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Expression of Heat Shock Protein HSP90 in Genomic-DNA of Chickpea (Cicer arietinum L.) Callus by Heat Shock Treatment

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Abstract:

This study was able to detect of the expression activity of heat shock proteins *HSP90* and heat transcription factors HSFs for the first time in callus cultures of chickpea, *Cicer arietinum* L., that exposed to abiotic shocks, grown on MS medium supplemented with 1.0 mg L^{-1} naphthalene acetic acid (NAA) and 2.0 mg L^{-1} benzyl adenine (BA). Heat shock proteins HSPs were constructed for increase of withstand long-term physical shocks, and production of resistant to heat chickpeas plants, this shock was enhancement of tolerance of chickpea callus to abiotic stresses (high - temperatures). Results enhanced the ability of chickpea callus to abiotic stresses bearing and induce of HSF genes to heat shock proteins *HSP90* production quickly to removing denatured proteins, avoid apoptosis, thus, supporting tolerance to the sudden action of these shocks. Expression activity of heat shock genes and transcription factors by determined based on polymerase chain reaction qPCR, that explained the gene activity increasing at shocks intensity increased,

And exposure period simultaneously with the increase in the recorded RNA and DNA concentrations. Expression of heat shock genes and transcription factors in the current study was determined by adopting the qPCR, results of which demonstrated an increase in gene activity with at the heat shock intensity increase, with increases of DNA, RNA concentrations. Nitrogenous bases sequences of thirty samples of callus of embryos, hypocotyl which exposed to heat shocks, and the comparison callus were determined by the specific polymerase chain reaction sPCR for detection of *HSP90* genes. The purified DNA of chickpea plant was match with genes in the NCBI Genetic Data Bank, with many mutations whose effects varied in the amino acids type, thus, contributed to the identification of heat shock proteins. Phylogenetic tree data confirmed the existence of a close relationship between the chickpea cultivar under study and the Indian and Egyptian cultivars registered in NCBI by adopting molecular evidence represented by the genes of heat shock proteins.

Keywords: Chickpea (*Cicer arietinum* L.), Heat stress, Heat shock protein *HSP*90, RNA sequencing, gene Expression

1. Introduction

Chickpea *(Cicer arietinum* L.) is considered one of the most important types of food legumes in the world in economic terms, and cultivated at a rate of 14.56 million/hectares with a productivity of 11.68 million tons in more than 55 countries (1). The legume family (Fabaceae) constitutes the third largest family with more comprises than 650 genera and 18000 species, the genus Cicer includes 43 cultivated species (2). The annual chickpea plant is self-pollinating 2n=16 (3).

Modern biological technologies have contributed to improving the production of leguminous crops and developing high-quality crop varieties. Legumes are among the crops sensitive to biotic and abiotic stresses (4). Plants deal with environmental stresses and cause a significant loss in production, because they pose a serious threat to plant survival. Despite this, plants have evolved a diverse set of adaptation mechanisms to resist environmental conditions through increased expression of heat shock genes (5). These genes activate metabolic pathways in plants to withstand the harmful effects of various stresses including temperature, drought, salinity, photooxidation,

Heavy metals, high intensity radiation, wounds and other environmental factors (6). Most of the genes responsive to heat stress (HS) are involved in primary and secondary metabolic processes, translation, transcription, regulation and response to biological processes in plants such as calcium transport, phytohormones, sugars and lipids or protein synthesis including phosphorylation (7).

Heat shock proteins (HSPs) are considered essential in acquisition of heat tolerance in plants (8). The sources mentioned the positive effects of heat shock in a number of leguminous plant systems and its stimulation of metabolic pathways in cells represented in building new proteins, as in chickpea and soybean plants (9). Heat shock proteins (HSPs) function as molecular chaperones, successfully involved in the quality and type control of protein within living cells to maintain cellular homeostasis under environmental stress conditions (10). Heat shock of plant cells contributed to improving the plant species through changes in the fluidity of cell membranes and the accompanying changes at the molecular level. The expression of genes related to the regulation of the transcription process and synthesis of various proteins (11). Therefore, some plants respond to such stresses by reprogramming their proteins to ensure a steady state of metabolic processes that help them to survive and function under stress conditions (12). The genetic basis of chickpea is expected to be an important factor in stress tolerance by restoring cellular homeostasis and its direct effect on cellular and physiological changes (13).

