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RESEARCH ARTICLE

Salt-related Genes Expression Pattern in Salt-Tolerant and Salt-Sensitive Cultivars of Cotton (*Gossypium* sp.) under NaCl Stress

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ABSTRACT: Salinity is one of the most important limitation factors in development of agricultural products. Cotton has a relative tolerance to salinity; however, salinity reduces its growth during germination and seedling stages. In this research, split-factorial design of time based on randomized complete block design with 3 replications was used. The real-time PCR results for, root, stem, and leaves of 14-day cotton seedlings of tolerant (Sepid) and sensitive (Thermus14) cotton cultivars with salinity levels from 0 to 16 ds.m^{-1} were analyzed at three time points, namely 0, 7 and 14 days after salinity stress. Selected genes for Real Time PCR reaction in current study were selected using Cytoscape 3.3.0 software. Results showed that the selected genes *GhERF2*, *GhMPK2*, *GhCIPK6*, *GbRLK*, *GhNHX1*, *GhGST*, *GhTPS1* and *Gh14-3-3* have positively responded to salinity stress and their expression in the root was higher than in stem and leaf. Moreover, the expression of tolerant genotype (Sepid) was higher than the sensitive cultivar (Thermus 14) one, however, a slight increase in sensitive genotypes was observed in a number of genes (*GhERF2* and *GhGST*) 14 days after starting the stress treatment.

KEYWORDS: abiotic stress, real-time PCR, Salt tolerance

INTRODUCTION

Salinity is one of the most important abiotic stresses, regarded as a serious constraint role for crops. At all, about 20% of the world's agricultural lands are influenced by salt and will be added to these statistics yearly. In addition, soil salinity is a great constraint for developing cultivable lands [53]. Water losses and salts accumulation are two factors resulting in ion toxicity in plant tissues. Also, oxidative stress and nutrient deficiencies are considered as secondary effects of this stress. Plants have several mechanisms regarding tolerance to salinity stress and Na^+ distribution. These mechanisms are mainly related to the control of Na^+ uptake and their distribution in plant organs including the adjustment of Na^+ transfer to the shoot, Na^+ flow from the shoot to the phloem, accumulation in the particular part of plant, secretion to the external surface of leaves and control of evapo-transpiration [27]. Understanding the molecular mechanism of plant reaction to salt stress can facilitate

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development of new cultivars having an increase in salt tolerance [52]. Cotton (*Gossypium hirsutum* L.) is an oil-fiber plant which is of global importance and has high commercial value. In fact, it is recognized as an important plant to study the molecular basis of plant reaction to salinity since it is compatible with semi-arid and subtropical conditions. Also, it is capable of growing in soils with high salt contents [42]. Although cotton is the second industrial plant tolerant to salinity, its growth and productivity is affected by high salinity, especially in germination and seedling stages [1].

Identification of genes involved in salt tolerance pathway are important for producing plant cultivars tolerant to salt stress using genetic engineering methods. Although many genes controlling response to high salinity concentration in the model plants have been identified, only a few of them have been investigated in cotton such as tonoplast antiporter Na^+/H^+ (*NHX1*) [64], transcription factors in dehydration-responsive element binding to the protein family (*DREB*) [22], ethylene responsive factors (*ERF2-6*) [6; 37, 38], NAC transcription factor family (*NAC1-NAC13*) [34, 48], metallothionein type 3 *MT3A* [67], mitogen-activated protein kinase (*MPK2*) [74], mitogen-activated protein kinase kinase (*MKK5*) [73], CCCH-type zinc-finger proteins (*ZFP1*) [24], CBL-interacting protein kinase *CIPK6* [28], transcription factors WRKY family (*WRKY17)* [68] and *WRKY39*-1 [60] and receptor-like kinase (*RLK*) [76].

Network analysis provides unique opportunity for gene/protein interactions and correlations through the data set within the framework of networks [11]. The network's topology can highlight key genes, as well as, genes that are linked to different networks [14]. Different approaches for discovering the network have been used in a gene set such as data mining from sources, simultaneous analysis of genes, promoter analysis, micro-RNA prediction, as well as, the representativeness of the GO (Gene Ontology)'s interaction. Similar to the network analysis, the GO analysis adds a new dimension to the biological discovery of information from a set of distinct expression genes. The GO categorization of significantly reduced or increased gene expressions is the first step in analysis of functional genomics for interpreting the transcriptome data. In the GO analysis, genes and proteins are classified into three major types, including biological process, molecular function, and cellular component. Finding key GO groups and selecting genes based on meaningful GOs are considered as new and reliable approaches for discovering genes. In addition, GO's comparison of a specific sample versus GO distribution of the whole genome (as a reference), by Fisher's exact test, shows groups with distinct functions in a particular sample. In addition, recent advances in the creation of GO networks provide unique opportunity to examine transcriptome in the context of functional interactions between different GO classes [17, 18]

The transcriptome analysis provides detailed information on the expression of genes at the mRNA level and is widely used to determine the main genes involved in responses to the stress. Recent advances in transcriptomics have greatly contributed to our knowledge of the ways in which the molecular regulation has been identified for cotton tolerance and adaptation to salinity stress, since several genes and miRNAs have been identified that play important roles in response to salinity stress [57]. However, their mechanisms are not well understood, because the studies have focused on either early transcriptional changes in response to salinity stress or transcription in the leaf or root yet, as a matter of fact, roots are the first organ to sense salt stress, but leaves are the main part of water loss in stress and play a major role in response to salinity stress [21].

This study aimed to determine the key genes involved in salinity stress in allotetraploid cottons of a tolerant and sensitive cultivars and then, evaluating the expression of these genes in root, stem and leaf tissues for 7 and 14 days under salinity and control conditions.

MATERIALS AND METHODS

Plant Material and Stress Treatment

The seeds of the Sepid (Salt tolerance) and Thermus14 (Salt sensitive) cultivars were received from Khorasan Razavi Agricultural and Natural Resources Research Center. The seeds were surface sterilized and sown in the greenhouse of Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) in August 16, 2016. Ten seeds of each cultivar were sown in 11 cm pots filled with sand (Sands were leached with 10% HCl). After emergence, plants were thinned to three plants per pot.

