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Identification of heat responsive genes in cotton

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Abstract

High summer temperature negatively affects cotton yield, and better understanding of genetic mechanisms of heat stress responses in cotton may facilitate development of new heat tolerant cultivars. We attempted to determine heat responsive genes in cotton using tolerant (Stoneville 453, BA 119) and susceptible (Nazilli 84S) cultivars. Twenty five expressed sequence tags (ESTs) were sequenced and studied for gene homology. Sixteen ESTs were significantly similar to known genes, whereas eight ESTs were similar to not annotated cDNA clones and 1 EST did not show homology to any known gene. Expression of some ESTs was analyzed by quantitative real-time PCR and IAA-ala hydrolase (IAR3), folylpolyglutamate synthase (FPGS3), and two not annotated ESTs (GhHS126 and GhHS128) were consistently up-regulated under both short- and long-term heat stress. Since cotton is considered relatively more heat tolerant than most of the other crops, it can be suggested that these genes and ESTs could play a significant role in heat tolerance. In addition, GhHS126 and GhHS128 might be parts of the new candidate genes for heat tolerance.

Additional key words: differentially expressed genes, *Gossypium hirsutum*, high temperature, real-time quantitative PCR.

Introduction

Heat stress negatively affects various physiological, biochemical, and growth processes in plants. The optimal daily average temperature for growth of cotton is 27 to 29 °C (Reddy *et al*. 2004), however, cotton is often cultivated in regions where daily temperature exceeds 40 - 45 °C (Rahman *et al*. 2004, Singh *et al*. 2007), particularly during reproductive stage. Since there is a strong negative correlation between high temperature and cotton yield (Ashraf *et al*. 1994, Oosterhuis 2002), heat stress is considered one of the most important elements for reduced cotton productivity. Plant tolerance to high temperature may be achieved through various mechanisms, including changes at the molecular, cellular, biochemical, physiological, and whole-plant levels.

The effect of high temperature is primarily on photosynthetic functions. Net photosynthetic rate and efficiency of photochemical reactions driven in thylakoid membranes are decreased by high temperature (Wang *et al*. 2006, Essemine *et al*. 2011), whereas dark respiration increases (Bednarz and Van Iersal 2001). However, plants evolved diverse physiological and molecular mechanisms to adapt to high temperature. The activity of antioxidant enzymes is altered in cotton in order to reduce oxidative damage (Gür *et al.* 2010). Gong *et al*. (2012) reported an increase in the activities of ascorbate peroxidase, catalase, peroxidase, superoxide dismutase, and glucose-6-phosphate dehydrogenase (G6PDH) in *Przewalskii tangutica* and tobacco calli after 40 °C treatment. Further, expression of heat shock protein (*HSP*) genes is significantly up-regulated in plants at high temperature (Vierling 1991, Tao *et al.* 2012). It is considered that HSPs have very important role at heat tolerance in plants.

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Abbreviations: ABCC3 - MRP-like ABC transporter protein; CIPK - CBL-interacting serine/threonine-protein kinase; CTL2 - chitinase-like protein; DD - differential display; DD-PCR - differential display PCR; EST - expressed sequence tag; FPGS3 - cytosolic folylpolyglutamate synthase; HSP - heat shock protein; IAR3 - IAA-ala hydrolase; LSm8 - *N*-α-acetyltransferase 38, NatC auxiliary subunit-like; PP2C - protein phosphatase 2C; psaB-rps14 - photosystem I P700 apoprotein A-ribosomal protein S14; RPS14 - 40S ribosomal protein S14; RT-qPCR - real-time quantitative PCR; TH1 - thiazole biosynthetic enzyme.
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Abiotic stresses alter expression patterns of numerous genes in plants (Seki *et al.* 2001, Singh *et al.* 2002). Differentially expressed genes are divided into four groups: *1*) unique, *2*) up-regulated, *3*) down-regulated, and *4*) suppressed. It is thought that particularly unique and up-regulated genes have a function in increasing stress tolerance (Yamaguchi-Shinozaki *et al.* 2002). Alteration in gene expression causes changes in physiological and biochemical processes of plants, and, as a result, plants may tolerate heat stress. Therefore, there have been numerous studies on identification of differentially expressed genes in plants under abiotic stresses. Plants have complex defense mechanisms controlled by multiple genes. Zhang *et al*. (2013a) identified 4 153 heat-responsive genes that were over-expressed 3-fold and more in rice flag leaf. In fescue, 299, 477, and 395 differentially expressed ESTs were identified at temperature of 39, 42 and 44 °C, respectively (Zhang *et al*. 2005). A total of 698 differentially expressed ESTs in fescue were grouped into 10 functional categories: cell maintenance and development; chloroplast and photosynthesis; metabolism; protein synthesis; signaling; stress related; transcription factors; transport; unclassified proteins; and others. A total of 519 differentially expressed ESTs are identified in cotton under water stress (Park *et al*. 2012). Nearly 70 % of the ESTs belonged to four major categories: stress/defense, gene regulation, metabolism, and unclassified. They found that heat shock protein-related and reactive oxygen species-related ESTs were induced by water stress. Xu *et al*. (2013) reported 2 634 (1 513 up-regulated + 1 121 down-regulated), 2 449 (1 586 up-regulated + 863 down-regulated), 2 271 (946 up-regulated + 1 325 down-regulated) and 3 352 (933 up-regulated + 2 419 down-regulated) genes differentially expressed after exposure to NaCl for 3, 12, 72, and 144 h, respectively. A total of 36 961 transcripts either up- or down-regulated were identified at 150 mM NaCl or 17 % PEG treatment in at least one tissue sample of cotton leaf, stem, and root (Zhang e*t al.* 2013b). Their study revealed tissue selective signaling and hormone crosstalk in response to salt and osmotic stresses in *G. arboretum*.