Among the families of stress-responsive genes, there are HSP90. genes Known to be involved in both biotic and abiotic stresses and heat shock protein (*HSP90*) The family is important, because it not only regulates responses against various biotic and abiotic stresses in plants, but also play an important role in various developmental processes (14). Expression *HSP90* against high temperatures is one of its best properties' responses. *HSP90* escorts are basically, and expressed in most organisms under normal conditions, while, expression increases significantly under stress. *HSP90s* play a vital role in plant development, stress response and disease resistance (15). Recent studies have indicated that heat shock protein *HSP90* is one of the most abundant proteins in living cells when exposed to abiotic stresses (16). This manuscript aimed to study the role of heat shock in chickpea callus growth, characteristics, and to identify heat shock genes *HSP90* in exposed tissues to these shocks.

2. Materials and Methods

Embryos and Hypocotyl Production

Cicer arietinum L. "Desi variety" seeds were sterilized by immersing in 96% ethyl alcohol for 2 min., submerging in sodium hypochlorate solution (NaOCl 5%) for 5 min., then washed four times with sterile distilled water. Sterilized seeds were transferred to the surface of 25 ml of solid basal MS medium free from growth regulators (17). in 100 ml glass containers and incubated in culture room 23°C, light intensity of 2000 lux at 8/16 light / hour. Sterile seeds and seedlings were used as source of embryos and hypocotyls, respectively.

Callus Initiation

Sterilized seedlings 12-15 days old were excised, and divided into small pieces of 1.5 cm length. Embryos and hypocotyls fragment of each seedling were cultured on agar solidified MS medium containing 3% sucrose, provided with 1.0 mg L^{-1} NAA and 2.0 mgl-1 BA (20) for callus initiation. The end cation was MS+NAA+BA cultures were kept at culture room conditions.

Embryos and hypocotyls callus exposed to heat treatment

A set of 30-day-old callus samples weighing 1 g were taken from hypocotyls and embryos and placed in sterile glass containers of 100 ml and exposed to each temperature of 30, 35, 40, 45 and 50 ° C. Exposure time 5, 10 min. named short-term heat shock, and in the other treatment it appeared at the same temperature but for 15, 20 min., that called long term heat shock (18). Use a hot waterbath in the required degrees for both treatments. After exposure, all samples are placed directly in a baker of room temperature water to reduce their temperature. After exposing all samples were cultivated in MS medium mentioned previously.

Assay *HSP90* genes expression in exposed callus to heat shock

The reverse transcription PCR was used to identify the cDNA strand by adopting mRNA as the genetic material isolated from callus to be the cDNA reverse amplification product. The basis for a second step was the quantitative amplification of the reverse amplification product by quantitative real time (qPCR) technique to assess the number of mRNA copies produced by expression of the target gene (Table 1).

Table 1: Sequence of primer set used in the study.

Gene	Sequence 5'-3'	initiator	size (bp.)
*HSP*90	GCCTCCGTGCTTTGGTAA	Forward	356 base pair
	TGCCAAGAACCTCACCAGTA	Reverse	

RNA isolation and RNA-seq

The extracted RNA was reverse transcribed to cDNA using Prime Script 1st strand cDNA synthesis kit Miniprep RNA extract kit (S. Korea) Gene all. The cDNA was performed on Rotor-gene Q real-time PCR system with SYBR Green PCR Kit (Wiz bioScript-TM cDNA synthesis Kit) and transcript specific primers. The cDNAs have been checked expression of heat stress marker (GMHsp90C2.1). This can validate for the plants applied heat stress (19). PCR primers were designed with the parameters of 17-25 nucleotide lengths, and 150-200 product size. PCR program was set as 5 minutes at 95°C, 40 cycles each of 20 seconds at 95°C, 30 seconds at 59°C. Melt curve analysis was performed at the end of every PCRs. Threshold cycle for each reaction was normalized with expression value of *Act B* (B-actin). Relative expression values were calculated by 2–CT method (19). All experiments were processed with three biological replicates to obtained high credibility of experiments.

Genetic determinants amplification and sequencing alignment

sPCR Master Mix was 10µl (Gene All, S.Korea), additional components were1µl (10µM) of each of forward and reverse primer set (Ella Biotech ,Germany), 4µl of gDNA (10ng\ml), the final volume was completed to 20µl with nuclease free water (4µl). The ITS1, ITS4 primer set that used in this research was listed in table one. Amplification steps were initial denaturation 95°C for 4min, then 35 cycles of each denaturation at 95°C for 30 sec., annealing temperature 60°C for 30 sec. to hybrid the primers, extension at 72°C for 30 sec., and final extension at 72°C for 5 min. Amplification products were purified using PCR purification Kit (Gene All, S.Korea),which was sequenced using genetic lyzer (Applied Bio-systems3500. USA). The sequence was aligned (Mega X-software) and assembled via National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST).

