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ANALYSIS OF EXPRESSION OF HEAT SHOCK PROTEIN-70 (HSP-70) GENE IN SALT STRESSED GROUNDNUT (*Arachis hypogaea* L.) PLANTS

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ABSTRACT

Quantitative real-time PCR (RT-PCR) has already been used to study the expression profile of several plant gene families. This study demonstrates the potential of RT-PCR to measure the expression of heat shock protein (HSP) genes in groundnut (*Arachis hypogaea* L.) that underwent salt stress followed by RT-PCR of *hsp70* mRNA using the housekeeping gene tubulin (*tub*) as a reference gene and the fluorogenic dye SYBR Green I. Expression of HSP70 gene was identified in salt stressed groundnut genotype TMV7.

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KEY WORDS

Heat shock protein, quantitative RT-PCR, SYBR Green, complementary DNA; deoxyribonucleic acid; heat shock protein; ribonucleic acid; tubulin gene.

INTRODUCTION

Different types of edaphic factors affect plant growth. Salinity is one of the most important stress factors which limit the growth and development of plant by altering their morphological, physiological and biochemical attributes, according to USDA Salinity Laboratory [1]. Drought and salinity are becoming particularly widespread in many regions and may cause serious salinization of more than 50% of all arable lands by the year 2050. Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins. As a consequence, these diverse environmental stresses often activate similar cell signaling pathways and cellular responses, such as the production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes. The ongoing elucidation of the molecular control mechanisms of abiotic stress tolerance, which may result in the use of molecular tools for engineering more tolerant plants is based on the expression of specific stress-related genes. These genes include three major categories: (i) those that are involved in signaling cascades and in transcriptional control, such as MyC, MAP kinases and SOS kinase, phospholipases and transcriptional factors such as HSF, and the CBF/DREB and ABF/ ABAE families (ii) those that function directly in the protection of membranes and proteins, such as heatshock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA) proteins, osmoprotectants and free-radical scavengers; (iii) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters[2,3].

Groundnut is an important commodity in many developing countries particularly in India where the nitrogen rich crop residues are also used as fodder. The production of groundnut in India needs to be increased from the current 8 million to 14 million by 2020 to meet the increasing demand of oil and confectionery industry [4].This is partially achieved by growing groundnut in lands considered so far as unsuitable for agriculture like rice (*Oryza sativa*)fallow affected by salinity. Little is known about the salinity tolerance of groundnut and a few attempts have been made to breed salinity tolerant groundnut varieties [5].

Like all HSP families, HSPs70 have been recognized as molecular chaperones playing a central role in cell biology and biochemistry [6, 7, 8]. They are encoded by a multigene family whose members are developmentally regulated and differentially expressed in response to temperature stress and other conditions that interrupt normal protein folding or favor protein denaturation[9]. In real-time PCR (RT-PCR), a fluorogenic dye is used to continuously monitor product accumulation. With this method, the initial concentration of template DNA is assessed by using the number of PCR cycles at which PCR amplification enters the exponential phase. Other methods such as Northern hybridization, RNase protection assay and in situ hybridization have been used for quantification of gene expression. However, RT-PCR has

many advantages over these methods, including time, sensitivity, specificity, ease of use and reproducibility [10, 11, 12, 13, 14]. The quantification is performed by comparing the expression of internal standards, mainly housekeeping genes, expression of which occurs in all nucleated cell types being necessary for cell survival [15, 16]. Although such synthesis often shows very little fluctuation, their use as internal standards should be carefully examined in relation to cell types and cell metabolism. The internal standard selected for this study was the housekeeping gene tubulin (*tub*).

Plant samples (leaf sample of salt tolerant groundnut variety, TMV7)
Primers (Table 1)
RNA Isolation – Trizol Reagent (Invitrogen®)
RNA Electrophoresis
RevertAid H Minus First Strand cDNA Synthesis Kit (MBI-Fermentas)
2 X PCR Master Mix – (MBI-Fermentas)
Quantifast SYBR GREEN PCR kit- Qiagen
Instruments:NanoDrop 8000 pectrophotometer.
Agarose Gel Electrophoresis
Mastercycler ep Gradient Real Plex
Thermalcycler ep Gradient
ChemiDoc XRS Gel Quantification

MATERIALS & METHODS

Table1
Primers used in relative quantification assay

SET	PRIMER	SEQUENCE (5'-3')
1	HS1f	GGA TTT CGA CAA CAG GAT GG
	HS1r	CCT TCT CGT TAC CCT CAC CA
2	TubA1f	GTG CAT TTC AAT CCA CAT CG
	TubA1r	CAG TGG GCT CAA GAT CAA CA