Although knowledge on differentially expressed genes by drought and salt stresses has increased currently, only limited molecular research has focused on heat stress responses in cotton. Better understanding of the cotton response to heat stress at molecular, cellular, and genetic levels would facilitate cotton breeders to generate more heat tolerant cotton genotypes. In this study, we attempted to identify heat responsive genes in two tolerant and one susceptible cotton cultivars by using differential display (DD) and RT-qPCR techniques.

Materials and methods

Two experiments were carried out to determine heat responsive genes in cotton (*Gossypium hirsutum* L.) under heat stress conditions. Experiment 1 was designed to identify differentially expressed genes by differential display technique. Experiment 2 was designed to determine heat responsive genes in cotton by RT-qPCR, using two tolerant (Stoneville 453 and BA 119) and one susceptible (Nazilli 84S) cultivars.

Experiment 1: Cotton cv. Stoneville 453 (commonly grown under hot and semiarid climatic conditions) was used to determine differentially expressed genes under heat stress. Stoneville 453 is considered as a heat resistant cotton cultivar having higher productivity than the other cultivars when heat stress occurs (Çopur *et al.* 2010). In our previous experiments, Stoneville 453 exhibited low cell injury according to cellular membrane thermostability (CMT) assay under heat stress (unpublished data).

Plants were grown in a growth chamber (*DigiTech DP16C,* Ankara, Turkey) with day/night temperature of 30/24 °C, 14-h photoperiod, irradiance of 350 μmol m^{-2} s^{-1}, and air humidity of around 60 %. The experiment was conducted with three replications containing three pots with one plant in each pot. Heat stress treatments were applied at the beginning of squaring stage. For differential display studies, cotton plants were divided into three groups as control, 38 °C, and 45 °C treatments. The control group plants was grown at above mentioned temperature. For the second group, temperature gradually increased from 30 to 38 °C within 1 h and 45 min for acclimatization and afterwards plants were kept at 38 °C for 2 h. Plants of the third group were firstly acclimated at 38 °C for 2 h, then temperature was gradually increased to 45 °C in 2 h, and then plants were kept at 45 °C for 2 h. Young leaves were collected from control, 38 and 45 °C treated plants and immediately frozen in liquid nitrogen. Frozen samples were stored at -80 °C until RNA extraction for differential display.

Total RNA was extracted from bulk of young leaves of three different plants from each treatment using *RNeasy*® plant mini kit (*Qiagen,* Hilden, Germany). DNA contamination was removed from the total RNA samples by treating with DNase I of *MessageClean* kit (*GenHunter*, Nashville, TN, USA) according to the manufacturer's instructions. The total RNA samples were quantified using a spectrophotometer (*Shimadzu UV-1601*, Duisburg, Germany) at 260 nm. RNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining. Total RNA was converted to cDNA by reverse transcription reaction using *RNAspectra*™ kit (*GenHunter*). Three RNA aliquots were taken from each stress treatment. For each RNA aliquot, three cDNA pools were synthesized. Totally nine distinct reverse transcription reactions were performed by combining three anchored primers (Table 1) and three RNA samples from stress treatments. Each reverse transcription mixture of a 20 mm^3 volume contained 200 ng of total RNA,

100 U *MMLV* reverse transcriptase, 0.2 μM of one anchored primers, 50 μM of each dNTP, 25 mM Tris-HCl (pH 8.3), 37.6 mM KCl, 1.5 mM $MgCl_2$, and 5 mM dithiotreitol (DTT). Reverse transcription reaction was performed at 37 °C for 1 h.

