TÜBİTAK

Turk J Biol
36 (2012) 746-756

doi:10.3906/biy-1207-54

Identification and isolation of salt-stress–responsive transcripts from *Gossypium arboreum* L.

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Received: 28.07.2012 ● Accepted: 31.07.2012

Abstract: Salinity has adverse effects on plant development and ultimately reduces production. Very few reports on the genes induced by salt stress in *Gossypium arboreum* are available. In this report salt-stress–responsive genes were screened from cotton leaves using the differential display (DD) technique, and gene expression was compared under control and salt stressed conditions. By using a 107 primer combination, 25 gene fragments were found to be up-regulated in response to salt stress. Out of 25 induced fragments, 12 were rejected as false positives after reamplification and quality control assay. The remaining 13 fragments were selected for cloning and genetic transformation. Sequence analysis using GenBank databases revealed that 5 fragments ranging from 300 to 600 bp have significant homology to well-known proteins (i.e. protein kinase, proton gradient regulation, yeast cadmium factor, proteinase inhibitors, and expressed protein). Real time PCR studies confirmed over-expression of the identified transcripts in salt stressed samples as compared to control. These studies will provide insight into the role of these genes in the comprehensive molecular mechanisms of salt tolerance in agriculturally important crops.

Key words: Cotton (*Gossypium arboreum*), differential display, salt-stress–responsive transcript

Introduction

Cotton (*Gossypium*), a significant commercial crop primarily known for fibre, is responsible for contributing 8.6% of the value added in agriculture and about 1.8% to GDP (1). *Gossypium arboreum* is related to diploid cotton and possesses many positive characteristics (tolerance to drought and resistance to salts, diseases, and insect pests) that upland cotton (*G. hirsutum*) cultivars are deficient in. These characteristics allow for cultivation of *G. arboreum* in arid or semiarid areas with minimal farming input applications. Moreover, due to these distinctive qualities it is considered an important gene pool source for improving recent cotton cultivars (2). *G. arboreum* variety FDH-171, which is well known for abiotic stress tolerance (drought), was used to further extend the study of salt stress (3).

Agricultural productivity is severely affected by joint abiotic stress factors, and high salinity is among these (4–6). Accumulation of high levels of salt may adversely affect irrigated lands, and this is now a major environmental concern (7). The mechanism involved is as follows: hyperosmolarity of the soil solution restricts water uptake by the roots, triggers transient changes in plant/water relations, and leads to water deficit (8). Secondly, the accumulation of saline ions in plant tissues leads to salt-specific toxic effects to plant metabolism (9). As a consequence, cellular ionic balances are disrupted; however, the physiological and biochemical responses in plants differ (10). The

effect of salinity on cotton is noteworthy [e.g., leaves, roots, and stems (11); fruiting performance (12); lint and biomass yield (13)].

Identifying the genes that demonstrate expression in response to abiotic stresses may allow for the introduction of several stress tolerance mechanisms into crops. Numerous genes and their products respond to abiotic stresses at the transcriptional and translational level (14,15). Plants have evolved diverse systems of adaptation in order to endure saline conditions. These systems include the production of osmoprotectants, i.e. proline, polyol, glycine, and betaine or late embryogenesis-abundant (LEA) proteins (16); extrusion and compartmentalisation of sodium ions (17); and adjustment of ion homeostasis (18). Nevertheless, under salt stress plant systems experience a composite chain of alterations in physiological and molecular areas which involve the harmonised communication of several genes. Thus, it is essential to attain a comprehensive understanding of the alteration in expression of a number of genes, as opposed to targeting only one at a time.

Although salt tolerance mechanisms have been intensively studied in model plants, only a few salt stress-inducible genes such as Na^+/H^+ antiporter, DREB, ERF, NAC, MAPK, PIP, and receptor protein kinase 1 (RPK1) were documented in cotton (19,20). Plants use proton gradients as the driving force of the secondary transport systems that control ion fluxes under salt stress (21), whereas V-ATPase establishes proton gradients across the membrane and the regionalisation of vacuolar Na^+ (22).