Procedure :

1.0 RNA Isolation

Total RNA was isolated from leaf tissue samples.100 mg of tissue was crushed in liquid nitrogen using mortar and pestle and tissue was converted into powder form.1 ml of Trizol reagent was added to tissue powder, mixed and incubated in ice for 15 minutes. The tube was centrifuged at 5000 rpm for 5 minutes, supernatant was transferred in new 1.5 ml tube and pellet discarded. 200 µl of chloroform was added to the supernatant, mixed by vortexing and incubated on ice for 10 minutes. For phase separation the tube was centrifuged at 13000 rpm for 15 min. Upper clear aqueous phase was transferred in new 1.5 ml tube and 500 µl of isopropanol was added. The tube was incubated in ice for 15 minutes and centrifuged at 13000 for 15 minutes. Isopropanol was discarded and the formed RNA pellet was washed with 70 per cent ethanol prepared in DEPC treated water. The tube was then centrifuged at 13000 rpm for 30 minutes. Ethanol was decanted and the tube was kept at room temperature for 15-20 minutes for pellet drying. Dried pellet was dissolved in 30 µl of DEPC treated water and the tube incubated at 65°C for 15 minutes. The RNA stock sample was quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 40 µg RNA

per ml. Purity of RNA was judged on the basis of optical density ratio at 260:280 nm. The RNA optical density ratio at 260:280 nm was 1.98 for total RNA.

2.0 Denaturing Agarose Gel Electrophoresis

1 gm of agarose was heated in 36.25 ml DEPC treated water, cooled to 60°C, and 5 ml of 10 X MOPS buffer was added followed by 8.75 ml of 37% formaldehyde, this was then mixed properly and casted in the AGE unit. RNA sample (2 µl) was mixed with 2 µl of 2X RNA loading dye, heated at 70 °C for 5 minutes, chilled on ice for another 5 minutes and mixed with 0.5 µl of 1% Ethidium Bromide and loaded on the gel.

The electrophoresis was carried out using 1X MOPS buffer. The image was captured using the ChemiDoc XRS instrument.

3.0 DNase treatment of RNA (Table 2)

Table2

Reaction set up for DNase treatment of RNA

Component	Volume (µl)
Total RNA	10
10 Reaction buffer	2
DNase 1	3
DEPC treated water	5
Incubate mixture at 37°C for 30 min	
Add 2 µl of 25 mM EDTA and incubate at 65°C for 10 min	

4.0 First cDNA synthesis using RevertAid H Minus First Strand cDNA Synthesis Kit

cDNA is perpared for two samples using H minus First Strand cDNA synthesis using the Random Hexamers primers as given in the protocol(Table 3).

Table 3

Reaction set for cDNA preparation

Components	Quantity (µl)
Total RNA Template	4
Primer (Random Hexamers)	1
DEPC -treated water	7
5x Reaction Buffer	4
RiboLock RNase Inhibitor (20u/ul)	1
10 mM dNTP mix	2
M-MuLV Rev. Transcriptase (20u/ul)	1
Total Volume	**20**

Added the following components in 0.2 PCR microcentrifuge tube in the order kept on ice. Mixed gently, centrifuged and incubated for 5 minutes at 25°C followed by 60 minutes at 42 °C. The reaction was terminated by heating at 70 °C for 5 minutes and kept hold at 4°C.

5.0 PCR Amplification of HSP-70 and Tubulin gene from cDNA

HSP-70 and tubulin gene were amplified from prepared cDNA using gene specific primers for optimization of cycling conditions and primer to template ratio. Table 4 and 5 show the representative set up of PCR reaction

Table 4

PCR reaction set up for amplification of cDNA

Components	Quantity
Nuclease free water	9.0µl
cDNA	1.0 µl
Forward Primer (10pmole /ul)	1.0µl
Reverse Primer (10pmole /ul)	1.0µl
2 X PCR master mix MBI Fermentas	13 µl
Total Volume	**25µl**

Table 5

PCR reaction set up for amplification of cDNA

Steps	PCR		
	Temperature °C	Time	Cycle
Initial Denaturation	95	8 min	
Denaturation	95	30 sec	
Annealing	46	30 sec	43 x
Extension	72	35 sec	
Final Extension	72	10 mins	
Hold	4	-	-

6.0 2% Agarose Gel Electrophoresis for quantification of PCR products

1 gm of Ultrapure Agarose was heated in 50 ml 1 x TAE, cooled to 60 °C and 1% Ethidium bromide was added @ 10µl /100ml concentration. 5 µl of PCR product was electrophoresed along with 1kb DNA molecular weight marker (Zipruler1 Fermentas).Electrophoresis was carried out at 80V for 30 minutes at room temperature.

