Biotechnology Journal International

24(3): 24-34, 2020; Article no.BJI.56712
ISSN: 2456-7051
(Past name: British Biotechnology Journal, Past ISSN: 2231–2927, NLM ID: 101616695)

Identification and Expression Analysis of Stress Responsive Genes in Lentil (*Lens culinaris*)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JS, PK, SK and AS conceived and designed the experiments. Authors AY, Himanshi, Shruti and SK performed the experiments. Authors AY and SK analyzed the data. Authors JS and PK contributed reagents/materials/analysis tools. Authors AY, Himanshi, Shruti, PK and JS wrote the paper. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2020/v24i330105
Editor(s):
(1) Dr. Ghousia Begum, CSIR-Indian Institute of Chemical Technology, India.
Reviewers:
(1) Bernard China, Quality of laboratories, Belgium.
(2) Mankesh Kumar, Bihar Agricultural University, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/56712

Original Research Article

Received 01 March 2020
Accepted 05 May 2020
Published 18 May 2020

ABSTRACT

Plants during their growth, experience periodic stress conditions both abiotic (adverse environmental conditions) as well as biotic (infection by pathogens). They appear to respond to these adverse conditions by modulating the expression of many genes. One of the pronounced effects of stress on plant is the enhanced synthesis of a set of proteins-termed ' stress proteins'. Lentil contains asset of genes/proteins which helps this crop to overcome abiotic stresses. In the present study, HSP70 (Heat Shock Protein), LEA (Late Embryogenesis Abundant) and Aldolase genes were identified and cloned in pTZ57RT vector followed by sequencing. Expression analysis was done through Q-PCR which was assessed by using cDNA from all the heat, drought and salinity stressed and unstressed lentil cotyledons. The highest level of transcript of HSP70 was realized upon exposure to heat at 45°C for 3 hour followed by at 45°C for 2 hour and lowest at 40°C for 1hour. LEA gene was identified under drought and salinity stress and highest transcript was at 20% PEG for 3 hour (drought stress) and in salinity stress highest transcript was at 150 mm for 6 hour. For Aldolase gene highest transcript was recorded after 3, 6 and 12 hr at 100 mM, 150

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mM, 200 mM of salinity stress respectively. From these studies it can be concluded that heat shock protein gene, LEA, and aldolase present in lentil which can be exploited in overcoming the abiotic stresses for obtaining the higher productivity in crop plants through genetic engineering.

Keywords: High stress; heat shock proteins; LEA; aldolase; lentil; Q-PCR.

1. INTRODUCTION

Plants are exposed to multiple environmental stresses throughout their life cycle. Abiotic stresses such as heat, drought (moisture), and high salinity affect most areas of the world and affect plants by directly reducing its survival in the natural environment and productivity in agriculture. These stresses produce extensive changes in the regulation of gene expression, gene activation/gene suppression, signal transduction pathways biochemical modulation and proteomic machinery which lead to the survival or death of the affected plants [1]. Adverse environmental conditions, such as high temperatures, drought and salt severely limit the growth and geographical distribution of many plants. At the same time, plants have evolved many mechanisms to tolerate these adverse conditions [2] and the regulation of gene expression is critical for these processes. Crop plants such as soybean, pea, maize, and wheat etc. adapt to theses environmental stresses by synthesizing HSPs (Heat Shock Protein), LEA (Late Embryogenesis Abundant) and aldolase genes in response to high temperature, drought and salt stresses.

Genes for Heat shock proteins (HSP) are present in plant under normal conditions, but are expressed only when plants are exposed to high temperature stress and are involved in protein folding, degradation of non-native proteins, assembly and translocation of native proteins in many normal cellular processes [3,4,5,6]. HSP are responsible for stabilizing proteins and membranes and are involved in protein refolding under stressful environments. They also play an important role in protecting plants against stress by altering normal protein conformation and thus maintain cellular homeostasis [7,8,9,10].

A Late Embryogenesis Abundant (LEA) protein is a group of hydrophilic and low molecular weight (10-30 kDa) proteins, which are involved in protecting higher plants from damage caused by environmental stresses, especially drought. LEA protein synthesis, expression and biological activities are regulated by many factors (e.g. developmental stages, hormones, ion binding, signal transduction pathways, cryoprotective, radial- scavenging, antifreeze functions) when expose to various stress factors, including drought, high-salinity stress, low- temperature, heavy-metal stress and perhaps also to biotic stresses.