Selection and subtraction has been performed to isolate differentially expressed mRNAs in particular tissues or cells through differential display (DD) (23). This method is more receptive to lower levels of expression than techniques that were already in use [restriction fragment length polymorphism (RFLP), restriction landmark genome scanning (RLGS), micro satellite, random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and florescent in situ hybridisation (FISH)].

Differential display involves the reverse transcription of mRNA with oligo-dT primers anchored to the beginning of the poly(A) tail, followed by PCR reaction in the presence of a second short primer that is arbitrary in sequence. The technique has the ability to highlight lower abundance transcripts (24) and has been used against biotic and abiotic stresses in different plants (25).

The present study aimed to identify and isolate differentially expressed transcripts in *Gossypium arboreum* under salt (NaCl) stress conditions. This is an initial step to pave the way for isolation and characterisation of novel gene(s) for their regulatory elements, which ultimately will lead to better understanding of the pathways crops have adapted under saline conditions.

Materials and methods

Plant cultivation and salt stress treatment

Seeds of a local variety of *Gossypium arboreum* (FDH-171) were obtained from the Cotton Research Substation, Raiwind, and delinted with concentrated H_2SO_4. As the H_2SO_4 was added, the seeds were stirred continuously with a spatula for 10–15 min until the surfaces of the seeds became shiny. Seeds were then washed 4–5 times with double distilled autoclaved water and stirred well to remove the acid. Seeds floating at the surface of the water were removed.

Seeds were germinated at 25±2 °C, and seedlings were hydroponically grown with Hoagland nutrient solution (26) in a greenhouse under a 13 h photoperiod and a light intensity of 1500 μmpl $m^2 s^{-1}$ at 50% humidity. Cotton leaves were used for identification of differentially expressed transcripts (3,27). For this purpose, an initial screening of different NaCl treatment concentrations was given to the seedlings (data not shown). Finally, 3–4 leaf stage seedlings treated with 150 mM NaCl for 24 h were selected for leaf harvest and RNA extraction, as previously reported (28). Seedlings without NaCl treatment were used as controls.

Differential display

Total RNA was isolated using 2 g of fresh leaf tissue with little modification of the protocol described by Jaakola (29). Differential display of mRNA was performed using a previously described procedure (23). First, strand cDNA was synthesised from 1 μg of total RNA using reverse transcriptase (Fermentas RevertAid H Minus First Strand cDNA Synthesis Kit, USA). The reaction mixture consisted of 1 μg

of total RNA, 1 µL of 1 µM oligo-dT (dT18) primer, 200U of MMLV reverse transcriptase, 2 µL of 10 mM dNTPs, and 4 µL of 5X first strand buffer [250 mM Tris (pH 8.3), 20 mM $MgCl_2$, 250 mM KCl, and 50 mM DTT]. Differential display PCR was performed in a 25 µL of reaction mixture, using 500 ng cDNA as a template. Each reaction mixture contained 1 µL of each anchored oligo-dT and arbitrary primers (10 µM), 1 µL of dNTPs (2.5 mM), 2.5 µL of 10X PCR buffer, and 5 units of Taq polymerase. The reaction was performed using a thermal cycler (ABI, Gene Amp® PCR System 9700) programmed to 95 °C for 5 min, annealing at 42 °C for 2 min, extension at 72 °C for 30 s, and denaturation at 95 °C for 30 s followed by a repeat of the annealing temperature for an additional 40 cycles. DDRT-PCR product was denatured with the same volume of gel loading buffer (95% formamide, 0.1% xylene cyanole FF, and 0.1% bromophenol blue) for 2 min at 90 °C. Then the denatured products (2 µL) were separated by electrophoresis at 200 V constant electric power on 6% polyacrylamide/7M urea DNA sequencing gel. Polyacrylamide gel was silver stained according to the Bio-Rad silver stain handbook. Gel was fixed with 8% acetic acid (v/v) and photographed by GrabIt software v 2.5 on gel document system (Ultra Violet Products). Randomly employing 19 arbitrary and 11 anchored oligo-dT primers, a total of 107 primer combinations were made (Table 1) (3,30). The difference in transcripts between salt stressed and control samples was recorded for the product obtained after using each set of primers.