Phylogenetic Analysis

Protein sequences of chickpea *HSP90* were aligned using phylogenetic tree was generated based on the MEGA software version 6.0. (Phoenix, AZ, USA) (20). and protein formation of *Cicer arietinum* heat shock 90 kDa protein gene using Expasy software (21).

3. Result

Detection of heat shock protein *HSP*90 gene expression activity in embryogenic and hypocotyls callus exposed to heat shock.

Gene expression of *HSP*90 (heat shock protein) samples were determined by RT-PCR techniques. These techniques revealed the activity of the *HSP*90 genes of the standard gene Beta Actin in the early stages of the amplification processes during the reaction (Fig.1).

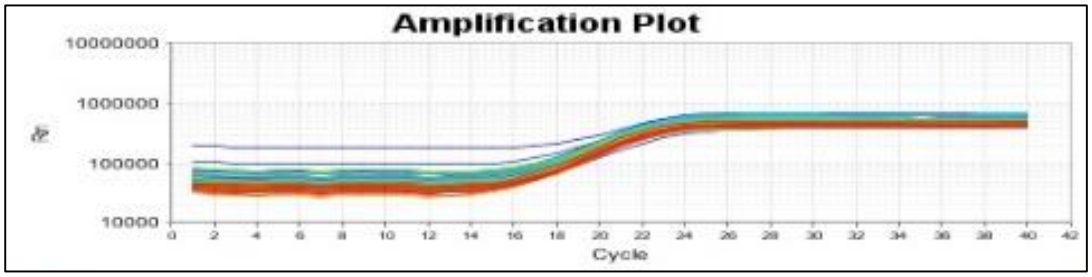


Fig.1: Expression of the β Actin gene standard for heat shock proteins *HSP90* of chickpea *Cicer arietinum* L.

Each of embryonic and hypocotyls callus exposed to heat shock and control samples was determined by adopting the reading of a Nano Spectrometer (nano, 2000, Thailand) at wavelength of 260 / 280 nm. Nuclear RNA in the callus exposed to heat shock 45 ° C / 15 and 20 min., additionally to non-exposed callus (Table 2).

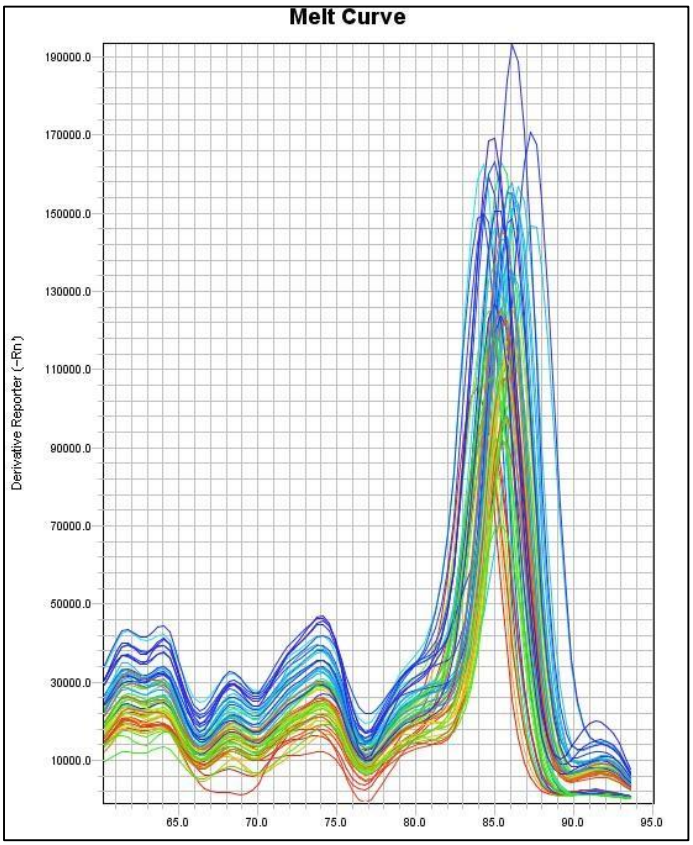
Table 2: Concentration and purity of RNA isolated from tissues of embryonic callus and hypocotyls callus of chickpea *Cicer arietinum* L. exposed and not exposed to heat shock.

Embryonic calli			Hypocotyls calli	
Samples °C/min	Purity 280 / 260	RNA (ng/µ) Hypocotyls	Purity 280 / 260	RNA (ng/µ) Embryo callus

control	1.9	6012	1.9	8149
30	1.7	6582	1.7	6635
35	1.9	7313	2	7283
40	1.8	9377	1.8	12462
45	1.7	9628	1.9	16539
50	1.8	11901	1.8	6175

Detection of *HSP*90 gene expression activity in embryonic and hypocotyls callus exposed to heat shock.