7.0 Real Time PCR (RT PCR)

In the present assay, Sample 1(Groundnut varietyTMV7, treated with 100mM NaCl) and sample 2 (Groundnut varietyTMV7, untreated) are relatively quantified using real time PCR.

HSP-70: Heat Shock Protein-70 (test gene)

TUB: Tubulin gene (Reference gene)

Real Time PCR reaction for both genes of both samples was performed in triplicate using Quantifast SYBR GREEN Real Time PCR Master mix (Table 6 & 7).

Table 6

Real Time PCR set up for relative quantification assay.

Components	Quantity
Nuclease free water	9.5µl
cDNA	1.0 µl
Forward Primer (10pmole /ul)	1.0µl
Reverse Primer (10pmole /ul)	1.0µl
TAQXpedite Real Time PCR Mastermix	12.5µl
Total Volume	**25µl**

Table 7

Real Time PCR set up for relative quantification assay

Steps	PCR		
	Temperature °C	Time	Cycle
Initial Denaturation	95	8 min	1x
Denaturation	95	30 sec	43 x
Annealing	60	30 sec	
Extension	72	35 sec	
Final Extension	72	10 min	1x
Melt Curve	95	15 sec	1x
	60	15 sec-	
		20 min	
	95	15 sec	
Hold	4		

Following were the formulae utilized in the calculation of fold of expression

1) Δ CT = CT target - CT House keeping

2) Δ Δ CT= ΔCT Test samples- ΔCT Calibrator samples

3) Fold difference= 2 -Δ Δ CT

RESULTS AND DISCUSSION

Molecular chaperones are key components contributing to cellular homeostasis in cells under both optimal and adverse growth conditions. They are responsible for protein folding, assembly, translocation and degradation in a broad array of normal cellular processes; they also function in the stabilization of proteins and membranes and can assist in protein refolding under stress conditions. A wide range of proteins has been reported to have chaperone activity. Moreover, many molecular chaperones are stress proteins and many of them were originally identified as heat-shock proteins (Hsps). Thus, abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and

oxidative stress are serious threats to agriculture and that result in the deterioration of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. It is most likely, being supported by experimental data in plants and other organisms, that Hsps/chaperones play a crucial role in protecting plants against stress and in the reestablishment of cellular homeostasis [17].

Initially, several pairs of primers were designed for *hsp70* and *tub* and tested using RT-PCR. The reaction was optimised by choosing the best primers and concentration of the remaining components of the master mix, by titrating SYBR Green I, and by optimising the thermal profile. During optimisation, it was necessary to determine the fraction of the RT-PCR signal that originated from primer dimer or by-product production. This was accomplished by plotting the melting curve. It was also found that the success of the RT-PCR experiment depends on the choice of PCR primers. Other researchers have used *tub* as reference gene in quantification studies [18, 19].

In the present study, the groundnut genotype TMV7 is treated with 100mM NaCl and the untreated groundnut genotypes were relatively quantified using real time PCR with HSP-70: Heat Shock Protein-70 as test gene and TUB: Tubulin gene as reference gene. Real Time PCR reaction for both genes of both samples was performed in triplicate using Quantifast SYBR GREEN Real Time PCR Master mix (Figure 3 & 4). The isolated RNA (Fig.1) was treated with DNase for cDNA synthesis which was done using Revert Aid H Minus First Strand cDNA Synthesis Kit .

1 2

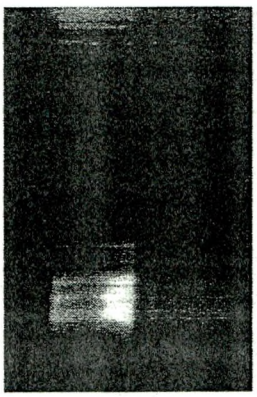


Fig
1 % denaturing agarose gel electrophoresis of RNA

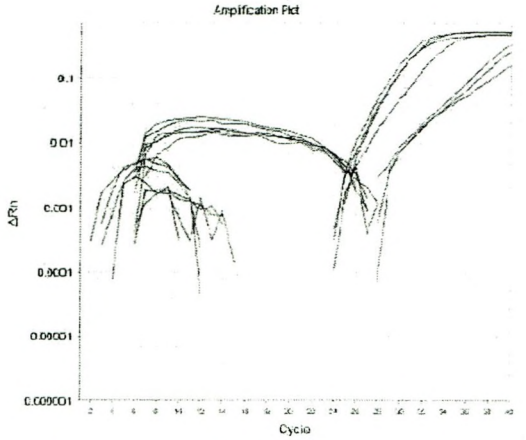


Figure 3

Amplification plot of relative quantification assay for groundnut varietyTMV7, treated with 100mM NaCl