Aldolase is also a key enzyme in plants, which is involved not only in glycolysis and gluconeogenesis in the cytoplasm, but also in the Calvin cycle in plastids. Aldolase catalyzes the reversible aldol condensation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) in glycolysis, gluconeogenesis, and the Calvin cycle [11]. Aldolases in plants have two isoforms, namely chloroplast aldolase and cytosol aldolase [12]. It may also play important role in sugar, abscisic acid (ABA) stress signaling in plants. The mRNA level of aldolases have been shown to increase in response to chilling and hydrogen peroxide stress in *Cadonopsis lanceolata*, high salinity, drought, and ABA stress in *Sesuvium portulacastrum* [13].

Legumes being one of the most important crops worldwide [14] are limited in terms of adaptability and productivity mainly by the abiotic stresses. Lentil (*Lens culinaris*) is an edible cold season food legume that encounters numerous biotic and abiotic stresses. It is highly remunerative crop which grows well under low input situation and provides a valuable and balanced protein source. Lentils can grow on various soil types, from sand to clay loam, growing best in deep sandy loam soils with moderate fertility and soil pH around 7.

In 2016, the global production of lentils was 6.3 million tonnes, led by Canada with 51% and India with 17% of the world total. In India total harvested area of lentil 1548106 acreage (hectare) and production was 1055536 tonnes in 2016 [15].

Lentil also contains a set of genes like Heat shock genes, LEA and aldolase gene etc. which protects it from stresses and maintain its productivity and physiological changes. Therefore, future efforts should be directed

toward the development of lentil cultivars with abiotic stress tolerance. Response to stress is complex and involves several factors including signaling, transcription factors, hormones, and secondary metabolites. Application of available approaches genomics, biotechnology and information technology may mitigate the detrimental effects of heat and drought through the use of agronomic management practices and the development of crop varieties with increased productivity under stress [16]. Hence, it is very important to identify stress tolerant genes from lentil.

2. MATERIALS AND METHODS

2.1 Plant material and Stress Treatments

The seeds of lentil were collected and were grown in growth chamber under regulated condition with temperature regime of 22-25°C and humidity of 100%. Plants were randomly selected into four groups (3 groups for the treatment & 1 group for control) at seedling stage (10 days old). Heat shock (HS) treatments were applied at 35°C, 40°C (moderate heat stress) and 45°C (extreme heat stress) for 1, 2 and 3 hr followed by overnight incubation. Moisture stress for LEA gene was given at concentration 20% PEG for 3 hrs, 6 hrs and 12 hrs and salinity stress (NaCl) for aldolase gene given at different concentrations of 100 mM, 150 mM and 200 mM for 3, 6 and 12 hrs. After each stress treatment, cotyledons were harvested and immediately frozen in liquid nitrogen and stored at -80°C till RNA was isolated [17].

2.2 Isolation and Cloning of HSP70, LEA and Aldolase Gene from Lentil

The primers used for the PCR analysis were designed by retrieving EST sequences of *Lens culinaris* from NCBI database www.ncbi.nlm.nih.gov trimmed by vecscreen http://www.ncbi.nlm.nih.gov/tools/vecscreen/ followed by preparation of contigs through Seqtrimnext www.scbi.uma.es/seqtrimnext. Further, contigs were used for designing gene specific primers specific to Heat Shock Protein/genes, LEA and Aldolase (Table 1). Total RNA was isolated (GeneJET plant RNA purification mini kit #K0801) and quantified, from 10 days old germinating lentil seedlings which were treated with heat, moisture and salinity stress. RNA integrity was verified in 1.2% agarose gel electrophoresis. After that cDNA were synthesized using gene specific reverse primer (RevertAid^{TM} minus first strand cDNA synthesis kit, Fermentas, Germany). Synthesized cDNA from lentil were used for PCR amplification of HSP70, LEA and Aldolase gene by using gene specific primers as given in Table 1. The amplified product was cloned in pTZ57R/T vector (Fermentas, Germany) and sequenced at DNA sequencing facility, Department of Biochemistry, South Campus, University of Delhi, New Delhi.

2.3 RNA Extraction and Quantitative Real Time PCR (qRT PCR)

For expression analysis through quantitative real time PCR, stresses were given to lentil cotyledon at different temperature and different concentration for different time variations and then total RNA was extracted using the GeneJET plant RNA purification mini kit #K0801, Thermo Scientific and quantification was done using spectrophotometer (SHIMADZU UV 1800). RNA integrity was verified in 1.2% agarose gel. cDNA synthesis was performed using gene specific reverse primers (RevertAid^{TM} minus first strand cDNA synthesis kit, Fermentas, Germany). cDNA was diluted to a final concentration of 1.0 ng μl^{-1}. Highly purified primers as given in Table 2 were used for the quantitative real time PCR amplification of HSP70, LEA and ALD for each stress conditions as well as for controls, expression measurements were performed using duplicate replications.