Differential display (DD) of cDNA was performed as described by Liang and Pardee (1992) with some modifications using *RNAspectra*™ kit (*GenHunter*). Each DD-PCR was combined with one of sixteen different arbitrary primers and one of three anchored primers (Table 1). Each reaction mixture of DD-PCR contained 2 mm^3 of cDNA from the previous step, 50 μM of each dNTP, 0.2 μM of one anchor primer, 0.2 μM of one arbitrary primer, 1 unit *Taq* DNA polymerase (*Bio-Rad,* Hercules, CA, USA), 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001 % (m/v) gelatin in a final volume of 20 mm^3. The PCR was carried out for 40 cycles with the following temperature profile: denaturation at 94 °C for 30 s, annealing at 40 °C for 2 min, and extension at 72 °C for 1 min. After 40 cycles, the PCR product was subjected to additional extension step at 72 °C for 5 min. DD-PCR products were separated by 5 % (m/v) denaturing polyacrylamide gel electrophoresis (PAGE) at 60 W for 2.5 h and visualized by silver staining.

Differentially expressed bands were excised from polyacrylamide gels and cDNA was eluted from corresponding gel slice. Extracted cDNA was reamplified under the same conditions as done previously but with 10 times less dNTP concentration. PCR products were cloned into PCR-TRAP cloning vector according to the manufacturer's protocol (*GenHunter*). Clones were sequenced bidirectional using Lgh (5′-CGACAA CACCGATAATC-3′) and Rgh (5′-GACGCGAAC GAAGCAAC-3′) primers by *GenHunter's* sequencing service. ESTs obtained from this study were compared with nucleotide and protein sequences from various databases by using *BLAST* algorithm (Altschul *et al.* 1997).

To confirm differential display results, a RT-qPCR method was used. Total RNA was extracted from young leaves through cetyltrimethylammonium bromide (CTAB) and LiCl according to Ginzberg *et al.* (2009) with some modifications. Genomic DNA contamination was removed from total RNA by DNase digestion using DNase set (*Qiagen,* Hilden, Germany). The pure RNA samples were quantified using spectrophotometer (*Shimadzu UV-1601*) at 260 nm. RNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining. Equal amount of pure RNA from each sample was converted to cDNA by reverse transcription reaction using *QuantiTect* RT kit (*Qiagen*) with wipeout gDNA stage according to manufacturer's instructions. Reverse transcription reaction was performed at 42 °C for 30 min. cDNA synthesis was checked by standard PCR amplification using cotton *actin* primers. Primer sequences (forward primer, 5′-ATTGTGAGC AACTGGGATGA3′; reverse primer, 5′-GTAGAT GGGGACGGTGTGAG-3′) of cotton *actin* gene (AF059484) were obtained from Sotirios *et al.* (2006). RT-qPCRs were performed with 6 mm^3 of 1:100 diluted cDNA, 3.25 mm^3 of each 2 μM specific primer (Table 2), and 12.50 mm^3 of 2× qPCR mastermix (*Fermentas,* Leon-Rot, Germany) in a total volume of 25 mm^3. Specific primers were designed from sequenced ESTs by using *Primer 3* program (http://fokker.wi.mit.edu/primer3/input.htm). Each RT-qPCR was performed with three technical replicates and the conditions consisted of an initial incubation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 52 °C for 15 s, and 72 °C for 20 s for all investigated ESTs. Then, RT-qPCR, melting curve analysis was used for determination of specific amplicon amplification. Relative amounts of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). *Polyubiquitin* gene (GhUBQ14, DW505546), a very stable reference gene in cotton (Artico *et al*. 2010), was used for normalization. Primer sequence of GhUBQ14 was taken from Artico *et al.* (2010).

Table 1. Primers used in mRNA differential display.

	Primer names	Sequence
Anchored primers	H-T_{11}G	5′-AAGCTTTTTTTTTTG-3′
	H-T_{11}A	5′-AAGCTTTTTTTTTTA-3′
	H-T_{11}C	5′-AAGCTTTTTTTTTTC-3′
Arbitrary primers	H-AP 1	5′-AAGCTTGATTGCC-3′
	H-AP 2	5′-AAGCTTCGACTGT-3′
	H-AP 3	5′-AAGCTTTGGTCAG-3′
	H-AP 4	5′-AAGCTTCTCAACG-3′
	H-AP 5	5′-AAGCTTAGTAGGC-3′
	H-AP 6	5′-AAGCTTGCACCAT-3′
	H-AP 7	5′-AAGCTTAACGAGG-3′
	H-AP 8	5′-AAGCTTTTACCGC-3′
	H-AP 49	5′-AAGCTTTAGTCCA-3′
	H-AP 50	5′-AAGCTTTGAGACT-3′
	H-AP 51	5′-AAGCTTCGAAATG-3′
	H-AP 52	5′-AAGCTTGACCTTT-3′
	H-AP 53	5′-AAGCTTCCTCTAT-3′
	H-AP 54	5′-AAGCTTTTGAGGT-3′
	H-AP 55	5′-AAGCTTACGTTA-3′
	H-AP 56	5′-AAGCTTATGAAGG-3′