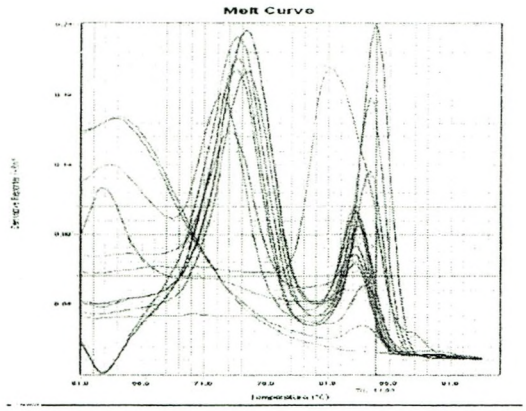
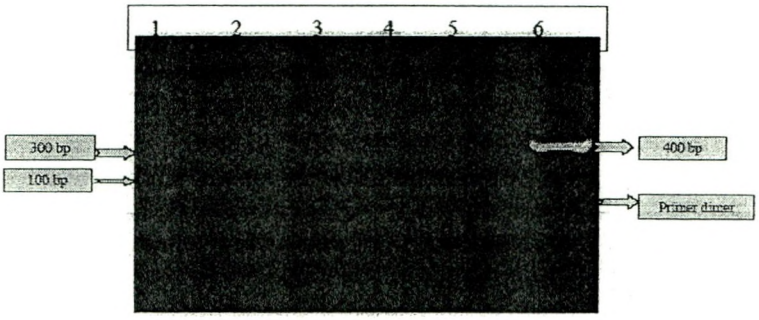


Figure 4

Melt curve analysis in relative quantification of groundnut varietyTMV7, treated with 100mM NaCl

HSP-70 and tubulin gene were amplified from prepared cDNA using gene specific primers for optimization of cycling conditions and primer to template ratio which is depicted in Figure 2. The results revealed the expression of HSp 70 gene in the groundnut variety TMV7 which has played the key role in tolerating the 100 mM sodium chloride stress during its growth period.



Fig

2 % Agarose gel showing amplification of genes from cDNA

Lane 1: DNA ladder (10000 bp- 100 bp)

Lane 2: Amplicon of 250 bp amplified with tubulin gene specific primer from Sample- 1(Groundnut varietyTMV7, treated with 100mM NaCl)

Lane 3: Amplicon of 350 bp amplified with HSP-70 gene specific primer from Sample-1(Groundnut varietyTMV7, treated with 100mM NaCl)

Lane 4: Amplicon of 250 bp amplified with tubulin gene specific primer from Sample-2 (Groundnut varietyTMV7, untreated)

Lane 5: Amplicon of 350 bp amplified with HSP-70 gene specific primer from Sample-2(Groundnut varietyTMV7, untreated)

Lane 6: Amplicon of 400 bp amplified using control sample.

CONCLUSION

Complex traits of abiotic stress phenomena in plants make genetic modification for efficient stress tolerance difficult to achieve. The discovery and use of new stress-tolerance-associated genes, as well as heterologous genes, to confer plant stress tolerance (including those unique to extreme-growth-environment organisms e.g. halophytes, thermophilic organisms), has been the subject of ongoing efforts to obtain tolerant plants. Due to noise during amplification relative quantification of the expression of HSP 70 in the groundnut genotype TMV 7 was possible, but the presence of HSP 70 gene was noticed during salt stress of 100 mM concentration which proved the tolerance of the groundnut genotype TMV7 to salt stress and this had paved the way for further analysis of HSP family in groundnut under stress and their quantification.

An ideal genetically modified crop should possess a highly regulated stress-response capability that will not affect the crop performance in the absence of stress. In this respect, conventional breeding and selection techniques will continue to make a contribution. While certain transgenic crops have already been moved from the laboratory, only a few stress-resistant transgenic crops have been evaluated in field trials under real stress conditions [20]. When facing the deleterious effects of drought and salinity, it is imperative that more crops, which are genetically resistant to abiotic stress, be designed, tested, and eventually released for application as new commercial varieties.

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