Split-factorial design of time based on randomized complete block design with three replications was used. Two cultivars (Sepid and Thermus14) were stressed in two salt concertation levels (0 and 16 dsm^{-1}); different tissues (root, stem and leaf) with form of factorial arrangement in sub-plot, as well as, sampling time in the main-plot. Conditions of greenhouse were 35/25 ºC and relative humidity was 60/70% (day/night). Plants were

daily irrigated via 150 ml half strength Hoagland solution [31] containing no NaCl until the first true-leaf stage has been emerged. Subsequently, 14 days after planting, seedlings were subjected to salt stress treatment (The following formula was used to determine the amount of salt required, In this formula TDS is: mg of salt required for one liter of distilled water and EC: intend Electric conductivity, $\mathrm{EC} = \mathrm{TDS}\ (mgl^{-1}) * 640$). Then sampling was started 0, 7 and 14 days after initiation of salt treatment. Leaves, roots and stems of the plants were transferred to -80 freezer until RNA extraction.

Construction of Gene Network

A regulatory network was constructed based on differentially expressed genes in cotton. The study of Peng *et. al.* identified genes displaying significant expression changes during NaCl treatment. Differentially expressed uni-genes (DEUs) were analyzed in 4-and 24-h transcriptome libraries with the control of two genotypes Nan Dan Ba Di Da Hua (salt-sensitive cotton) and Earlistaple7 (salt-tolerant cotton) [57].

Moreover, topological features, including betweenness and closeness centrality, as well as, edge between centrality were calculated using the constructed network and Cytoscape software version. 3.3.0 [59]. Between is a quantitative measure of the centrality of an entity in a complex network based on computing shortest paths of all-pairs in the graph [5; 32; 43]. Furthermore, in a graph representing a biological network, closeness centrality is the average of the shortest distance from one vertex to all other vertices [5; 43]. Edge-between centrality is defined as the frequency of an edge that places the shortest paths between all pairs of vertices and is considered as a standard measurement of the influence of a node or a linkage in network [58; 70].

RNA Extraction and Real Time PCR Reaction

Total RNA was extracted from leaves, stem and leaf samples using the modified LiCl method [41]. Total RNA concentration and quality were checked using Shimadzu 2600UV/Vis spectrophotometer, which were confirmed by %1.2 denaturing agarose gel electrophoresis. The cDNA was synthesized from total RNA (1µg) of samples, according to the manufacturer's instructions using the High-Capacity cDNA Reverse Transcription Kit (Thermo fisher Scientific). The Real Time PCR reactions were conducted using the Maxima SYBR Green qPCR Master Mix (2X) (Thermo fisher Scientific) in a 12.5 µl reaction

Table 1: Sequences of primers used for real-time PCR.

Gene Name	Primer Sequence (5´→3´)	Tm
GhERF2	GTCCATGCAGTTCGATGGTC ACCCTAACCCCTTTCCTTGG	63.90 66.32
GhMPK2	GCCTTAATCAACTAATATGTGCTA CTGAATCCTGCTGAACTATTG	59.93 58.38
GhCIPK6	GGTGGGAAGTCATTGTTTGATG AAAGCAGCCCACTACCACAA	65.08 63.94
GbRLK	GGGATTGCTTACTTGCACGA CAAAGCTCTGGTGCTGCATA	64.66 62.64
GhNHX1	TTCGGATTGCTCAGTGCTT CAGCCAGCATGTAAGAGAGG	62.33 61.29
Gh14-3-3	AGCCAGAGCAGTAGTGAGG GTAGGGAGCCACTCAGGGGCAC	57.78 72.37
GhGST	AACTCTTGTTGCCGGGTTCT GTGTCGGTGGTCATCCCAAA	65.08 67.02
GhTPS1	AATGGGGAACCGCCTGATTT AATTTCGGGAGAAACGGGGA	69.57 70.13
GhUBQ14	CAACGCTCCATCTTGTCCTT TGATCGTCTTTCCCGTAAGC	63.64 64.28

volume (containing 1 µl cDNA reaction mixture, 6.25 µl Maxima SYBR Green qPCR Master Mix (2X), 0.3 µl of each primer and 3.95 RNase & DNase-free water). The reaction conditions were prepared according to the following protocol (10 min at 95 °C, 40 cycles of 95 °C for 15s and annealing temperature (depending on primer used (Table 1)) for 1 min, 55 °C for 5seconds). For all samples, three technical and three biological replicates were considered. The final values of the threshold cycle (Ct) were the mean of all replicates. Relative expression levels of Ct values were calculated using the $2^{-\Delta\Delta CT}$ formula [46]. *GhUBQ14* was used as the internal control of genes for normalization of Real Time PCR data. The data were compared and analyzed using student's t test (α=0.05). Expression numerical values are represented as FC ± SD.

RESULTS

Regulatory Network of Salt-responsive Genes in Cotton

We used differentially expressed genes obtained from transcriptome sequencing (mRNA-seq and small RNA-seq) of cotton leaves under salt stress in Nan Dan Ba Di Da Hua (salt-sensitive cotton) and Earlistaple7 (salt-tolerant cotton) to construct a gene regulatory network. [57]. The network was centered on *NHX1*(Na^+/H^+ antiporter that catalyzes the exchange of Na^+ for H^+ across vacuole membranes and has basic role in hemostasis ions in salt stress), *MPK2* (a Mitogen-activated protein kinase which has a positive effect on tolerance to salt and drought stresses), *CIPK6* (specific