Isolation, reamplification, and confirmation of differentially expressed transcripts

From the gel, 25 differentially expressed products were isolated. The identified bands were selected and cut out. Gel slices were soaked in 50 µL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] followed by heating at 100 °C for 5 min, and then DNA was eluted. Confirmation PCR was performed and the product was run again on the same gel. Then eluted fragments were precipitated in ethanol, purified, and resuspended in 25 of µL of sterile distilled water. The eluted fragments were reamplified in 25 µL of PCR mixture using the same set of arbitrary and anchored primers that generated differential product. Confirmed and reamplified PCR fragments (10 µL) were resolved on 1.5% agarose gel then excised and further eluted by DNA extraction kit (Fermentas). Excised fragments were cloned using Dual Promoter TA Cloning Kit pCRII Vector (Invitrogen) according to manufacturer's instructions.

DNA sequencing

The DNA sequence was determined using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Branchburg, NJ, USA) and a DNA sequencer (ABI PRISM 3700). All sequences were compared to ESTs and protein databases by BLAST algorithms (31). A deduced amino acid sequence homology between a known sequence and an EST was counted as significant with maximum identity shown by BLASTN. A homology study was made on the basis of biotic or abiotic stress tolerance—especially salt stress—well known proteins, and homology to plants species relevant to cotton.

Reverse transcription PCR analysis

Reverse transcriptase PCR was carried out with specific primers designed from a known *G. arboreum* sequence (Table 2). Protocol for total RNA extraction and synthesis of cDNA was carried out as described above. The same concentration of cDNA as in DD-PCR was used for real time (RT) PCR reaction. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene internally expressed in cotton was used as internal control. PCR was performed under following temperature profile: 94 °C for 5 min; 94 °C for 30 s (40 cycles); 55 °C for 30 s; and 72 °C for 45 s, after cycling a last step at 72 °C for 10 min.

Quantitative real time PCR

The Real Time ABI 7500 system (Applied Biosystems Inc, USA) with Maxima™ SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) was used to perform RT-PCR. GAPDH was used as the housekeeping gene to normalise data. Real time PCR was programmed as denaturation at 95 °C for 3 min and 95 °C for 30 s, followed by annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and a repeat of the annealing temperature for an additional 40 cycles. The last extension was performed at 72 °C for 10 min. The relative gene expression analysis was by SDS V3.1 software (Applied Biosystems Inc., USA). Each reaction was performed in triplicate.

Table 1. Primers sequences for DD-PCR.

5'Arbitrary primers	**Sequence**
P1	5'-ATTAACCCTCACTAAATGCTGGGGA-3'
P2	5'-ATTAACCCTCACTAAATCGGTCATAG-3'
P3	5'-ATTAACCCTCACTAAATGCTGGTGG-3'
P4	5'-ATTAACCCTCACTAAATGCTGGTAG-3'
P5	5'-ATTAACCCTCACTAAAGATCTGACTG-3'
P6	5'-ATTAACCCTCACTAAATGCTGGGTG-3'
P7	5'-ATTAACCCTCACTAAATGCTGTATG-3'
P8	5'-ATTAACCCTCACTAAATGGAGCTGG-3'
P9	5'-ATTAACCCTCACTAAATGTGGCAGG-3'
P10	5'-ATTAACCCTCACTAAAGCACCGTCC-3'
A1	5'-AAGCTTGATTGCC-3'
A2	5'-AGCTTCAAGACC-3'
A3	5'-AAGCTTTATTTAT-3'
A4	5'-AAGCTTCGACTGT-3'
A5	5'-AAGCTTGCCTTTA-3'
A6	5'-AAGCTTCTTTGGT-3'
R1	5'-AAGCTTCGACTGT-3'
R2	5'-AAGCTTTGGTCAG-3'
R3	5'-AAGCTTCTCAACG-3'
3'Anchored primers (oilgo-dt primers 30mer)	**Sequence**
T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
T5	5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
T6	5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
T7	5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
T8	5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
B1	5'-AAGCTTTTTTTTTTTTTA-3'
B2	5'-AAGCTTTTTTTTTTTTTG-3'
B3	5'-AAGCTTTTTTTTTTTTC-3'