Experiment 2: To strengthen reliability of expression of investigated genes under heat stress and to determine heat responsive genes, two tolerant (Stoneville 453 and BA 119) and one susceptible (Nazilli 84S) cotton cultivars were studied under long-term heat stress. BA 119 was chosen as a heat tolerant cultivar since it exhibited better yield under high temperature of field conditions (Çopur *et al.* 2010) and it had less electrolyte leakage in CMT assay (unpublished data). Nazilli 84S was determined a susceptible cultivar among tested seventeen cotton cultivars as a result of our CMT assay (unpublished data). To identify correlation between genes and heat tolerance, stress treatment study was carried out with three replications including three pots with two plants in each pot. Three cultivars were grown under the same

conditions as mentioned above for Stoneville 453 and exposed to heat stress at the beginning of squaring stage. Ambient temperature was increased from 30 to 38 °C and plants were kept at day/night temperature of 38/33 °C for 24 h. After acclimatization, temperature were increased to 45 °C and plants were exposed to 45 °C for 2 h. Young leaves were harvested from control and 45 °C treated plants and immediately frozen in liquid nitrogen. Frozen samples were stored at -80 °C until RNA extraction.

Expression of several genes was investigated by RT-qPCR. Total RNA was extracted from young leaves through CTAB and LiCl according to Ginzberg *et al.* (2009) with some modifications. Genomic DNA contamination was removed from total RNA by DNase digestion using DNase set (*Qiagen*). The pure RNA samples were quantified using spectrophotometer (*Shimadzu UV–1601*) at 260 nm. RNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining. Equal amount of pure RNA from each sample was converted to cDNA by reverse transcription reaction using *QuantiTect* RT kit (*Qiagen*) with wipeout gDNA stage according to manufacturer's instructions. Reverse transcription reaction was performed at 42 °C for 30 min. cDNA synthesis was checked by standard PCR amplification using cotton *actin* primers. Primer sequences (forward primer, 5′-ATTGTGAGCAAC TGGGATGA-3′; reverse primer, 5′-GTAGATGGGGA CGGTGTGAG-3′) of *actin* gene (AF059484) were obtained from Sotirios *et al.* (2006). A RT-qPCR was performed with 6 mm^3 of 1:100 diluted cDNA, 3.25 mm^3 of each 2 μM specific primer (Table 2), and 12.50 mm^3 of 2× qPCR mastermix (*Fermentas*) in a total volume of 25 mm^3. Specific primers were designed from sequenced ESTs by using *Primer 3* program. Each RT-qPCR was performed with three technical replicates and the RT-qPCR conditions consisted of an initial incubation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 52 °C for 15 s, and 72 °C for 20 s for all investigated ESTs. After RT-qPCR, melting curve analysis was used for determination of specific amplicon amplification. Relative amounts of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). *Polyubiquitin* gene (GhUBQ14, DW505546) was used for normalization.

Table 2. cDNA-specific primers used for quantitative real-time PCR.

EST	Acc. No.	Homologous gene	Forward primer sequence	Reverse primer sequence
GhHS23	JK749842	CTL2	5′-AGCTTGACCTTTTGGGAGTT-3′	5′-ACAACCAACACAAACTGCTG-3′
GhHS26	JK749843	TH1	5′-TGGAACATATGTGGGAAGCA-3′	5′-AAGGCATGCAAGGAGAAGAA-3′
GhHS83	JK749849	psaB-rps14	5′-GCTTGATTGCCTCTACATCG-3′	5′-TGCCATAATGGTTCAGTTCC-3′
GhHS93	JK749850	ABCC3	5′-CGGTCATAACAATCGCTCAC-3′	5′-GCTGTGCAAACGATGAAGAC-3′
GhHS96	JK749852	CIPK	5′-TGAGGTGGGAAGTCATTGTT-3′	5′-CTTAAAGCAGCCCACTACCA-3′
GhHS97	JK749853	IAR3	5′-AACGATAAGGACTTGCACGAG3′	5′-AATACCCAGGAAACGCCTCT-3′
GhHS100	JK749854	PP2C	5′-GGTCAGGTCTTTGCAGTTCC-3′	5′-GAGCAGCAGTGGAGAAAGGT-3′
GhHS101	JK749855	PK HT1	5′-CACACTCACACCTCCTCCAT-3′	5′-CCTTTGCCCGAACAGATAAC-3′
GhHS106	JK749856	LSm8	5′-CTCCACCAAGGAAGGTGTTC-3′	5′-CAAGTCGAGTGCAGAATCCA-3′
GhHS110	JK749857	-	5′-ACTCAATTGGATGTGACGAAAC-3′	5′-AGAAACTACAATCACCCGAACT-3′
GhHS126	JK749859	-	5′-TGGGATGACTGGTTATGCTT-3′	5′-CAAATCTATCGCTCCTTCCA-3′
GhHS127	JK749860	FPGS3	5′-TCAACGCTTCCTCTCATTTC-3′	5′-TGAAATTGCCTCATCCAGTT-3′
GhHS128	JK749861	-	5′-TTGGGATGACTGGTTATGCT-3′	5′-CAAATCTATCGCTCCTTCCA-3′
GhHS129	JK749862	RPS14	5′-GTCAGCACTTAGAGCCCTTG-3′	5′-AAATGTGCAGCAAGAGAACC-3′
GhHS150	JK749863	RNA helicase 38	5′-CTCCACCAAGGAAGGTGTTC-3′	5′-CAAGTCGAGTGCAGAATCCA-3′
-	DW505546	GhUBQ14	5′-TATTTGAACGGAGGAAAAGG-3′	5′-AAATCCGCTGGCAAATTAC-3′