Quantitative PCR was performed in 25 µl reactions using gene specific primers, 2 µl of cDNA as template, 0.33 µl primers and the 1 µl iTaq ^{TM} universal SYBR Green SuperMix (BioRad, UK). Reactions were performed on the IQ5-Real-Time PCR system (BioRad, UK). The thermal profile for HSP70 was: 2 min at 95°C, followed by 40 cycles each consisting of 95°C for 10 s, 50°C for 30 s and 68°C for 30 s. For LEA and ALD 2 min at 95°C, followed by 40 cycles each consisting of 95°C for 10 s, 58°C for 30 s and 68°C for 30 s. The expression levels of Cox gene [18] was used as internal control for normalization of cDNA template quantity using Cox specific primers (Table 2). Data analysis was performed using software provided by BioRad, UK. The comparative Ct (2-$\Delta\Delta$Ct) method [19] was used to calculate the changes

Table 1. List of gene specific primers for different stress used for PCR amplification of genomic DNA

Primers	Sequences (5'-3')	Base pairs	Tm
HSP70-F	AACTTAACATGGATCTATTCA	21	50°C
HSP70-R	ACCCACATTTAATCAATAACT	21	52°C
LEA-F	ATGAAAAGGCAAAGCAAG	18	50°C
LEA-R	TAGTCTTGTACTTGGACT	18	50°C
ALD- F	TCCATAGATCAAAATGGCC	19	43°C
ALD-R	TCAGAGAACTTGGATATCAGA	21	43°C

Table 2. List of primers used in quantitative real time PCR

Primers	Sequences	Base pairs	Tm
HSP-70Fq	GTGTCTGAGGGATGCAAAGA	20	51°C
HSP-70Rq	CTCGTCTGGGTTGATACTCTTG	22	54°C
LEA-Fq	CCATTTCCTGGTACCCTTCG	20	58°C
LEA-Rq	CCATTTCCTGGTACCCTTCG	20	58°C
ALD-Fq	CTGCTGCTAATCAGGTGGAA	20	58°C
ALD-Rq	CTGGTGAGCCTAGAGGAGAATA	22	58°C
Cox-Fq	GATTCGTTCGGACCTATTGCCA	22	48°C
Cox-Fq	AACTGCTAAGRGCATTCC	18	46°C

in gene transcript as a relative fold difference between an experiment and calibrator sample.

2.4 *In-Silico* Characterization of HSP70

The cloned sequences were used for *In-Silico* characterization. The nucleotide sequence of HSP70, LEA and ALD was used for similarity search using BLASTn tool (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and converted in to amino acids and protein sequences by BioEdit tool or Expasy tool (http://web.expasy.org/translate/). These genes sequences reported from different plant sources, retrieved from NCBI nucleotide database were aligned using clustal W alignment (http://www.genome.jp/tools/clustalw/) [20] and phylogenetic tree was constructed using Neighbour-joining method with the help of MEGA5 tool [21].

3. RESULTS AND DISCUSSION

3.1 Sequence Identification of HSP70, LEA and Aldolase Gene from Lentil (*Lens culinaris*) and *In silico* Characterization

An amplicon of ~1000 bp, ~266 bp, and ~750 bp for HSP70, LEA and ALD respectively was amplified from lentil by PCR using gene specific primers (Fig. 1) and sequenced HSP70, LEA and ALD gene were used for BLASTn homology analysis. The HSP70 amplified product showed maximum resemblance with HSP70 from *Pisum sativum* 90%, *Cicer arietinum* 87%, *Cicer arietinumx* 82%, *Medicago sativa* 82%, *Medicago truncatula* 81%, respectively. For LEA maximum resemblance from *Medicago sativa* 99%, *Pisum sativum* 99%, and *Oxytropis lambertii* 99% and for ALD 100% homology resemblance from *Medicago truncatula*, *Vicia faba* and *Glycine max.* Then the sequence was submitted to NCBI Gen Bank with accession number LC156096 (HSP 70), MF421716 (LEA) and MF421715 (ALD). Similar procedures were followed by Pavli et al. [22] while studying HSP90 in *Sorghum bicolor* L and Kumar et al. [23], identified Faba bean VfHsp17.9 gene. Zhao et al. [24] while studying *LEA* in Agropyron mongolium Keng identified *Mongolia wheatgrass* LEA gene.