Table 2. Primers sequences for RT-PCR.

Transcript	Forward primer	Reverse primer	Amplicon length (bp)
P5T7-a	5'-ATGGATGCTCGTATCGAAGG-3'	5'-TTCTGATGGACCCCTTTGTC-3'	170
P8B2-b	5'-CGTTGGGAAGAAAAGGTTCA-3'	5'-CATCACCAATTCATGCAAGG-3'	173
A6B3-b	5'-AAGAAGGGGAAGGAGAAGGA-3'	5'-AGCTGATAGGCTTACCCCAAC-3'	175
P10T8-a	5'-CAACAACTCCACCGTTCCTT-3'	5'-CAGCGTTATGGTGATGCAGT-3'	162
P3T9-b	5'-ACTAAATGCTGGTGGGCAAA-3'	5'-CGTTGCTATCAACCCAAACC-3'	150
GAPDH	5'-TGGGGCTACTCTCAAAGGGTTG-3'	5'-TGAGAAATTGCTGAAGCCGAAA-3'	162

Results and discussion

Cotton has worldwide importance as a primary source for natural fibre production (32), and it is greatly affected by high salinity. Therefore, this study was aimed at identifying the transcripts related to salt tolerance in cotton. Differential display method facilitates up- and down-regulation, presence and absence of fragments, qualitative and quantitative differences, and signals of varying intensity (33). Induction or repression is compared in mRNA populations with a small number of products but provides quick output (5,34). Using 11 anchored and 19 arbitrary primers, 25 gene fragments were induced while 35 were repressed in response to salt stress. Out of 25 induced fragments, 12 were rejected as false positives on reamplification and quality control assay. This technique has limitations such as variable (low) reproducibility, a significant incidence of false positives (35,36), under representation and redundancy of mRNA signals (37), frequent priming by the G/C rich 5' primer at both ends (38,39), and a bias for high copy number mRNAs (37,40). The remaining 13 fragments were selected for further analysis. Sequence analysis using GenBank databases revealed that 5 fragments ranging from 300 to 600 bp have significant homology with known genes (Figure 1). The other 8 cDNA fragments showed no or low homology with known genes.

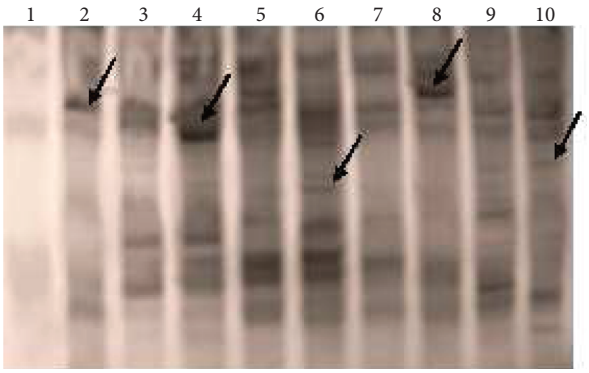

Figure 1. Differential display (DDRT-PCR) from leaves of *Gossypium arboreum.* Odd numbered lanes represent control sample, and even numbered lanes represent salt stressed samples. RT-PCR reactions were conducted using anchored primers and arbitrary primers (Table 1). cDNA fragments, which appeared to be differentially expressed in treated samples, are indicated by arrows (lane 2: P10T8-a, lane 4: P8B2-b, lane 6: A6B3-b, lane 8: P5T7-a, lane 10: P3T9-b).