Samples of RNA samples extracted from the genetic repertoire were amplified for the purpose of stabilizing the genetic expression of HSP90 genes in RNA samples extracted from embryonic callus and hypocotyls callus under study. High temperatures degree stimulated the activity of HSP90 gene expression and the melting curve of HSP90 heat shock protein genes, which shows the absence of dimer in the callus samples under study (Fig.2).



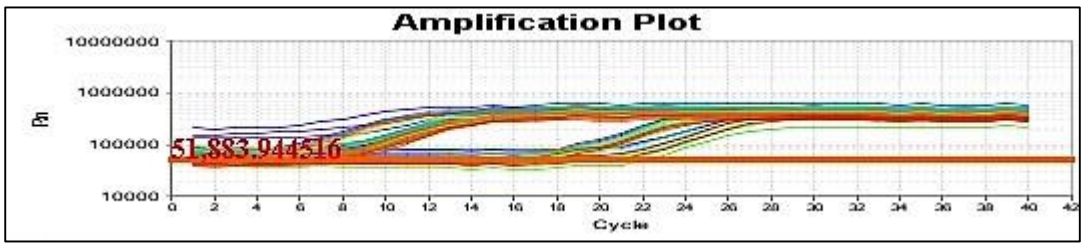


Fig. 2: Melting curve and genetic expression of heat shock protein *HSP90* genes of embryonic and hypocotyls calli of *Cicer arietinum* L. exposed to heat shock.

The achieved results (Table 3) demonstrated a high increase in the activity of the *HSP90* gene responsible for shock proteins in terms of increase in expression levels of the Δct gene when callus exposed to heat shock with a selected duration 5-20 min. For the shock of the embryonic callus exposed to 45℃ and 50℃, compared to samples exposed to low temperatures and after thirty days of exposure to both STHS and LTHS which recorded a slight increase in activity of *HSP90* gene. Long-term heat shock achieved an increase in the amount of RNA, as it was noted that its purity was suitable for performing simultaneous sequence replication qPCR.

Table 3: *HSP90* gene expression activity of embryonic callus of chickpea *Cicer arietinum* L. exposed to heat shock

Embryonic callus C / min°	Beta actin	*HSP*90	Δct
control	18.83878	23.09563	0
30 / 10	19.12056	20.55118	-2.826241493
35 / 5	19.41429	10.53783	-13.13332415
40 / 20	19.31317	9.936205	-13.6338253
45 / 15	18.9709	9.407584	-13.82017231
50 / 15	19.29653	7.027575	-13.82017231

Whereas, data of qPCR products indicated that heat shock supported high levels of Δct and stimulated the *HSP90* gene for hypocotyls callus.

It was noted that the activity of the gene stimulating heat shock proteins increase at its highest levels, and the amount of RNA increased with highe temperatures and exposure time compared to the rest of treatments and controls (Fig.3).

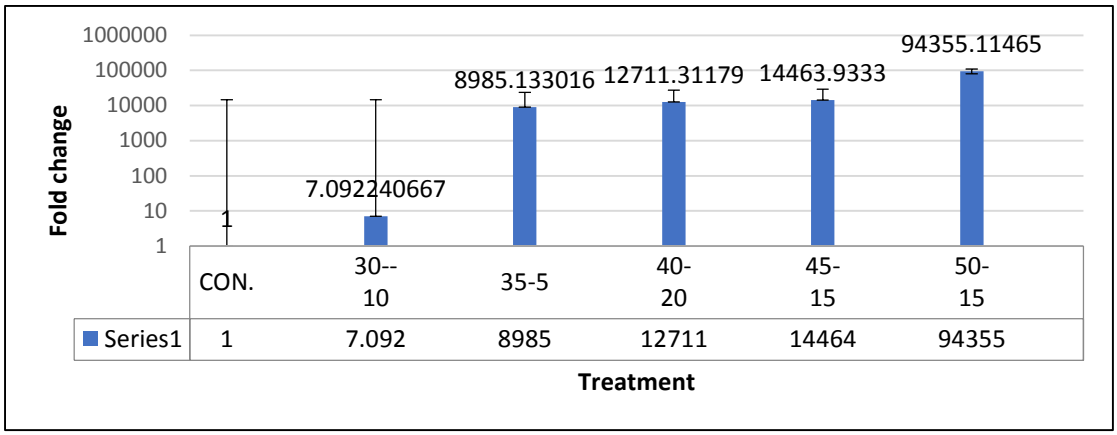
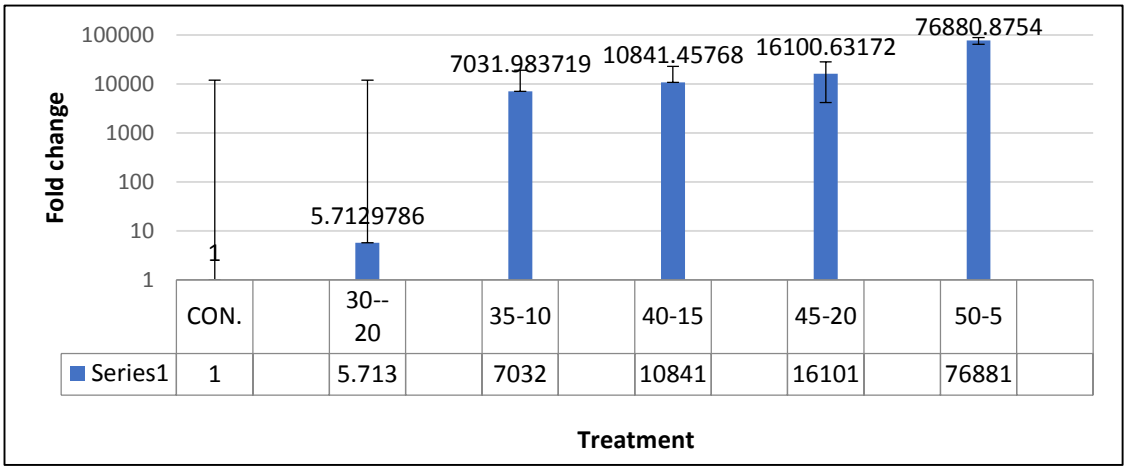