Results

Stoneville 453 was used as the preliminary plant material in order to identify differentially expressed genes in cotton. Gene expression profile of heat stress treated (38 and 45 °C) Stoneville 453 was compared with control by using DD technique. A total of 147 differentially expressed EST bands were scored on the gels. Among them, 41 ESTs were either down-regulated or suppressed, whereas 106 ESTs were either up-regulated or unique for heat stress. Eighty four ESTs of 147 scored bands were excised from the gels and amplified by PCR using the same primer pairs as mentioned in Materials and methods. Among 84 ESTs, 39 ESTs generated only one band on agarose gel. These products were cloned into PCR-TRAP vector and 25 cDNA fragments were sequenced bidirectional. The sequences of the ESTs were compared with nucleotide and protein sequences from various databases by using basic local alignment search tool (*BLAST*). Sixteen of twenty five ESTs showed significant similarity to known genes, while 8 ESTs were similar to not annotated cDNA clones and 1 EST did not show homology (Table 1 Suppl.).

For confirmation experiment, growing conditions and

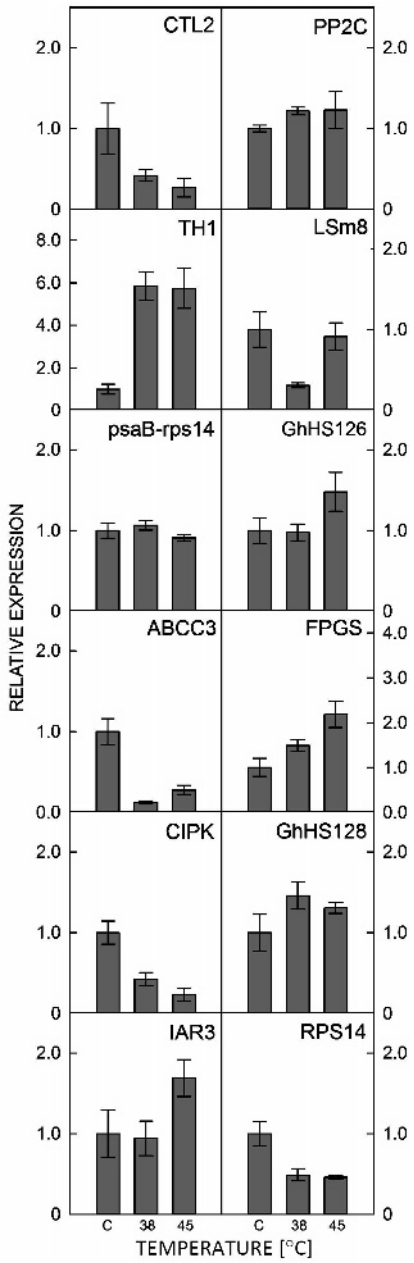

Fig. 1. Relative expression of heat responsive genes in cv. Stoneville 453 under control conditions and heat stress of 38 or 45 °C confirmed by RT-qPCR. Means ± SE of three replications.

heat treatments of Stoneville 453 were the same as in DD studies. RT-qPCR analysis was used to validate expression patterns of twelve genes determined in DD studies. As a result of RT-qPCR, expression patterns of 6 genes *TH1*, *ABCC3*, *IAR3*, *FPGS*, *GhHS126*, and *GhHS128* were in agreement with that of DD analysis, whereas 4 genes *CTL2*, *CIPK*, *LSm8*, *RPS14* showed contrary expression pattern as compared to DD experiment. However, expressions of *psaB-rps14* and *PP2C* were not altered by heat stress in cotton. According to RT-qPCR analysis, gene expressions of *TH1*, *IAR3*, *GhHS126*, *FPGS*, and *GhHS128* were increased by heat stress. In contrast, *CTL2*, *ABCC3*, *CIPK*, *LSm8*, and *RPS14* genes were down-regulated (Fig. 1).

Expressions of fifteen genes were investigated in three different cotton cultivars under long-term heat stress. Plants were exposed to heat stress for approximately 28 h, which is different heat treatment than in DD study (see Materials and methods). RT-qPCR analysis was used to determine accurate expression pattern of investigated genes under heat stress and to identify heat responsive genes in three cotton cultivars. As a result, 11 of the 15 ESTs were up-regulated 2-fold and more in at least two cotton cultivars, whereas 2 ESTs (*CTL2* and *RPS14*) were down-regulated. In addition, expression of two ESTs (*TH1* and *PP2C*) was not affected significantly by long-term heat treatment (Fig. 2).