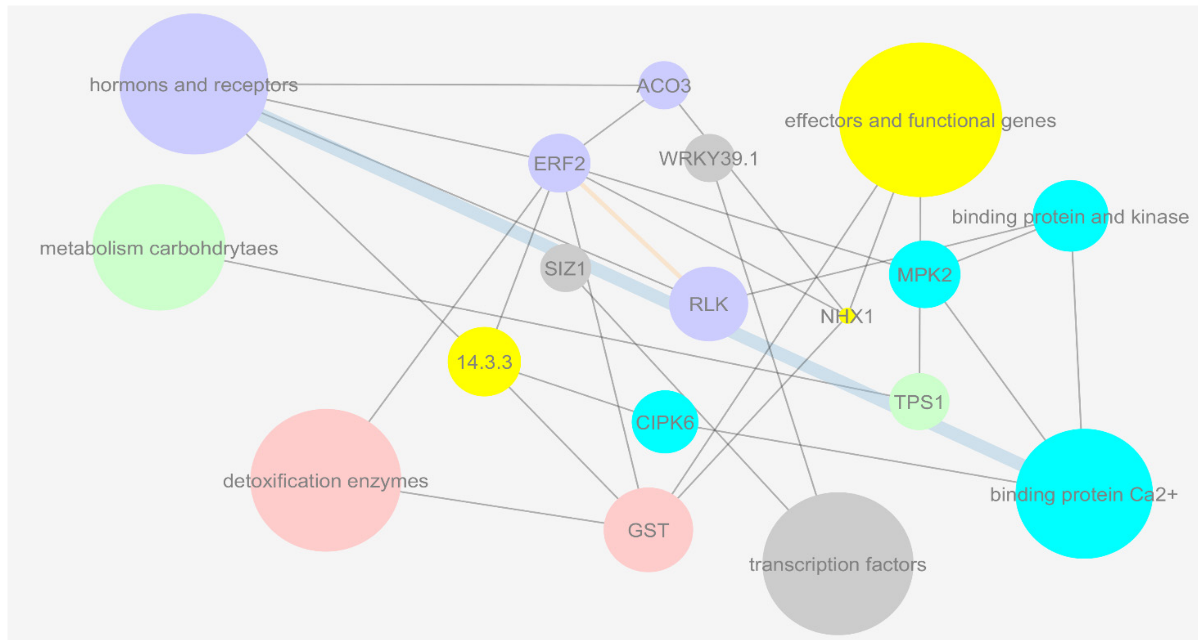

Fig 1. Constructed regulatory network based on DE transcripts in cotton under salt visualized using Cytoscape 3.3.0. Size (high= genes with low values collapsed in big node) and each color represent family of salt tolerant genes

Ser/Thr protein kinases, which are activated through interaction with calcineurin B-like protein (CBL), this complexes play an important role in signal transduction in biotic and abiotic stresses, as well as developmental processes by detoxification reaction oxygen species (ROSs)), *TPS1* (trehalose-1-phosphate-synthase creates tolerance abiotic stresses (drought, salinity and cold)), *GST* (glutathione S-transferase that has a positive role in tolerance to drought and salinity stress), *14-3-3* (a type of PR protein, is involved in defense systems against attack of pathogens, salinity, high temperature, drought, heavy elements, cold stresses), *RLK* (a component of the receptor kinase that acts as a receptor and detects stress when it is subject to saline stress) and *ERF2* (a factors responding to ethylene, which is considered as secondary messages responding to stress) as DE genes during salt stress treatment.

As illustrated in Fig. 1, nodes with bigger size (*NHX1*, *MPK2*, *CIPK6*, *TPS1*, *GST*, *14-3-3*, *RLK* and *ERF2*) indicate higher betweenness centrality. Similarly, edges with higher thickness manifest the high value of edge betweenness centrality. The closeness centrality of each node is presented using gradient size-coding system. The big nodes such as *NHX1*, *MPK2*, *CIPK6*, *TPS1*, *GST*, *14-3-3*, *RLK* and *ERF2* have the highest closeness centrality, while small ones indicate the lowest value of closeness centrality in the graph (Fig. 1).

Expression analysis of Salt-responsive Genes

- *Receptor, GhRLK* gene expression:

Comparison of *GhRLK* expression patterns in the different times showed significant differences in gene expression rates. The increasing in *GhRLK* transcript levels was observed at 14th day after stress initiation. *GhRLK* expression in treated tolerant cultivar was induced by treatment with 16ds.m^{-1} NaCl (2.85-fold higher than sensitive cultivar). In root tissue relative expression demonstrated the highest amount, especially in tolerant cultivar, and the stem and leaf tissue showed lower expression, especially in sensitive cultivar (Fig. 2).

- Transcription factor, *GhERF2* gene expression:

To investigate the expression pattern of *GhERF2*, we monitored the level of the relative expression in three tissues and under stress treatments in different time points by Real Time PCR. The results are shown in Fig3. We found that *GhERF2* level expression was considerable in three tissues both of cultivars in non-stress treatment. This pattern suggests *GhERF2* are constitutively expressed during vegetative growth. The expression of *GhERF2* gene could be significantly induced by salt stress, and expression levels increased obviously after 7th day after stress initiation. Relative expression *GhERF2* in tolerant cultivar was 1.52-fold higher than sensitive cultivar.

Relative expression in root tissue demonstrated the highest amount of expression, especially in tolerant cultivar, however, stem and leaf tissue indicated lower expression levels. Although, unlike other genes (*GhRLK*, *GhMPK2*, *ChCIPK6*, *GhNHX1*, *Gh14-3-3* and *GhTPS1*) relatively significant expression gene in sensitive cultivar was observed, but it was lower than the tolerant cultivar (Fig. 3).

- Signaling pathway: *GhCIPK6 and GhMPK2* gene expression:

GhCIPK6: Real Time PCR was performed to determine the expression levels of *GhCIPK6* in different tissues (leaf, stem and root) for 7 and 14 day periods under salinity condition. The result showed that *ChCIPK6* expression level increased when the plant was exposed to the stress for a 14 days' period in tolerant cultivar.