Clustal W alignment and phylogenetic analysis was also performed with other HSPs, LEA, and ALD reported from plant sources showed that Cicer arietinum (XM_004492061), *Oryza sativa* Indica (EEC71767), *Pisum sativum* (U43011), and *Medicago sativa* (AY830127) were highly similar and also showed that presence of evolutionary families of HSP70, LEA and ALD are *Pisum sativum* (Accession No. U08820) and *Medicago sativa* (AY830127) respectively (Fig. 2.A, B, C) similarly Kumar et al. [25] and Zhang et al. [26] reported on f HSP90 gene from wheat.

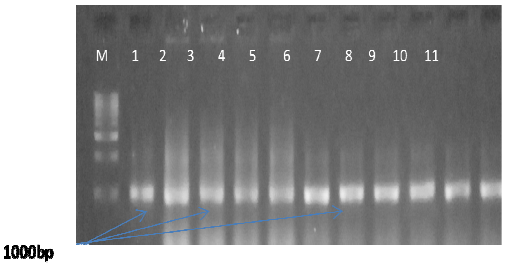

Fig. 1.1: PCR based amplification of *HSP* gene amplified from cDNA of Lentil (from Left to Right: M- Marker (Genei Cat# 612652070501730), Lane-1-5 stressed at 35°C, Lane 6-8 stressed at 40°C, and Lane 9-11 stressed at 45°C.

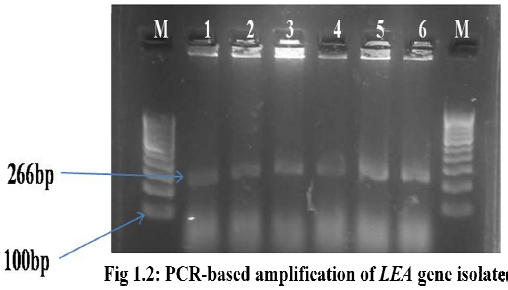

Fig 1.2: PCR-based amplification of *LEA* gene isolated from cDNA of lentil from left to right : M- Marker, 1-6 stressed *LEA gene* , M-100bp ladder, Genei (Cat# 612652070501730).

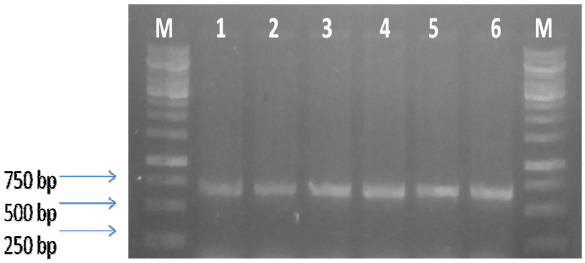

Fig.1.3PCR-based amplification of *aldolase* gene amplified from cDNA of Lentil (from Left to Right: M-Marker, Lane 1-6 stressed at 150 mM Salt concentration .

Fig. 1. PCR based amplification of HSP70, LEA and ALD

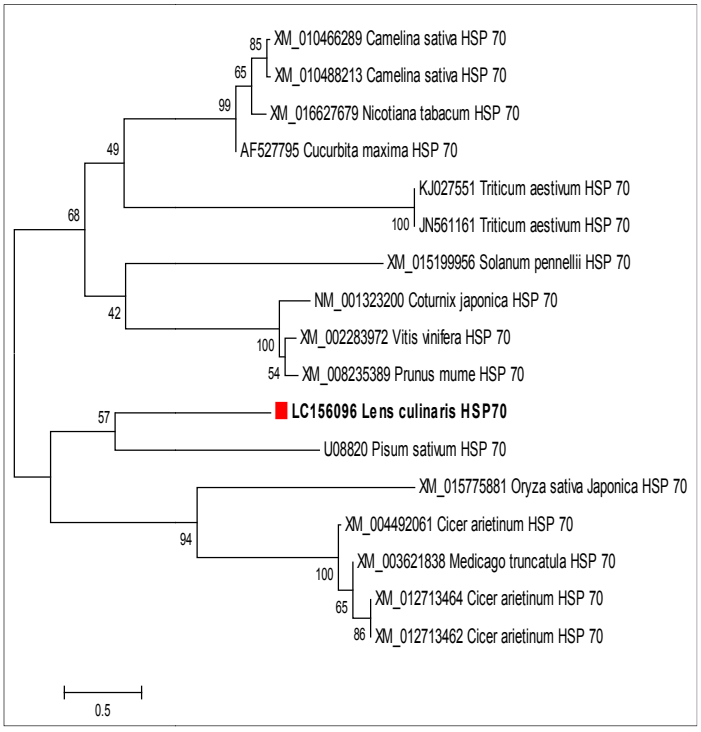

Fig. 2A. Phylogenetic tree constructed by Neighbour-joining method based on Nucleotide sequence of HSP70 gene of Lens culinaris. Numbers above branches denotes the bootstrap value with 500 replications