Fig. 3: Histogram of *HSP*90 gene expression activity of the embryonic callus of chickpea *Cicer arietinum* L. exposed to heat shock.

Data of qPCR products (Table 4) indicated that heat shock supported high levels of Δct and stimulated *HSP*90 gene of hypocotyls callus (Fig.4). It was noted that the activity of the gene stimulating heat shock proteins increased at its highest levels, and the amount of RNA increased with higher temperatures and exposure time compared to the remaining of the treatments and comparisons.

Fig. 4: Histogram of the *HSP*90 gene expression activity of the hypocotyl's callus of chickpea *Cicer arietinum* L. exposed to heat shock.



hypocotyls callus C / min°	Beta actin	*HSP*90	Δct
control	18.83878	23.09563	0
30/20	19.03487	20.77749	-2.514243126
35/10	18.83764	10.31478	-12.77971601
40/15	19.05466	9.907241	-13.40427113
45/20	19.32304	9.605064	-13.97482967
50/5	19.12325	7.149771	-16.23033714

Table 4: *HSP*90 gene expression activity of the hypocotyl callus of chickpea *Cicer arietinum* L. subjected to heat shock.

Molecular investigation of the genes of heat shock proteins *HSP*90 in embryogenic and hypocotyls calli of chickpea *Cicer arietinum* L.

Isolation of genomic DNA

The concentration of deoxygenated DNA extracted from samples of embryogenic and hypocotyls calli subjected to physical shock and control samples were determined by adopting the reading of a nano spectrophotometer (Nano 2000, Thailand) at wavelength of 260/280 nm and DNA concentration in callus was superior. subjected to heat shock 40°C / 20 min.

Nitrogenous base sequences for PCR products

Sequencing technology aimed to determine the relative positions of nitrogenous bases of DNA pieces obtained from the specialized replication reaction of DNA samples extracted from callus samples demonstrate to confirm the diagnosis of chickpea callus by adopting sequence alignment of the nitrogenous bases of the HSP90 genes.

Molecular expiantion of the *HSP*90 gene.

The results of PCR indicate performed for DNA samples isolated the extracted DNA products were obtained from samples of one size (356) base pair (Fig.5) and it has the ability to encode 237 amino acids in chickpea plant.