Progress in extensive genome projects has produced comprehensive data regarding structural classification and functional categorisation of abiotic stress-responsive genes in crops (41). A transcript designated P5T7-a showed homology (55%) with protein kinase APK1B, a chloroplast precursor, in *Oryza sativa* subsp. *japonica* (Figure 2a). A receptor protein kinase 1 (RPK1) gene has been isolated from *Arabidopsis thaliana*. A mitogen-activated protein kinase (MAPK) cascade of signalling network is found in all eukaryotic organisms and regulates fundamental aspects of plant biology including cell division and the commencement of developmental pathways induced by abiotic stresses such as water deficit, salinity, and temperature (42). Development of stomata is ideal for investigating the discrete aspects of

a- Transcript P5T7-a showing homology with Protein kinase APK1B, chloroplast precursor, putative [*Ricinus communis*]

GENE ID: 8261650 RCOM_0000910

Score = 155 bits (393), Expect = 2e - 36
Identities = 86/156 (55%), Positives = 109/156 (70%), Gaps = 1 4/156 (9%)
Frame = +2

```
Query  11   TKDLTARSDVYSFGVVLLEM LTGKRAVDKNRPSREHNLVDWAKPYLTSKRKFFQIMDAR  190
            T  LT +SDVYSFGVVLLEM ++G+RA+DKNRPSRE NLV+WA+PYL +KRK FQ+MDAR+
Sbjct  255  TGHLTKKSDVYSFGVVLLEMISGRRAIDKNRPSREQNLVEWARPYLGNKRKIFQVMDARV  314

Query  191  EGQYTPEAALKAAYLAVQCLSTEPKVRPKMTAVVKALEQLQDSGGDKGV----HQN--AA  352
            EGQY++ ALK A LAVQC+S EP+ RPKM VVKALEQL +S ++G H++
Sbjct  315  EGQYSLKDALKVANLAVQCISPEPRFRPKMEEVVKALEQLLESNDNEGSRGSRHESLRKV  374

Query  353 VHS  SHPTSRYALKKNPDGL--------QPSASPLST  436
              +S+ RY K + + PSASPL T
Sbjct  375 NRN  SNNGPRYHRKSTAETCDGKAASYPR PSASPLRT  410
```

b- Transcript P8B2-b showing homology with proton gradient regulation 5 [*Cucumis melo*] and [*Cucumis sativus*]

gb|ABY56089.1|
gb|ABY56101.1|
Length=127

Score = 148 bits (374), Expect = 2e - 34
Identities = 73/85 (86%), Positives = 79/85 (93%), Gaps = 0/85 (0%)
Frame = +3

```
Query  18   GAGKGVRAQPMMKNVNEGKGLFAPVVVVTRQIVGKKRFNQLRGKAIALHSQVINEFCKSI  197
            G GK VR++PMMKNVNEGKG+FAP+VVVTR I+GKKRFNQLRGKAIALHSQVI EFCKSI
Sbjct  43   GMGKPVRSRPMMKNVNEGKGVFAPLVVVTRNIIGKKRFNQLRGKAIALHSQVITEFCKSI  102

Query  198  GPDTKQKQGLIRLAKKNGERLGFLA  272
            G D KQ+QGLIRLAKKNGERLGFLA
Sbjct  103  GADGKQRQGLIRLAKKNGERLGFLA  127
```

c- Transcript A6B3-b showing homology with Ycf2 [*Gossypium hirsutum*]

ref|YP_538978.1|
ref|YP_538997.1| Ycf2 [Gossypium hirsutum]
sp|Q2L949.1|YCF2_GOSHI RecName: Full=Protein yc f2
gb|ABC73669.1| hypothetical chloroplast RF2 [Gossypium hirsutum]
gb|ABC73690.1| hypothetical chloroplast RF2 [Gossypium hirsutum]
Length=2298

GENE ID: 3989170 ycf2 | Ycf2 [Gossypium hirsutum] (10 or fewer PubMed links)