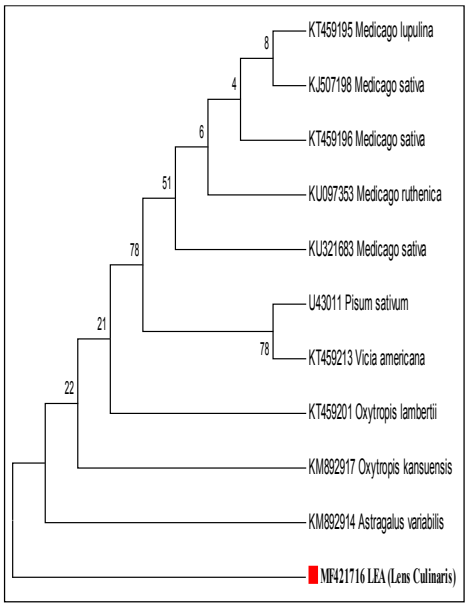

Fig. 2B. Phylogenetic tree constructed by Neighbour-joining method based on Nucleotide sequence of LEA gene of Lens culinaris (MF421716)

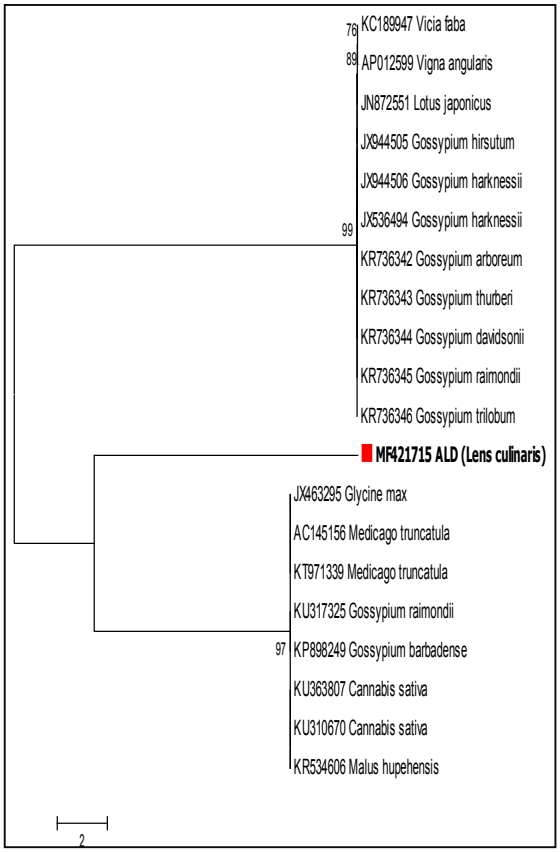

Fig. 2C. Phylogenetic tree constructed by Neighbour-joining method based on Nucleotide sequence of aldolase gene of Lens culinaris

3.2 Transcript profiling of HSP70, LEA and Aldolase Gene

The relative expression of HSP70, LEA and ALD of heat, drought and salinity stressed versus control (unstressed) from lentil cotyledons was calculated using BioRad IQ-5 software and revealed that gene is highly expressed in response to stress in *Lens culinaris*. Different expression levels were obtained at different temperatures (35, 40, 45°C), drought (20% PEG) stress and different concentration of salinity (100 mM, 150 mM, 200 mM) for various time periods. Here, *Cox* gene was used as internal control.

The relative fold expression profiling of HSP 70 gene in *Lens culinaris* cotyledon at different temperature and different time variations showed 0.48, 1.33, 1.0 at 35°C for 1, 2 and 3 hour, 0.068, 0.59, 0.8 at 40°C for 1, 2, and 3 hour and 0.46, 1.6, 4.8 at 45°C for 1, 2,and 3 hour respectively (Fig. 3A, B, C). Similarly, Hu et al. [27] showed the expression profiling of heat shock proteins and heat shock factors under abiotic stresses in rice.