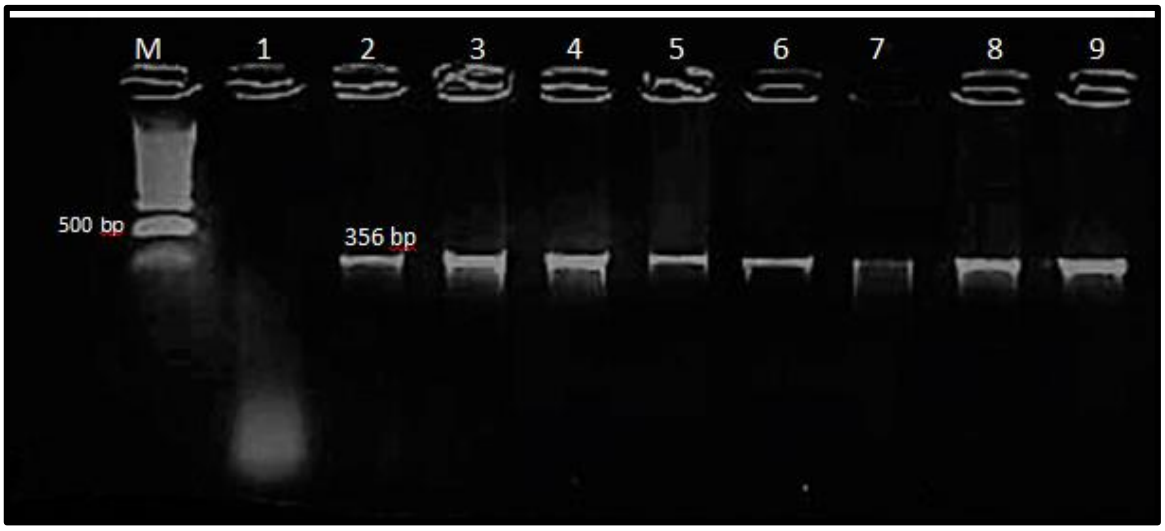


Fig. 5: Bundle of DNA bands of *Cicer arietinum* L. amplified for the *HSP*90 gene.

- **Lane (M) represents the molecular scale**
- **Lane (1) represents the control**
- **Lane (2 - 9) represents a positive result for the presence of the bands representing the gene *HSP*90 (356) base pair of the embryo and hypocotyls callus of chickpea.**

The results showed the alignment of the nitrogenous bases of 356 nitrogenous bases of chickpea calli samples with their complementary nitrogenous bases of *HSP*90 gene, as follows:

The sequences of embryogenic callus samples subjected to heat shock were entered into the NCBI-BLAST program available at https://blast.ncbi.nlm.nih.gov/Blast.cgi and the MEGA6.0+ BLAST program to analyze these sequences and show their affinity with the existing sequences. Results of analysis showed a significant similarity of -99% between these sequences and the sequences recorded in the Gene Bank with the number XM_004491473.2. (Table 5) shows a comparison of the DNA sequences of the samples of the embryonic callus, as it was noted that there are areas of replacement there were mutations of the type of transition at the site (573) that did not affect the genetic code and then the type of amino acid that constitutes the shock protein *HSP90*.

Table 5: Analysis of the DNA sequences of *HSP*90 genes from chickpea *Cicer arietinum* L. embryonic callus exposed to heat shock.

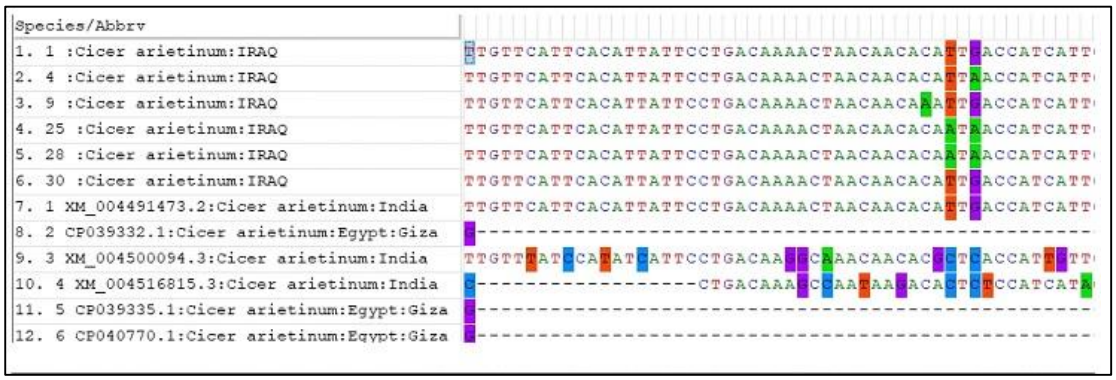
Source: Cicer arietinum heat shock 90 kDa protein								
Sample °C / min	**Type of substitution**	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Identities**
Control	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%
/ 10 30	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%
35 / 5	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%
/ 20 40	Transition	402	G\A	TTG\TTA	Leucine\ Leucine	Silent	ID: XM_004491473.2	99%
/ 15 45	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent		
/ 15 50	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%

Analysis results (Table 6) of nitrogenous bases sequences of the heat shocked hypocotyls callus samples subjected to heat shock and their convergence with the existing sequences recorded in the gene bank. The areas of substitution between the nitrogenous bases were observed between callus sample subjected to heat shock 35º C/10 min and registered in NCBI. It was a substitution site 398 and the mutation affected the genetic codes and then the encoded amino acids, as amino acid threonine was replaced by lysine. While the mutations in the callus samples exposed to heat shock did not affect the type of amino acids encoded.