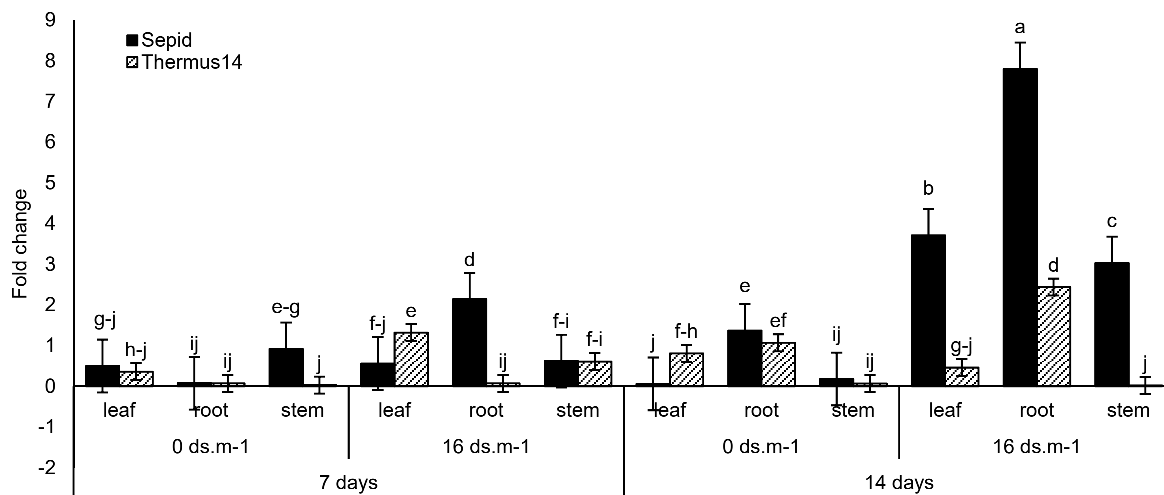

Fig 2. Expression pattern of *GhRLK* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

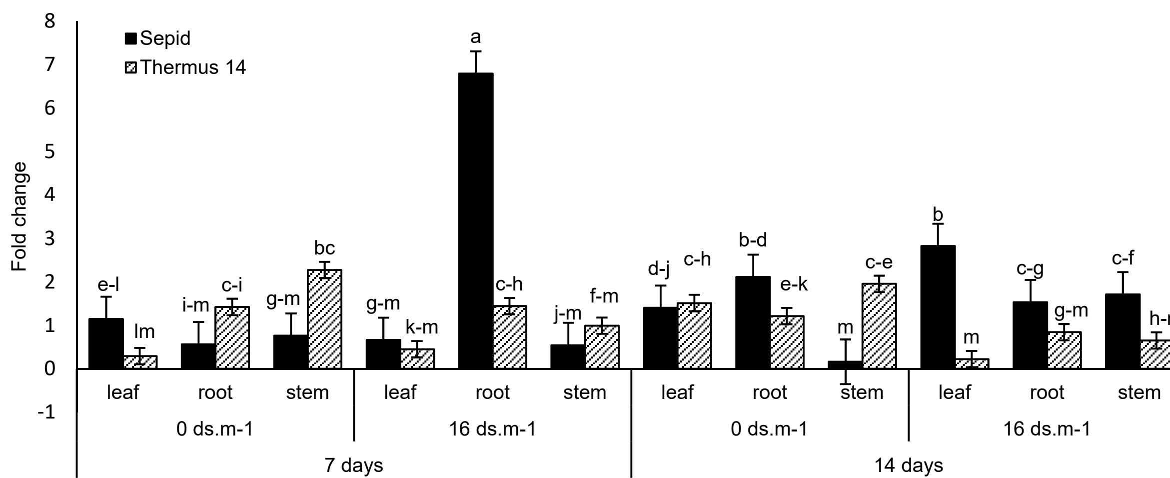

Fig 3. Expression pattern of *GhERF2* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in leaf, stem and root tissues and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

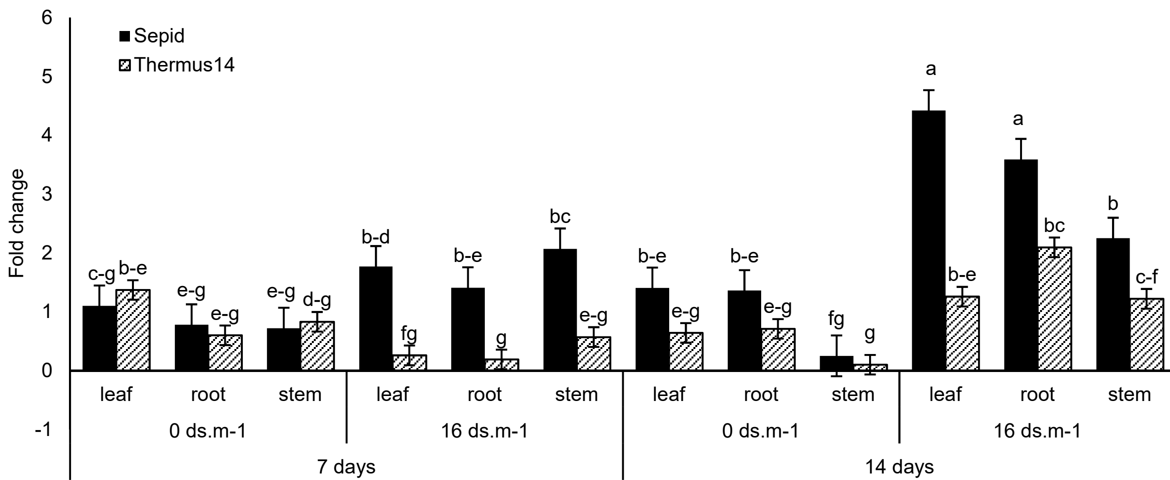

Fig 4. Expression pattern of *GhCIPK6* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