The RT-qPCR results exhibited that *ABCC3* and CIPK were up-regulated in three cotton cultivars under long-term heat stress, whereas they were down-regulated in Stoneville 453 by short-term treatment at 38 and 45 °C. *IAR3*, *FPGS*, *GhHS126*, *GhHS128* were up-regulated under both long-term and short-term heat stress (Figs. 1 and 2). *IAR3* was over-expressed 3.6-, 6.2- and 8.0-fold in Stoneville 453, BA 119, and Nazilli 84S, respectively, under long-term heat stress (Fig. 2). Up-regulation of *FPGS3* was 2.8-, 11.7-, and 9.9-fold in Stoneville 453, BA 119, and Nazilli 84S, respectively. *GhHS126* was up-regulated 3.6-, 2.7-, and 6.2-fold in Stoneville 453, BA 119, and Nazilli 84S, respectively. Similarly, *GhHS128* showed 3.1-, 4.2-, and 5.9-fold over-expression in Stoneville 453, BA 119, and Nazilli 84S. The other five genes over-expressing two folds and more in at least two cotton cultivars by heat stress were *LSM8*, *psaB-rps14*, *RNA helicase 38*, *PK HT1-like*, and *GhHS110*.

Discussion

Tolerant cultivar Stoneville 453 was used in order to identify differentially expressed genes under heat stress by DD technique. Although DD technique has some limitations, it produces powerful and useful results for identification of stress-related genes. However, DD results needs to be confirmed by Northern blot, semi-quantitative RT-PCR, or real time qPCR. Therefore, to confirm differential expression of ESTs obtained from the DD study, 12 differentially expressed ESTs were analyzed by RT-qPCR in Stoneville 453. Similar expression pattern was observed for six out of 12 ESTs, whereas contrary expression pattern was in another four as compared to DD results. In addition, expression of two ESTs was not altered significantly in leaves of Stoneville 453 by heat stress. Earlier researchers observed similar differences in expression results between DD or cDNA-

AFLP and expression confirmation experiments (Ueda *et al.* 2002, Liu and Baird 2003, Escalettes *et al.* 2006). This discrepancy in expression of some ESTs is probably due to some limitations of DD technique such as some false positive results. In addition, three different cotton cultivars (Stoneville 453, BA 119, Nazilli 84S) were exposed to longer heat stress (28 h) and expression of 15 ESTs was investigated by RT-qPCR to determine heat responsive genes.