The relative expression of LEA gene showed 0.38, 0.2, 0.1 for 20% PEG at 3, 6, 12 hour (Fig. 4). Same was done by Wang et al. [28] analyzed the expression profile of LEA

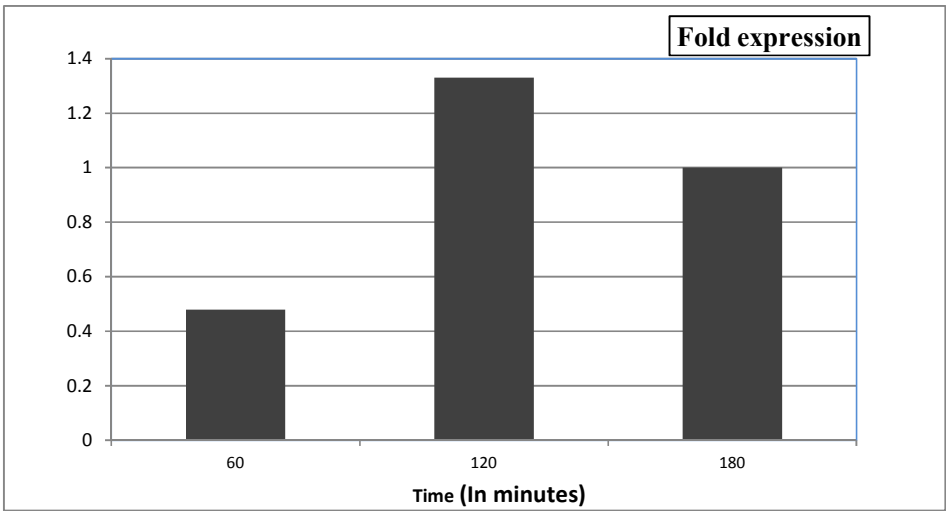

Fig. 3A. Accumulation of HSP70 gene transcript in stressed and unstressed lentil cotyledons subjected to heat stress at 35°C for 60, 120, 180 minutes

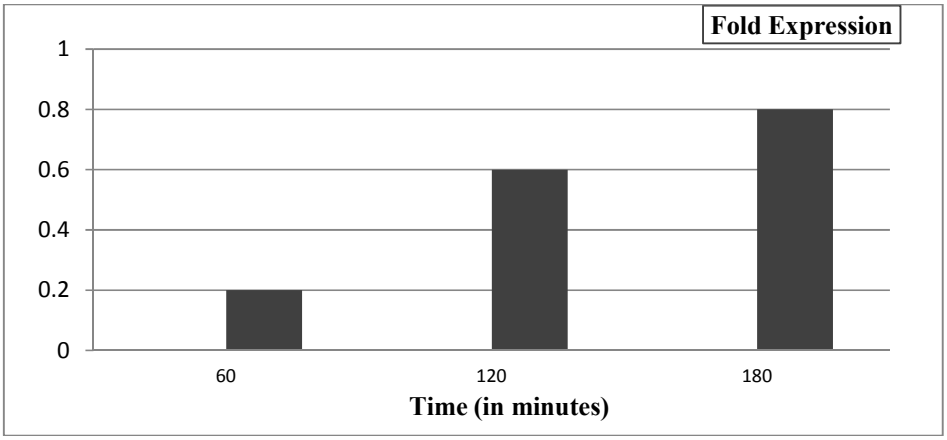

Fig. 3B. Accumulation of HSP 70 gene transcript in stressed and unstressed lentil cotyledons subjected to heat stress at 40°C for 60, 120, 180 minutes

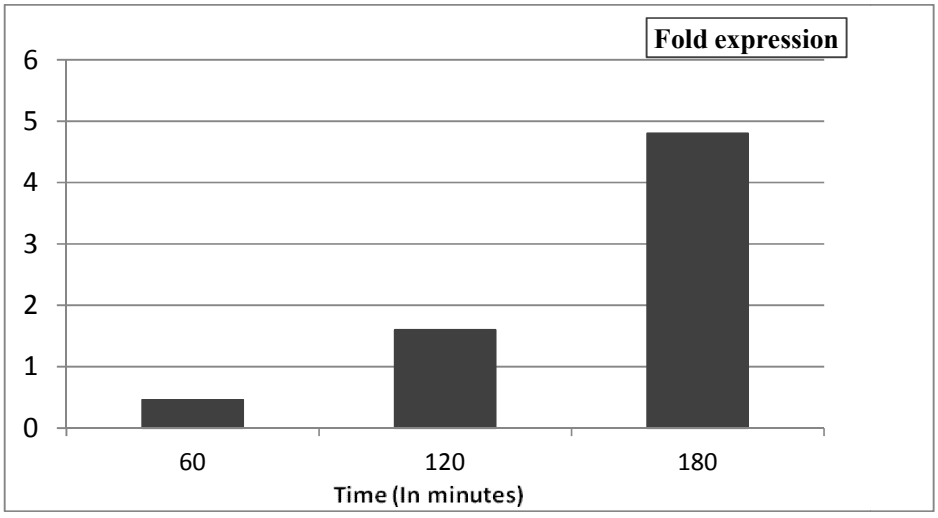

Fig. 3C. Accumulation of HSP 70 gene transcript in stressed and unstressed lentil cotyledon subjected to heat stress at 45°C for 60, 120, 180 minutes

gene by using cDNA in rice (*Oryza sativa* L.) when subjected to drought and salinity stress and also measured the transcript level of the TaSOS1 (*T. boeticum* and *Aegilops crassa*) gene by Quantitative RT-PCR [29].