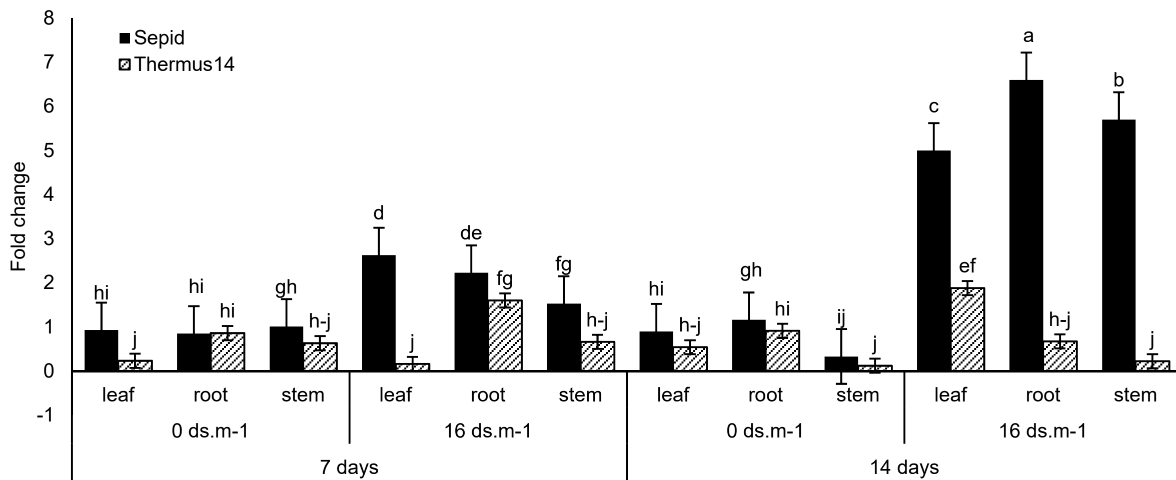

Fig 5. Expression pattern of *GhMPK2* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

Relative expression *GhCIPK6* in tolerant cultivar was 2.14-fold higher than sensitive cultivar. So salinity stress increased the *GhCIPK6* gene expression. Relative expression of three studied tissues in tolerant cultivar were at the highest level at 16 ds.m^{-1} 14 days after starting the stress. Although, a significant change was observed relative expression of three tissues in sensitive cultivars at 16 ds.m^{-1} salinity level at 14th day after stress initiation, but in all cases it was lower than the tolerant cultivar (Fig. 4).

GhMPK2: To study the effect of salinity stress on expression of *GhMPK2*, cotton cultivars seedling (Sepid and Thermus 14) stressed with NaCl concentration (8 and 16 ds.m-1)) for 7 and 14 day periods. Then real time analysis was performed in three tissues (leaf, stem and root). Relative expression of *GhMPK2* was significantly increased (3.42-fold higher than sensitive cultivar) after salinity stress. These result suggest that *GhMPK2* may be induced by salt stress when the plant was exposed to the stress for a 14-day period.

As is illustrated in Fig 5, the mentioned values indicated that the expression of stress-responsive genes such as *MPK2* has been increased at 14th day after stress initiation. Values indicated that the relative expression of

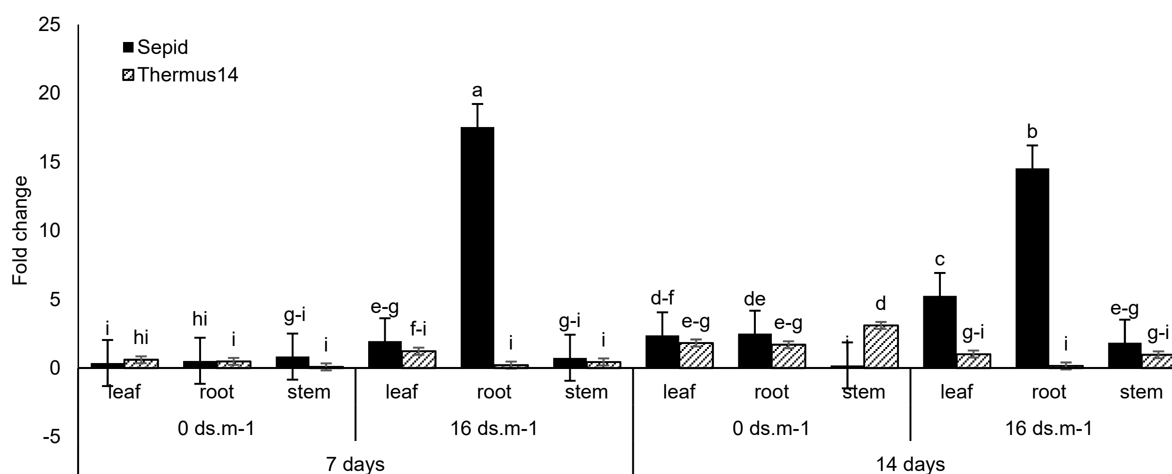

Fig 6. Expression pattern of *GhTPS* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

the three considered tissues in tolerant cultivar at salinity level up to 16 ds.m^{-1} was at its highest level 14 days after treating with salinity condition. Although a significant change was observed for the relative expression of three tissues in sensitive cultivars at a salinity level of 16 ds.m^{-1}; 14th day after stress initiation, it was in all cases lower than that of tolerant cultivar (Fig. 5).

- Functional genes: PR protein *(Gh14-3-3),* Transporter *(NHX1),* Carbohydrate metabolism *(GhTPS)* and Detoxification *(GhGST)*

GhTPS: The result of real time PCR analysis showed that the *GhTPS* gene expression was induced significantly by salt treatment at 7th day after stress. This expression in tolerant cultivar (Sepid) was higher than sensitive cultivar (Thermus 14). In salt condition, the *GhTPS* expression level increased to 4.10-fold in tolerant cultivar. Values in tolerant and sensitive cultivars indicated that relative expression *of TPS* increased in tolerant cultivar when the plant was exposed to the stress for a 7-day period.

Relative expression in root tissue showed the highest level, especially in tolerant cultivar, but in stem and leaf tissues showed lower expression, especially in sensitive cultivar (Fig. 6).

GhGST: Real Time PCR patterns for the *GhGST* gene under treatments (different concentrations of NaCl) and different time courses in two cotton cultivars are shown in Fig 6. Sepid had significantly higher expressions of *GhGST* at 14th after stress initiation, while sensitive cultivar (Thermus 14) showed high expressions of *GhGST* in stress and non-stress treatments but it was lower than tolerant cultivar (Sepid). The increment in tolerant cultivar was1.91-fold higher relative to control plants. In addition, result showed that the *GhGST* gene expression increased after treatments of NaCl in both Sepid and Thermus 14 cultivars. Relative expression in root tissue with the salinity level of 16 ds.m^{-1} had the highest level in tolerant cultivar. Although a significant relative expression gene was observed in sensitive cultivar unlike other genes (*GhRLK*, *GhMPK2*, *ChCIPK6, GhNHX1, Gh14-3-3* and *GhTPS1*) which had low expressions, its value was steal less than that of tolerant cultivar (Fig. 7).