Table 6: Analysis of the DNA sequences of *HSP*90 genes from chickpea *Cicer arietinum* L. hypocotyls callus exposed to heat shock.

Source: Cicer arietinum heat shock 90 kDa protein								
Sample C / ° min	**Type of substitution**	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Identities**
Control	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%
30/20	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%
35/10	Transvertion	398	C\A	ACA\AAA	Threonine\ Lysine	Missense	ID: XM_004491473.2	99%
40/10	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent		
40/15	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%
45/20	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%

50/5	Transition	573	G\A	GAG\GAA	Glutamic acid\ Glutamic acid	Silent	ID: XM_004491473.2	99%



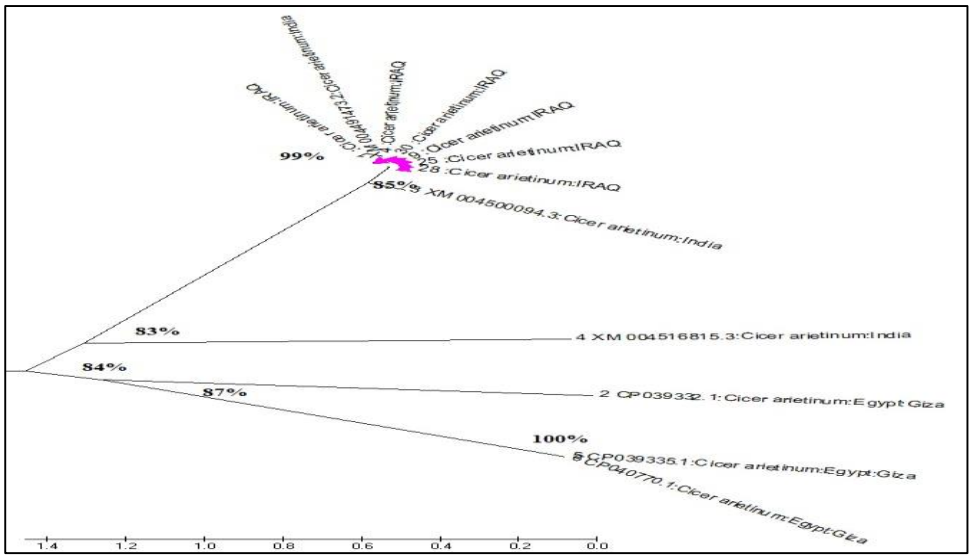


Fig. 6: Neighbor-joining tree *Cicer arietinum* heat shock 90 kDa protein gene.

It was noted from the genotype tree diagram that the standard cultivars registered in NCBI were from countries far from Iraq and the region in general. This indicates that the current study was one of the first studies at the local level that concerned itself with the genetic characterization of chickpea calluses based on the genes of heat shock proteins HSP90, as compared with international varieties, the presence of any genetically diagnosed local chickpea cultivar was not recorded in the gene bank. This results of the current study were able to reveal *HSP*90 genes from the chickpea genetic tree based on genome sequence information available in the NCBI Gene Bank which has the potential to genetically improve tolerance to abiotic stresses.

The heat shock protein HSP90 was drawn by inserting an alignment sequence of the nitrogen base sequences of the genes of heat shock proteins *HSP90* for selected samples of chickpea callus and based on the mutations induced in the Expasy program as shown in the figure below.