The relative expression profiling of Aldolase gene in *Lens culinaris* cotyledon was analyzed and fold expression was 0.96, 0.72, 0.07 at 100mM for 3, 6, and 12 hour, 1.0, 0.61, 0.32 at 150 mM for 3, 6, and 12 hour and 0.61, 0.7, 0.2 at 200 mM for 3,6, and 12 hour (Fig. 5 A.B.C). Similarily, Fan et al*.,* 2009, measured the transcript level of the *aldolase* gene by Quantitative RT-PCR [13]. Yang et al. [30] conducted an experiment of expression analysis of *SaCSD1,* which shows similar results in *SaCSD1* [30].

Hence, high transcript level of HSP 70, LEA and ALD were observed at 45°C for 3 hour, 20% PEG for 3 hour, 150 mM salinity for 6 hour and 150 mMm salinity for 3 hour respectively. This indicates that HSP, LEA and ALD genes of different metabolic pathways have their expression tightly regulated by heat, drought and salinity stresses in lentil. Moreover, the data show that the dynamics and the expression level

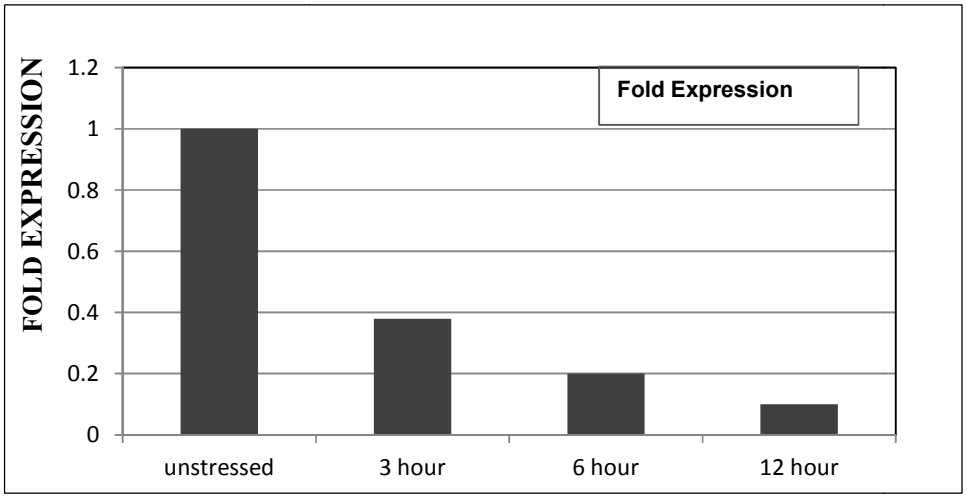

Fig. 4. Accumulation of LEA gene transcript in stressed and unstressed lentil cotyledons subjected to drought stress of 3, 6, 12 hr

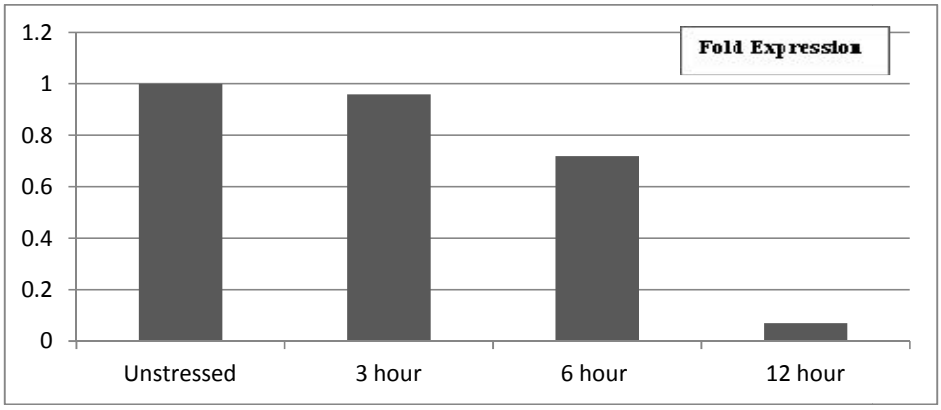

Fig. 5A. Accumulation of Aldolase gene transcript in stressed and unstressed lentil cotyledon subjected to salt stress at 100 mM for 3, 6 & 12 hr