GhNHX1: To determine the induction expression of *GhNHX1*, we firstly extracted total RNA from roots, stems and leaves of 14-day-old seedling of salt tolerant and salt sensitive cultivars of cotton for real time PCR. The result showed the *GhNHX1* gene expression was highly induced by salt stress in all organs. Secondly, the *GhNHX1* expression level were assayed in different salt-treated seedlings and seedling at different time points. When treated with different concentration of NaCl, the expression level was increased in tolerant cultivar seedling treated with higher concentration of NaCl (2 -fold higher than sensitive cultivar). Values in tolerant and sensitive cultivars indicated that the expression of stress-responsive genes such as *NHX1* increased when the plant was exposed to the stress for a 7 and 14 day periods.

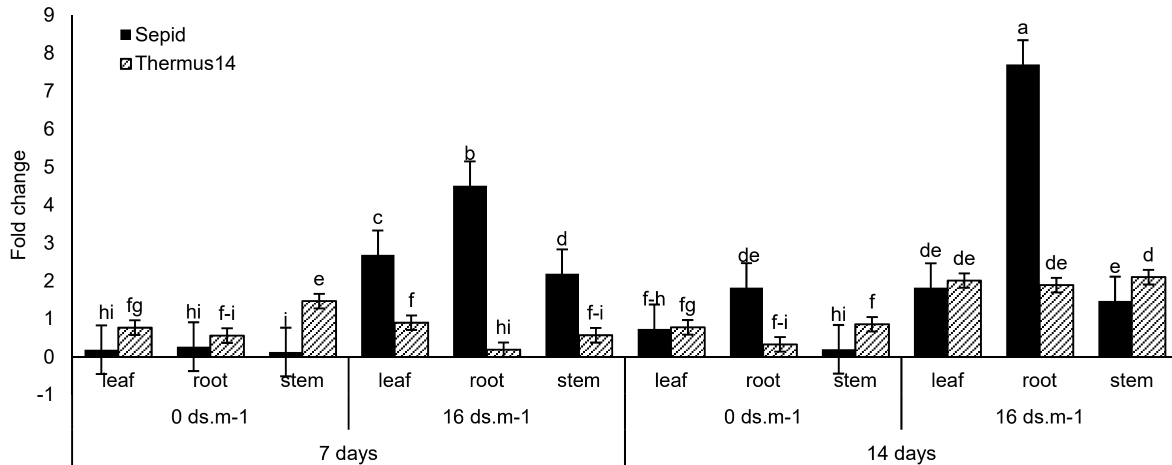

Fig 7. Expression pattern of *GhGST* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

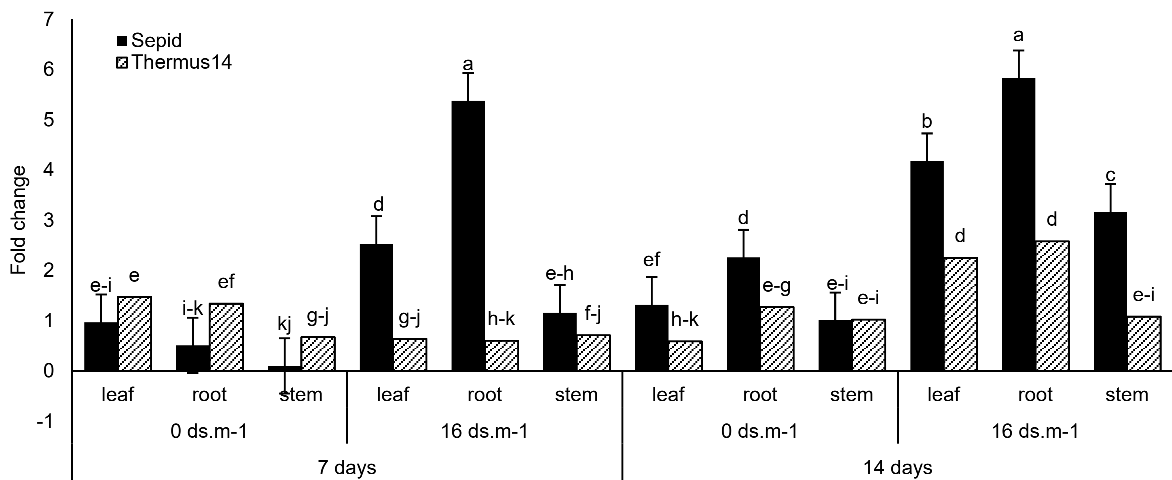

Fig 8. Expression pattern of *GhNHX1* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

Regarding the tolerant cultivar, the relative expression in the root tissue with the salinity level up to16 ds.m^{-1} showed the highest amount in both time courses. The relative expressions in the other tissues were less than that of in root tissue (Fig. 8).

Gh14-3-3: By using real time PCR, the expression pattern of *Gh14-3-3* gene was analyzed in different NaCl concentration, tissues, cultivars and time points (Fig.9). Under normal growth condition expression *Gh14-3-3* appeared to be very low in both of cultivars (tolerant and sensitive), but salt stress greatly increased the expression of *Gh14-3-3*. Tolerant cultivar showed an approx. 1.87-fold increase of expression level of *Gh14-3-3* higher than sensitive cultivar. Values in tolerant and sensitive cultivars indicated that the expression of stress-responsive genes such as 14-3-3 increased when the plant was exposed to the stress for a 7 and 14 day periods. In salt tolerant cultivar, with the salinity level up to 16 ds.m^{-1}, the relative expression of all tissues were in their highest levels of salinity in both studied time courses, while in the same cultivar, the relative expression, no salinity treatment, shows the lowest value in most tissues (Fig. 9).

