factors and genes can be upregulated or downregulated to produce specific gene products, such as proteins or RNA (Gupta & Huang, 2014) [10].

The synthesis of various protein assemblies is induced when organisms are exposed to stressors; these are known as stress-induced proteins, including heat shock proteins (HSPs), and are common phenomena in all living organisms that rely on them as molecular cofactors (Al-Wahaibi, 2011; Gupta *et al.*, 2010) [3, 11]. Protein stability depends on a set of these companion proteins that contribute to the assembly of polypeptide chains, ensure their proper folding, and transport within the cell. Exposure to stress causes damage to protein structure, and this is where the role of molecular cofactors comes into play, with increased production to prevent incorrect protein folding, as well as to facilitate the breakdown and rebuilding of damaged proteins (Finka *et al.*, 2016) [9]. Previous studies suggested that sHsps could potentially exert key role in plant adaptation to environmental stresses. MsHSPs exhibit increased expression in the salt-treated maize plants, to improve the mitochondrial electron transport effectiveness (Hamilton & Heckathorn, 2001) [12]. The results are consistent with those Al-Kutaibi *et al.*, (2021) in durum wheat plants under salt stress (200 mmol NaCl). The authors found that under salt stress the genes are likely up regulated such as family of HSPs including HSP101, HSP70 and HSP26. 3, and HSP17. 8. Particularly, the M23 and T11 highest increase of protein contents compared to the other varieties which clearly indicates enhanced resistance to salt stress. The results that gained by Subba *et al.*, (2013) [22] on chickpea plants treated under osmotic stress, showed the accumulation of HSPs. sHSP in drought resistant cultivars against susceptible ones at the initial stage of growth period. The expression of heat shock

genes in wheat leaves at either 0 mM or 50 mM concentration in control treatment can be attributed the sHSP effect during growth and development under both normal and stress conditions and processes such as photosynthesis, cell membrane stability, and the plant's response to heat stress.

Escobar *et al.* (2021) [8] demonstrated that *Arabidopsis thaliana* plant with decreased expression of M-sHSPs showed significant growth disturbances, leading to reduced plant size and adverse effects on physiological performance, including photosynthetic efficiency and heat stress response, while the formation of ROS and accumulation causes increased permeability of cellular membranes, emphasis the cellular damage resulting from the decline of M-sHSPs. The reduced gene expression following treatment with 50 mM NaCl may result from this stress level not being insufficient to stimulate an increase in gene expression. The reaction of HSPs is dependent upon nature of stress, whether biotic or abiotic, the plant genotype, the duration of exposure, and the developmental stage of the plant (Dodds & Rathjen, 2010) [6].

Conclusion

The findings of this study demonstrated that HSP 23.6 gene regulation in wheat leaves under normal growth conditions. Gene expression also increased in response to salt stress conditions generated by sodium chloride (NaCl). This illustrates the significance of this protein's expression in regularly growing plants and its role in aiding plants to cope with salinity stress.

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