Score = 72.4 bits (176), Expect = 2e - 11
Identities = 51/54 (94%), Positives = 52/54 (96%), Gaps = 0/54 (0%)
Frame = +1

```
Query  4     LLWSKPGNPLYMMQNGSCSIVDQRNLYekyesefeegegeg VLDP*QIEEDLFN  165
             LL S+PGNPLYMMQNGSCSIVDQRNLYEKYESEFEEGEGEG VLDP QIEEDLFN
Sbjct  2035  LLRSEPGNPLYMMQNGSCSIVDQRNLYEKYESEFEEGEGEG VLDPQQIEEDLFN  2088
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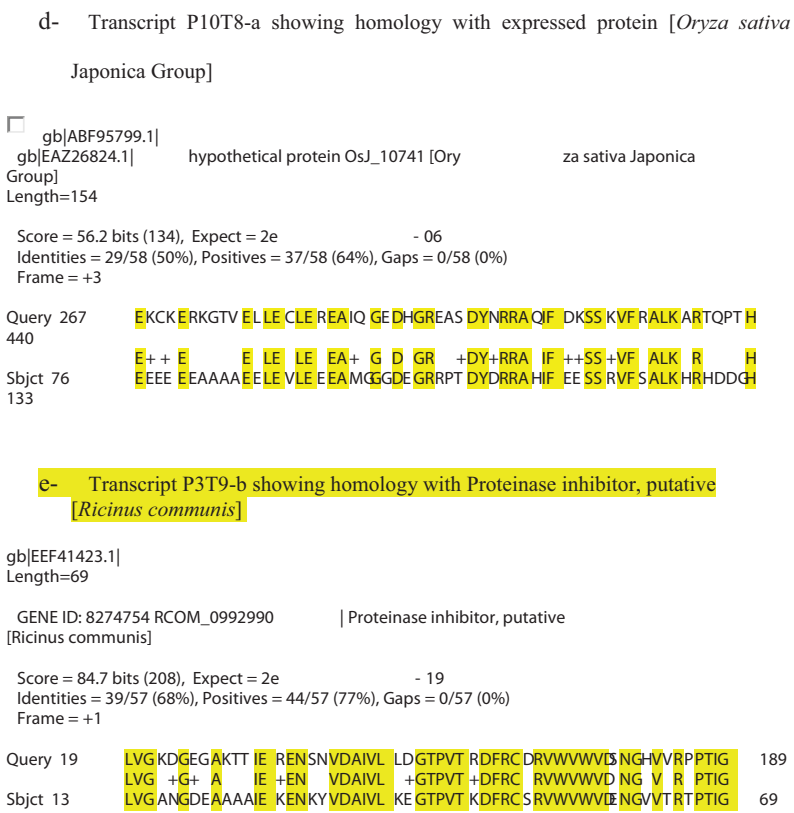

Figure 2. Amino acid sequence homology of 5 clones with known genes. The deduced amino acid sequences were aligned using NCBI BLAST pairwise alignment algorithm programs (http://www.ncbi.nlm.nih.gov/BLAST/).

MAPK signalling networks because stomata restrict the invasion of pathogens and the effects of many abiotic responses. Therefore, a rapid regulation and modulation of developmental processes in response to abiotic stresses and stomatal development by using a common MAPK module is promising (43). It has been observed that RPK1 was commonly expressed in the root, shoot, leaf, and flower of *Arabidopsis*, and mRNA levels increased under salt stress (250 mM NaCl) with the passage of time (20). The present results suggest that RPK1 may function as an osmoregulator in cotton under salt stress. Diverse features of abiotic stresses in plants including water deficit, salinity, chilling/freezing, hormone effect (ethylene, ABA, GA3, and IAA), injury, insect pest response, and cell physiology generate the MAP kinase cascades that may be concerned with different signal transduction mechanisms (44).

A fragment named P8B2-b has high homology (85%) with proton gradient regulation (PGR5) in *Cucumis melo* (Figure 2b). Plants use proton gradients as the driving force of secondary transport systems that control ion fluxes under salt stress (21). Plasma membranes and vacuolar proton transporters play essential roles in plant salinity stress tolerance by maintaining the trans-membrane proton gradient that assures control over ion fluxes and pH regulation (45). Increasing solute concentration in the vacuoles of plant cells would increase the vacuolar osmotic pressure, with a concomitant decrease in water potential, favouring water movement from soil into plant root cells, improving Na^+ accumulation in the vacuoles, and reducing toxicity in the cytoplasm, leading to higher salt tolerance in the plant (46). Overexpression of AVP1, which encodes a vacuolar H^+ pyrophosphatase, an H^+ pump, has increased drought/salt tolerance in *Arabidopsis*, tomato, and cotton (47–49). Halophytic plants have been shown to increase pump activity under salt stress conditions more drastically than glycophytes (50), but little is

known about the regulatory circuits that lead to either increased protein amounts or activity under salt stress. Therefore, the main proton pump in the outer cell membrane is essential for many physiological functions.