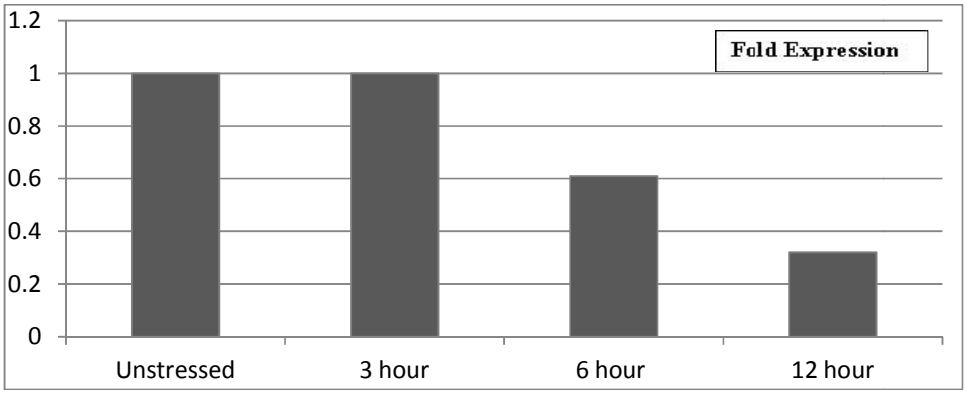

Fig. 5B. Accumulation of Aldolase gene transcript in stressed and unstressed lentil cotyledon subjected to salt stress at 150 mM for 3, 6 & 12 hr

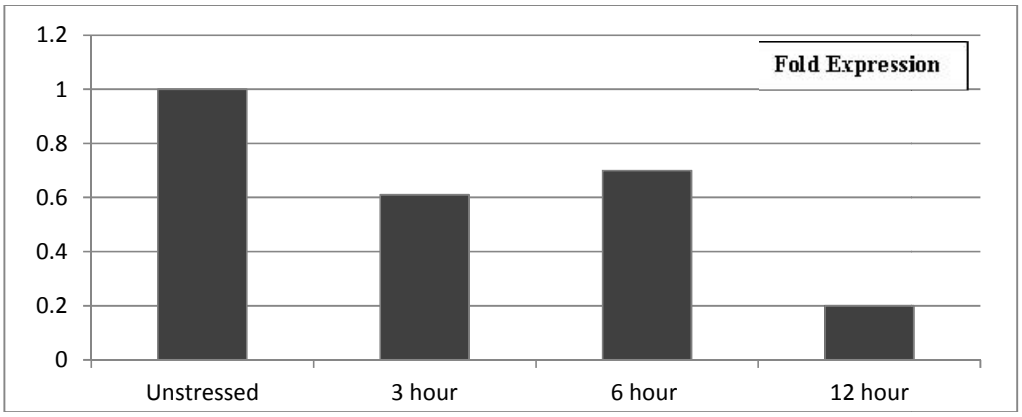

Fig. 5C. Accumulation of Aldolase gene transcript in stressed and unstressed lentil cotyledon subjected to salt stress at 200 mM for 3, 6 & 12 hr

can change drastically depending on the stress system. Our work has shed light on the gene expression, involved in lentil metabolism during abiotic stress condition.

Stresses are major environmental factors that affect the geographical distribution of plants in nature, limit plant productivity in agriculture, and threaten food security. The adverse effects of

these abiotic stresses are exacerbated by climate change, which has been predicted to result in an increased frequency of extreme weather. As plants show specific changes in gene expression, metabolism, and physiology in response to different environmental stress conditions for protecting itself from adverse environmental condition. There are some genes like HSP70, LEA and ALD which expresses under heat, drought and salinity stress. Here, we have cloned a full length gene of HSP70, LEA and ALD from lentil (*Lens culinaris*) and the expression analysis showed that the abundance of HSP70, LEA, and ALD transcript in stressed lentil cotyledons compared to unstressed lentil cotyledons in response to different abiotic stresses for different time variations. The transcript profiling showed that high expression of HSP70 was at 45°C for 3 hr followed by at 45°C for 2 hr, LEA gene expression was highest at 20% PEG for 3 hr, ALD gene expression was highest at 150 for 3 hr followed by 100 mM for 3 hr.

4. CONCLUSION

Several other stress responsive genes have been identified, if these genes would successfully introduce into other crops which are sensitive to adverse environmental conditions, can create a transgenic crops with enhanced stress tolerance. Therefore, future research is needed to explore more opportunity to the better understanding of plant responds to the abiotic stress so that abiotic stresses tolerant capability can be develop in sensitive crop plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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