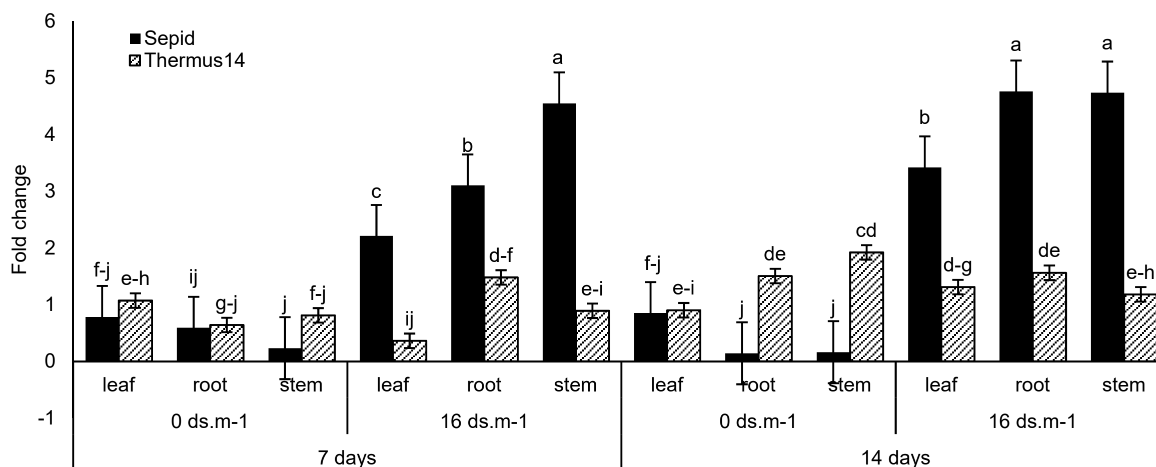

Fig 9. Expression pattern of *Gh14-3-3* gene using Real Time PCR under salt stress in cotton cultivars (Sepid and Thermus14), time points (7th and 14th day after salt stress initiation), in three tissues (leaf, stem and root) and two level salt stress (0 and 16 ds.m^{-1}), mean with same letter don't have different significant in p-value 0.05.

DISCUSSION

Receptor: *GbRLK* Gene Expression

According to the present study, in root tissue relative expression of *GbRLK* demonstrated the highest amount, especially in tolerant cultivar, and the stem and leaf tissue showed less expression, especially in sensitive cultivar. Results of previous studies have shown that the expression of *GhRLK* gene is induced by external factors such as abscisic acid, salicylic acid, methyl jasmonate, mock drought condition and high salinity. In Arabidopsis, overexpression of *GbRLK* of cotton increased the tolerance to ABA, drought and salinity stress in transgenic plants [76]. Other RLKs such as *ARCK1* [61], *GHR1* [33] and *RPK1* [54] in Arabidopsis and *OsSIK1* [55] in rice, contribute to tolerance in abiotic stresses. In addition, the expression of this gene plays a vital role in biotic stresses, including resistance to fungi species *Verticillium dahliae* [77].

Transcription factor: *GhERF2* Gene Expression

In current study, relative expression of *GhERF2* in root tissue demonstrated the of level highest expression, especially in tolerant cultivar, however, stem and leaf tissue indicated lower expression, especially in sensitive cultivar. In the other studies of *ERFs*, it has been shown that *AtERFs* [19], *CaERFLP1* [15] and *JERFs* [72] are induced by ethylene, ABA, salinity, drought and cold stresses. Analysis of expression patterns of *GhERF2*, *GhERF3* and *GhERF6* showed that the expression of these genes is significantly promoted by drought, cold, ethylene and ABA [37].

Signaling pathway*: GhCIPK6 and GhMPK2* Gene Expression:

GhCIPK6: In this study, relative expression of studied tissues in tolerant cultivar were at the highest level at 16 ds.m^{-1} 14 days after starting the stress. Although after 14 days a significant amount for the relative expression of three tissues in sensitive cultivars at 16 ds.m^{-1} salinity level was observed, but in all cases it was lower than the tolerant cultivar. In Arabidopsis, *AtCIPK24* (*SOS2*) interacts with *AtCBL4* (*SOS3*) to transduce Ca^{2+} signal, thereby activates a plasma membrane-localized Na^+/H^+ antiporter (*SOS1*) and vacuolar H^+ -ATPase to maintain ion homeostasis during salt stress [25; 45]. Similar *CBL/CIPK* networks seem to exist in other plants, such as in rice which was indicated by the presence of 10 CBLs and 30 CIPKs as well as a conserved SOS pathway [47, 65]. Transgenic plants with over-expressing *BnCIPK6* in Arabidopsis indicated enhanced high salinity but low tolerance to phosphate, and activation of *BnCIPK6* (*Brassica napus*) made transgenic Arabidopsis plants hypersensitive to ABA [9]. Ectopic expression of a *Ca-CIPK6* (*Cicer arietinum*) indicated enhanced salt tolerance in transgenic plants with this gene [62]. The expression of *AtCIPK6* is induced by ABA, osmotic and salt stress, and it interacts with *AtCBL1/3/4* to confer the

salt tolerant in chickpea [40]. He *et al.* has shown that overexpression of *GhCIPK6* enhances tolerance to salt and drought stresses and reduces ABA sensitivity in transgenic Arabidopsis plants [28].