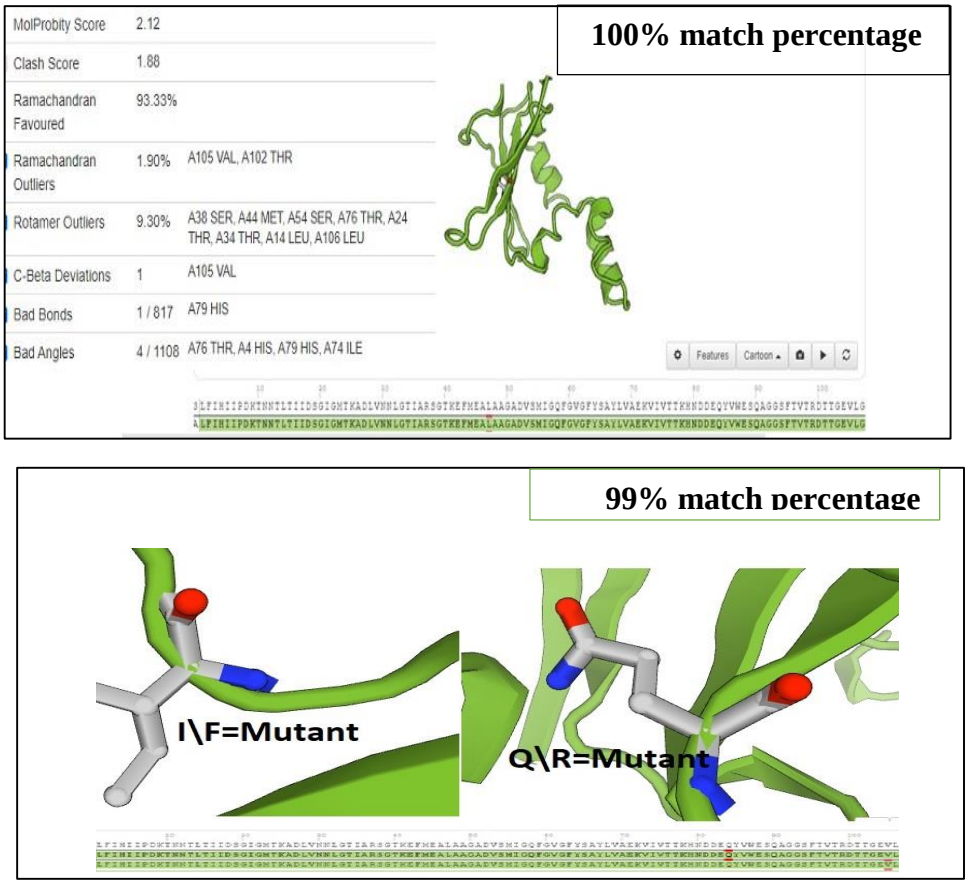


Fig. 7: Conformation of protein from chickpea *Cicer arietinum* L. heat shock 90 kDa protein gene

4. Discussion

The success of the current study in finding an integrated protocol for obtaining chickpea plants by means of plant tissue culture technique is due to the ex vivo response of chickpea plants combined with the appropriateness of the selected hormonal interventions. Moreover, the need to genetically improve chickpea by obtaining cell lines resistant to environmental stresses (22). Physical shock provokes multiple responses that include a series of chemical and molecular changes,

And that multiple stress-responsive mechanisms work in concert and synergistically to avoid cellular damage (23). This is due to the increase in the construction of heat shock proteins *HSP90*, as cells respond to stress to increase their genetic expressions that encode for the construction of these proteins this is due to their stimulation of heat shock factors HSFs, which play an important role in activating the *HSP*90 gene group. The construction of shock proteins HSP90 was also stimulated when tissue cultures of soybean and Arabidopsis were exposed to a degree 40 centigrade (15). The heat shock in the current study resulted in an increase in the accumulation of specific shock proteins HSPs, and synthesis synthesis of a group of proteins is an important event to adapt to heat stress. This is due to the increase and abundance of a specific number of enzymes that stimulate the synthesis of amino acids such as 5-methyl tetrahydroptroyltri glutamate- homo cysteine methyl transferase- Cystathionine gamma- Synthase., thereonine synthase (8). Among the indicators that explain the construction of heat shock proteins HSPs is that they stimulated the activity of genetic expression and encode its construction by activating a group of genes of shock proteins *HSP*90, especially at temperatures of 45 º C or less, as they provided protection for the callus from damage and restored its damaged cells to their physiological activity, and preserved the structure of damage-induced proteins (24).

In this study obtaining callus tissues that are resistant to abiotic shocks is represented by the activity of genes of heat shock proteins *HSP*90 in mitochondria of callus tissues, which have a vital role in heat tolerance through the mutations formed and activating the transcription of target genes *HSF*s. This affect the stress tolerance mechanism as they are considered a regulator. Key to signal perception, transmission and control of expression of effector genes (25).

Confirmation the diagnosis of *HSP*90 genes in callus of chickpea *Cicer arietinum* L. It proved the presence of heterogeneity at the nitrogen base level at site 250 represented by mutations R and G. A study (26) showed thmentionede discovery of induced mutations of the genes of heat shock proteins *HSP90* in rice, which showed a characteristic tolerance to HS stress. The shock proteins are able to tolerate a large numbers of mutations without undergoing significant changes in function.

The exploration of genes of heat shock proteins *HSP90* through molecular analysis of the genetic relationship between species of the genus Chickpea has a great affinity of up to 99%,

while it was found that there is a level of convergence of 83% among the other chickpea species under study, which indicates bio genetic diversity and that the standard chickpea cultivars The records in the National Center for Biotechnology Information (NCBI) were from countries far from Iraq and the region in general.

5. Acknowledgment

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