Among ESTs identified, *GhHS93* and *GhHS94*

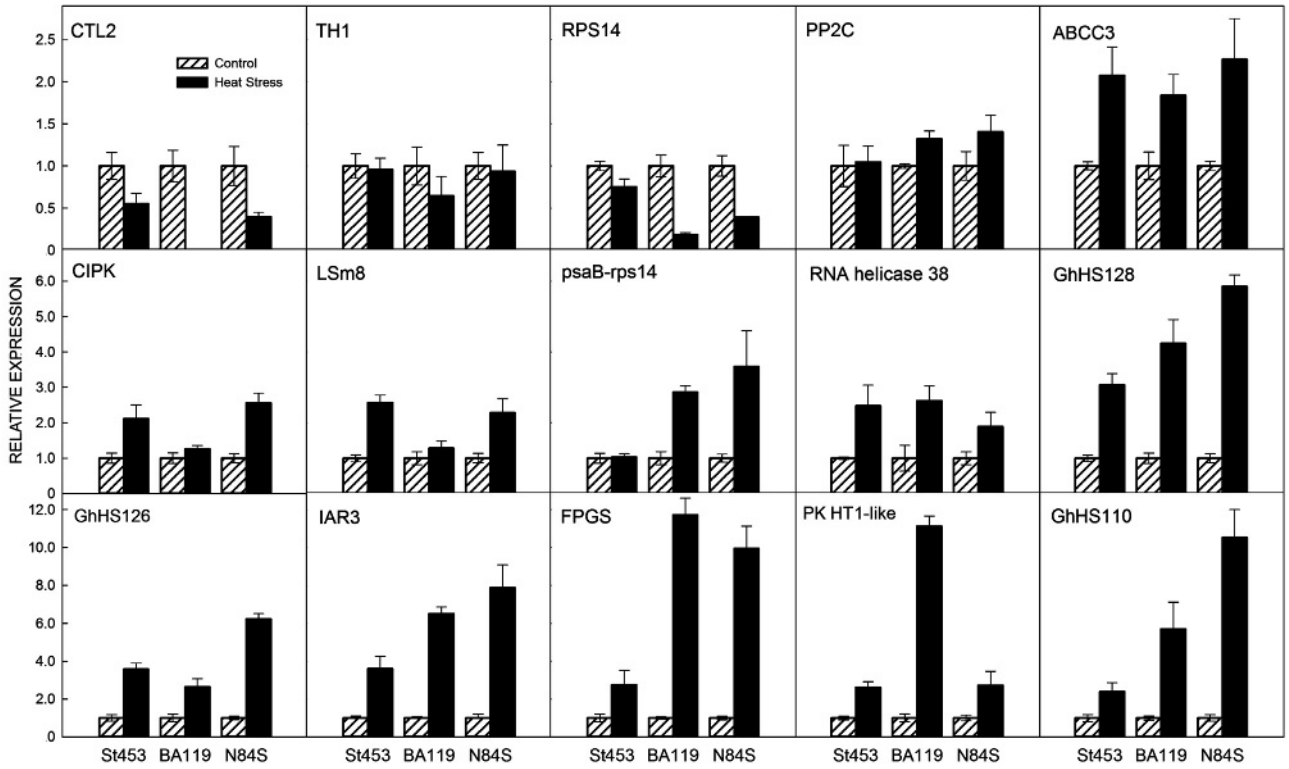

Fig. 2. Relative expressions of heat responsive genes in Stoneville 453 (St453), Beyazaltın 119 (BA119), and Nazilli 84S (N84S) under control conditions and 45 °C heat stress. Means ± SE, $n = 3$.

showed high similarity with *ABCC3* gene. *ABCC3* gene encodes glutathione sulfide-X (GS-X) pumps (Sánchez-Fernández *et al.* 2001) that are the member of ATP-binding cassette (ABC) protein superfamily, known as the biggest protein family. Plant ABCC3 is localized in the vacuolar membrane (Dunkley *et al.* 2006) and transports ions, conjugated molecules, and chlorophyll metabolites into the vacuole. Most of ABC proteins existing in bacteria, fungi, plants, and animals are membrane proteins and have a role in the transport of a broad range of substances across membranes. ABC transporter is down-regulated in root of cotton under water deficit (Park *et al.* 2012). There is limited information about expression pattern of *ABCC3* under abiotic stresses, particularly heat stress. In the present study, differential expression of *ABCC3* was observed in cotton under heat stress, however, its expression pattern showed contrary response to short and long-term heat stress. *ABCC3* was over-expressed 2.1-, 1.8-, and 2.3-fold in Stoneville 453, BA 119, and Nazilli 84S, respectively, under long-term heat stress, whereas it was dramatically down-regulated by short-term treatments at 38 and 45 °C in Stoneville 453. Due to such a variable response to heat stress, further investigations are indispensable for identification of a role of *ABCC3* in heat tolerance.

The sequence of *GhHS96* was similar to *G. arboretum* cDNA clone (BM359757.1) that was homologue to calcineurin B-like protein (CBL) interacting serine/ threonine protein kinase (CIPK). CIPK proteins might play an important role in control of energy balance. CIPKs are involved in control of sugar and nitrate metabolism to optimize growth (Halford *et al.* 2003). CIPKs having 25 members in *Arabidopsis* interact with CBL protein and mediate stress responses (Kolukisaoglu *et al.* 2004, Cheong *et al.* 2007). Most probably, CIPKs are the part of calcium regulated signaling pathway in plants. In *Arabidopsis thaliana*, *CIPK9* is transcribed almost in all tissues, except roots, and its transcription is highly increased by abscisic acid (ABA) treatment. In spite of that, expression of *CIPK9* is slightly increased under high NaCl and drought stress (Gong *et al.* 2002). In addition, *CIPK9* has critical role in *Arabidopsis* at low potassium supply and it is required for low potassium tolerance (Pandey *et al.* 2007). Transcription of *CIPK3* is increased by drought, wounding, cold, NaCl, and ABA

treatments. In addition, CIPK3 has a molecular bridge role between gene expression in cell and calcium signal induced by ABA and stress in *Arabidopsis* (Kim *et al.* 2003). Many biotic and abiotic stress factors alter Ca^{2+} content in plant cell and CIPK appears to be part of Ca^{2+}-dependent signal transduction pathway (Weinl and Kudla 2009). *CIPK* was responded to short and long-term heat stress cotton cv. Stoneville 453, however, its expression was down-regulated at short-term heat, whereas up-regulated at long-term heat. The reason of this discrepancy was most likely the difference in the stress conditions. Similarly, our not shown results from *in-silico* microarray analysis using *Genevestigator* (Hruz *et al.* 2008) indicated that transcription of *CIPK9* was down-regulated 2.01-fold under 37 °C for 30 h, whereas in *Arabidopsis* it was up-regulated 2.11- and 4.18-fold under 37 °C for 1 and 2 h, respectively.