The transcript A6B3-b has high homology (94%) with Ycf2 in *Gossypium hirsutum* (Figure 2c). The chloroplast Ycf2 gene plays a vital but unknown function in the higher plants: gene silencing or reduction in mRNA-synthesis–induced cell death (51). Plastids are organelles derived from the independent living cyanobacteria present in almost every plant cell and include a complete complex of proteins essential for photosynthesis (52). Chloroplast genomes of the land plants are highly conserved in organisation, gene order, and content across taxa. They have circular genome DNA of about 115–165 kb and are organised into large and small single-copy regions that are separated by an inverted repeat (53). *Arabidopsis thaliana* plants expressing yeast cadmium factor 1 (Ycf1)1 have improved salt or xenobiotic chemical (1-chloro-2, 4-dinitrobenzene, CDNB) tolerance. In addition, the extent or degree of tolerance may be the result of sequestration of salts or deposition of xenobiotic chemical into the vacuoles (54). Our results suggest that YCF-related transcript is possibly being used to advance salt and xenobiotic chemical tolerance in crops. Discovering the complete sequence of the chloroplast genome may unlock new opportunities for both basic and applied studies of this technology. Chloroplast genomes are generally inherited maternally; therefore, this information is useful for plant cytoplasmic breeding and tracing out parentage in interspecific hybrids. In comparison to nuclear genetic engineering, higher expression of transgenic insertion is obtained due to high copy numbers of chloroplast genomes within a single plant cell. This information is also appropriate for phylogenetic studies at an evolutionary level because of the lower rates of silent nucleotide substitution (55). This knowledge may increase our range of methods for advancing the capacity of plants/crops to survive and tolerate different abiotic stresses.

Transcript P10T8-a is up-regulated under salt stress with the function still incompletely known or designated as novel (Figure 2d). Although there are many other sequences which have higher scores than this sequence (ABF95799), their proteins are uncharacterised, hypothetical, and based only on predictions. Other types of genes that form salt-stress–relevant proteins such as SalT, which is organ-specific, and 15-kDa glycine-rich cytosolic protein were also isolated from rice plants. Although the function of this protein is unknown, it is recognised in the regulation of high salt, drought, abscisic acid, and proline (56). Another novel rice gene, RAB21, a glycine-rich protein (16.53 kDa) with a duplicated domain structure, has been reported. RAB21 mRNA and protein accumulated in rice embryos, leaves, roots, and callus-derived suspension cells when subjected to stress under NaCl, ABA, and drought. Characterisation and mapping of the RAB21 gene determined that the proximal promoter region included various GC-rich repeats (57). Some of the ESTs characterised for genes with unidentified roles were induced under salt stress in rice cultivars CSR27 and PB1 (T2D1-167) (58). An unidentified protein (JG294130) is abundantly expressed in the genome, and its mRNA is up-regulated by NaCl stress. Therefore, it may be valuable to observe and characterise its role for further research.