GhMPK2: the result of this study indicated that the relative expression of the three considered tissues in tolerant cultivar at salinity level up to 16 $ds.m^{-1}$ was highest level at 14th day after stress initiation. According to amino acid sequencing, MAPK contains 11 domains (I–XI) that are necessary for the catalytic function of serine/threonine protein kinase, and domains VII and VIII of MAPKs are well conserved [30] MAPKs carry either a Thr-Glu-Tyr (TEY) or Thr-Asp-Tyr (TDY) phosphorylation motif at the active site, which can be classified into four major groups (A, B, C and D) based on the presence of TDY and TEY motifs [35]. Activation of *MAPKs* have been detected when plants are exposed to various stresses such as salinity [49, 51], drought, cold [39] and pathogens [29, 75]. In Arabidopsis, of group B, *MPK4* is involved in osmotic stress response pathways activated by hypoosmolarity [13]. *MPK3* and *MPK6* (group A, MAPKs) are reported to be strongly activated by abiotic stress [3]. In rice, overexpression of *OsMAPK5* (group A, MAPK), leads to enhanced tolerance to drought, salt, and cold stresses [66]. In maize, a group of *ZmSIMK1* (Group B, MAPKs) is induced by drought, salt, and ABA. Overexpression of *ZmSIMK1* in Arabidopsis results in constant expression of stress-responsive marker genes (*RD29A* and *P5CS1*) and increased tolerance to salt stress [23]. Zhang *et al.* has shown that *GhMPK2* is responsive to salt, drought and ABA Treatment, and ectopic expression of *GhMPK2* positively indicates salt and drought tolerance [74].

Functional genes: PR protein *(Gh14-3-3),* Transporter *(NHX1),* Carbohydrate metabolism *(GhTPS)* and Detoxification *(GhGST)*

GhTPS: In current study, relative expression of this gene in root tissue had the highest level, especially in tolerant cultivar, but in stem and leaf tissues showed less expression, especially in sensitive cultivar. In a study on the *TPS* family in *G. raimondii*, *G. arboreum* and upland cotton cultivars, *TPS1* in *G. arboreum* and upland cotton have been induced by salinity stress in root, leaf and stem tissues [50]. Response of *TPS1* have also been reported in the other plant such as coconut [71], maize [36] and rice [44] and its expression induced by salt, temperature and drought stress.

GhGST: in this project, *GhGST* expression induced by salt stress. The results corroborate the ideas of the previous researchers such as: They showed that overexpression of *GSTs* improved abiotic tolerance in some plants. Such as: 35 of 56 *SbGSTUs* in Sorghum indicated significant response to abiotic stresses including cold, PEG and high salinity [10]. The expression of *GmGSTL1* from soybean in transgenic Arabidopsis could alleviate the symptoms under the salt stress [7]. Expression of *GmGSTL1* from soybeans in Arabidopsis transgenic plants reduced the effects of salinity stress [7]. In another experiment, in *G. raimondii* and *G. arboreum*, all *GSTs* expression in root was increased by salinity treatments, while only a slight increase was observed in stems, leaves, and cotyledon [12].

GhNHX1: In current project, *GhNHX1* expression level increased by salt stress. The promoted expression of the antiporter genes by salt stress have been reported in glycophytes including *Arabidopsis thaliana* [2], and halophytes, *Mesembryanthemum crystallinum* [8] and *Atriplex gmelini* [26]. Antiporter is also activated by salt stress in salt-tolerant plants, such as barley (*Hordeum vulgare*) [20] and *Beta vulgaris* [4]. These results indicated that the vacuolar Na^+/H^+ antiporters play a leading role in responding to salinity stress in salt-tolerance of a wide variety of plants. Wu *et. al.* indicated that the expression of *GhNHX1* in cotton seedlings was induced by salt stress. Also, the *GhNHX1* expression levels would be different with respect to differences in soil salinity [64].

Gh14-3-3: In current project, *Gh14-3-*3 expression level induced by salt stress in different salt levels, tissues and time points. In Arabidopsis (13 members), tobacco (11 members) and tomato (12 members), many *14-3-3* protein isoforms have been found. So far, *14-3-3* proteins to be involved in a large range of abiotic signaling processes and to interact with many target molecules, including plasma membrane H+-ATPase, ion channels ascorbate peroxidase (*APX*) and abscisic acid (ABA), All these targets are very important for plants adapting to salinity [16, 56. 63, 69].

CONCLUSION

All of selected genes induced by salt stress but some of them (*GhGST*, *GbRLK*, *GhCIPK6* and *GhMPK2*) showed high expression at 14th day after stress initiation and other ones, *GhERF2* and *GhTPS1,* demonstrated high expression at 7th day after stress initiation. So there was

different reaction for each gene when sensed salt stress. Between tissues, root expression level showed higher than stem and leaf because plant roots are the primary organ involved in salt tolerance. Plant roots perceive and respond to their environment using signaling systems, particularly adapted to salinity in soils.

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بررسی بیان ژن‌های مرتبط با شوری در دو رقم حساس و متحمل پنبه تحت تنش شوری

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چکیده

شوری یکی از مهم‌ترین فاکتورهای محدودکننده توسعه محصولات کشاورزی است. اگرچه به طور کلی پنبه دارای تحمل نسبی به شوری است. ولی شوری سبب کاهش رشد آن در مرحله جوانه‌زنی و گیاهچه‌ای می‌گردد. در این تحقیق نتایج Real Time PCR در قالب طرح اسپلیت فاکتوریل در زمان بر پایه طرح بلوک های کامل تصادفی با سه تکرار، سه بافت ریشه، ساقه و برگ گیاهچه‌های ۱۴ روزه پنبه، دو رقم پنبه متحمل (سپید) و حساس (ترموس ۱۴)، دو سطح ۰ و ۱۶ دسی‌زیمنس بر متر در سه زمان ۰، ۷ و ۱۴ روز بعد از تنش شوری آنالیز شدند. ژن‌های انتخاب شده برای واکنش Real Time PCR در این پژوهش خروجی نرم‌افزار Cytoscape 3.3.0 بوده است. نتایج نشان داد که ژن‌های انتخاب شده *GhERF2*، *GhMPK2*، *GhCIPK6* ، *GbRLK*، *GhNHX1*، *GhGST*، *GhTPS1* و *Gh14-3-3* به تنش شوری پاسخ مثبت داده‌اند و بیان آنها در ریشه بیشتر از سایر بافت ها بوده است بعلاوه میزان بیان در ژنوتیپ متحمل سپید از ژنوتیپ حساس بیشتر بوده است. . اما افزایش کمی در ژنوتیپ حساس (ترموس ۱۴) بعد از ۱۴ روز اعمال تنش در ژنهای *GhERF2* و *GhGST* دیده شده است.

کلمات کلیدی: تنش غیرزیستی، Real time PCR، تحمل به شوری