IAR3-like GhHS97, *FPGS-like GhHS127*, *GhHS126*, and *GhHS128* were consistently up-regulated under both long-term and short-term heat stress. IAA is the most abundant auxin in plants and acts in almost all stages of plant development from embryogenesis to senescence. IAA-Ala hydrolase encoded by *IAR3* gene is an enzyme that hydrolyzes inactive IAA-Ala conjugate to release free bioactive IAA. It was observed that transcription of *IAR3* is differentially expressed under various abiotic stresses. In the leaves of *Arabidopsis*, *IAR3-like* cDNA is up-regulated by wounding, jasmonic acid, and ABA (Titarenko *et al.* 1997). In addition, *IAR3* is over-expressed in *Arabidopsis* under drought stress (Catala *et al.* 2007, Kinoshita *et al.* 2012) and according to Kinoshita *et al.* (2012) the function of *IAR3* in drought tolerance is indispensable. In the present study, expression of *IAR3* was increased 1.7-fold in Stoneville 453 at 45 °C. As a result of long-term heat stress, *IAR3* was over-expressed 3.6-, 6.2-, and 8.0-fold in Stoneville 453, BA 119 and Nazilli 84S, respectively, under long-term heat stress. Since *IAR3* was consistently up-regulated in cotton under heat stress, we thought that *IAR3* gene might have a function in heat tolerance of cotton.

GhHS127 shows significant homology with *FPGS3*. In plants, isoform of folylpolyglutamate synthase (FPGS) are located in chloroplasts, mitochondria, and cytosol and these isoforms are encoded by different genes *FPGS1*, *FPGS2*, and *FPGS3*, respectively (Ravanel *et al.* 2001). FPGS catalyzes the attachment of glutamate residues to the folate for the synthesis of folylpolyglutamate. Folylpolyglutamates are important substrates for most of the folate-dependent enzymes that are involved in metabolism for purine, pyrimidine, and in amino acid synthesis. Although, the physiological and metabolic importance of folate polyglutamylation has been well documented in other organisms, the functional significance of compartmentation of FPGSs in plants is unclear. However, it is most likely that monoglutamate folates are transported among the mitochondria, cytoplasm, and chloroplasts, and subsequently polyglutamylated by the FPGS to a suite of mono- or dual-targeted FPGS proteins (Mehrshahi *et al.* 2010). In addition, FPGS1 is required for normal meristematic activity and cell expansion during postembryonic root development in *Arabidopsis* (Srivastava *et al*. 2011). To our knowledge, there is no reference available about regulation of *FPGS* gene expression under abiotic stresses. Our results showed that expression of *FPGS3* was consistently increased in all three cotton cultivars under heat stress. *FPGS3* was up-regulated 2.8-, 11.7-, and 9.9-fold in Stoneville 453, BA 119, and Nazilli 84S, respectively. As a result of short-term heat stress, expression of *FPGS3* was increased 2.2-fold in Stoneville 453. To our knowledge, this is the first finding on up-regulation of FPGS3 in response to heat stress in plants. Further investigations are needed in order to understand the function of FPGS for heat tolerance in plants. In addition, *GhHS126* and *GhHS128* having no homology with known genes could be considered as new candidate genes for heat tolerance. In this study, *GhHS126* and *GhHS128* were consistently up-regulated in all three cotton cultivars under heat stress.

A previous study exhibited that some genes (coding cysteine proteinase, low molecular mass heat shock protein 17.9, S-like ribonuclease, and senescence-associated protein) had higher expression in a heat-sensitive fescue than heat-tolerant one under heat stress (Zhang *et al*. 2005). Likewise, most of up-regulated genes by heat stress were expressed more in susceptible cotton cultivar than at least one of two heat tolerant cotton cultivars (Fig. 2). It is possible, that heat sensitive plants did not acquire thermotolerance by pretreatment at moderate heat stress, whereas thermotolerance was induced in tolerant genotypes during acclimatization stage. For this reason, heat sensitive plants had to struggle to survive at early stage of high temperature stress. Thus, some signal transduction and stress responsive genes might be up-regulated more in Nazlli 84S than in Stoneville 453 and BA 119. An increase in gene expression at early response (from minutes to hours) is probably due to rapid induction of signal transduction. Despite, late response (after days) triggers differential expression of some genes for acclimatization and resisting to stress.

In conclusion, *IAR3*, *FPGS3*, *GhHS126*, and *GhHS128* genes were consistently up-regulated in three cotton cultivars under both short- and long-term heat stresses. Since they were up-regulated at least 2-fold, we suggest that they are heat responsive genes and have probably a role in heat tolerance. In addition, seven more heat responsive genes (*ABCC3*, *CIPK*, *LSM8*, *psaB-rps14*, *RNA helicase 38*, *PK HT1-like*, and *GhHS110*) that were over-expressed 2-fold and more in at least two cotton cultivars under long-term heat stress, were identified. However, further investigation is needed to lighten the functions of these genes at heat tolerance.

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