Transcript P3T9-b was up-regulated under salt stress. This transcript has good homology (68%) with proteinase inhibitor (Figure 2e), which plays a very important role under abiotic stress. Proteinase inhibitor accumulates in tomato leaf during salt stress and helps the tolerance mechanism (59). Moreover, proteinase inhibitors (PIs) are potential components of gene stacks for the protection of important agricultural crops against biotic stress such as insect damage (60).

The expression levels of these transcripts were studied and validated by real-time PCR. The internally-expressed GAPDH gene in cotton was used as a housekeeping gene to normalise expression (61). Results showed that all 5 transcripts have over-expression in cotton leaves under salt stress (NaCl, 150 mM) compared to control leaves (Figures 3, 4). Compared to control, there was 3-times more expression in transcripts P10T8-a, 2.5-times more in P5T7-a, 3.5-times more in P3T9-b, almost double in A6B3-a, and 2-times more in P8B2-b. All of these transcripts were submitted to the NCBI GenBank EST database (Table 3). Plant responses to salt stress

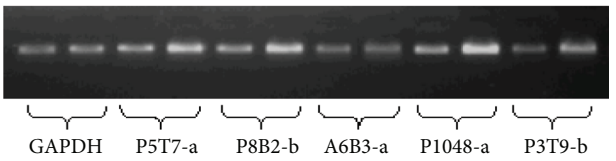

Figure 3. Reverse transcription PCR expression analysis of cotton transcripts with salt stressed and control leaves. Even bands (from left to right) indicate salt stressed samples, and odd numbers indicate control samples. The GAPDH gene was used as housekeeping control. PCR product was analysed on 1.8% (w/v) agarose gel.

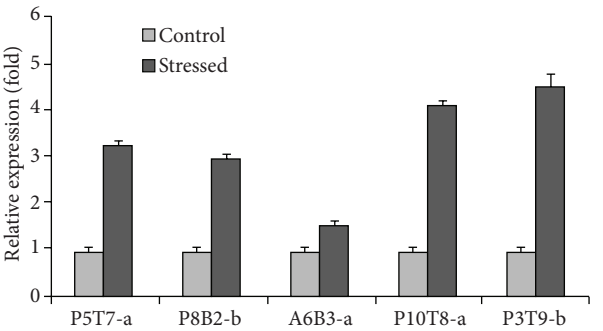

Figure 4. Relative expression of transcripts under control and salt stressed conditions. The GAPDH gene was used as internal control (housekeeping gene), and gene expression is indicated as a fold increase relative to the same transcript under control conditions.

Table 3. Clone identification and homology with known genes.

Clone identification	Size (bp)	GenBank Accession #	Homology
P5T7-a	590	dbEST JG294129	Protein kinase APK1B, chloroplast precursor, GenBank #XP002533148.1
P8B2-b	438	dbEST JG294131	Proton gradient regulation, GenBank #5ABY56089.1
P10T8-a	572	dbEST JG294130	Expressed protein, GenBank #ABF95799.1
A6B3-b	316	dbEST JG294141	Ycf2 (*Gossypium hirsutum*), GenBank #YP538978.1
P3T9-b	340	dbEST JG294132	Proteinase inhibitor, GenBank #XP002521006.1

differ with plant genotype, growing stage, and extent of the stress that induces responses for adjustment in gene expression in salt-stress–relevant biosynthetic pathways (27).

Conclusion

In this study differentially expressed transcripts were detected by applying the DD technique in cotton plants under salt stress and normal conditions. Under salt stress 5 identified cDNA transcripts showed higher levels of expression. These results indicate that *G. arboreum* variety FDH-171 is competent enough for up-regulation of some specific genes during salt stress, and these findings are of immense importance for the selection of cotton lines with salt-stress–tolerant characteristics.

Acknowledgements

The authors thank the Higher Education Commission (HEC) of Pakistan for partially funding the project.

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