Comparative Transcript Profiling and Multiplex qRT-PCR Analysis Between Salt-Tolerant and Sensitive Wheat Genotypes

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Abstract: Identification of candidate genes combined with gene expression profiling carries importance to facilitate the molecular basis of salt stress response in plants. Here, cDNA-AFLP was used to compare the transcribed sequences among two bread and two durum wheat genotypes with different levels of salt tolerance. Transcript derived fragments (TDFs) screened on polyacrylamide gels and 36 salt stress induced unique fragments were detected in salt tolerant bread wheat genotype (Alpu cv.). The fragment size of these 36 TDFs was ranged between 99bp to 252bp. Full sequence information of 14 TDFs were obtained after cloning, then GeXP analyzer-based multiplex qRT-PCR assay was performed on leaf tissue derived from 12 TDFs. Targeted gene expression levels of two TDFs (TDF4-GT066302 and TDF11-GT066301) were showed clear upregulation in salt tolerant bread wheat genotype (Alpu cv.) and they were matched with hypothetical proteins. Especially, gene expression level of GT066301 was increased as 3.28 fold at 27th hours of salt stress for salt tolerant genotype. According to blastx similarity results, out of 14 sequenced fragments, two TDFs were closely matched with "cytochrome P450 monooxygenase" protein while four of them matched with *Oryza* "hypothetical" and "unknown" proteins. Outputs of this study might ensure comparative data for hypothetical protein gene expression and new useful alleles in response to salt stress in wheat.

Keywords: cDNA AFLP, Hypothetical proteins, Multiplex qRT-PCR, Salinity, TDF, Wheat.

INTRODUCTION

Salt is a defective compound for plant growth and it has been accepted as one of the main environmental bottlenecks for global agricultural practices. Plants undergo a variety of physiological and molecular rearrangements to cope with negative effects of salt [1]. Today, studies conducted to find out the effects of salt on wheat and other major cereals are still an ongoing work. Basically, there is a large durum and bread wheat genotype diversity for responding to the salt stress [2], and salt-tolerant genotypes have been employed to isolate the conferring tolerance related genes [3].

On the other side, physiological screening studies in wheat indicated the efficient role of sodium excluders and high affinity potassium ion transporters [4]. To examine this, a *Nax*2 locus from wheat A genome progenitor introduced to durum wheat [5, 6]**.** In addition, wheat D genome is closely covered by genes conferring salt tolerance. So, it is one of the examples of carrying an important potassium/sodium transporter gene (*Kna*1) [7, 8]**.** Significanly, the role of potassium (K^+) has been found in wheat roots and K^+ flux accepted as a physiological key marker that could be used to identify salt tolerant plants [9]. Thus, these traits highlighted the hexaploid wheat as an important crop for understanding the salt stress response.

Moreover, transcript profiling on wheat has been showed many up and down regulated genes [10, 11]. There are different classes of methods released for transcriptome screening. One of them is cDNA Amplified Fragment Length Polymorphism (AFLP) that was used as a PCR based genome wide gene expression tool [12, 13]. The advantage of this technic is no need to previous sequence information [14]**.** In plants, transcript profiling studies have been reported in diverse species such as barley [15]**,** rice [16]**,** foxtail millet [17, 18], *Brachypodium* [19] and wheat [20, 11, 21-23]. In detail, identification of cadmium-regulated genes in *Brassica juncea* L. [24], aluminum-regulated genes in rice [25] and gene expression analysis under cold stress in chickpea [26] have been investigated with transcript profiling.

In this study, cDNA AFLP was used to screen the transcript differences between leaf tissues of two durum and two bread wheat genotypes which have varying levels of salt response. Gene expression levels of salt stress induced and salt tolerant genotype derived TDFs were comparatively analyzed by using multiplex qRT-PCR in all genotypes. Findings suggested a positive correlation between hypothetical proteins and salt tolerance in wheat.

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MATERIALS AND METHODS

Plant Material and Experimental Set-Up

In this study, two bread wheat; (*Triticum aestivum* L. cvs.) Alpu (salt-tolerant), ES14 (salt-sensitive) and two durum wheat; (*Triticum durum* L. cvs.) Meram (salt-tolerant), C1252 (salt-sensitive) genotypes were used. Seeds were provided from International Agricultural Research Institute, Konya-Turkey. Four week-old seedlings were transferred into half strength Hoagland's nutrient solution at pH 6.0 [27]. Aeration in nutrient media was ensured by air pumps in growth chamber at 22^{o}C, with photoperiod of 16hrs light/8hrs dark. Salt concentration was gradually increased at daily periods until reaching the application concentration of 150mM NaCl. Previously, this salt concentration has been approved in several reports and caused exact differences at physiologic, cellular and molecular level in wheat [28, 6, 29, 30]. Leaf tissues from ten independent seedlings were harvested at 8th and 27th hours and immediately frozen in liquid nitrogen and stored at -80 ^{o}C until RNA extraction.

Total RNA Isolation and cDNA AFLP Analysis

Total RNA from leaf tissues were isolated with Trizol Reagent (Roche). Formaldehyde Agarose gels (1%) and NanoDrop Photometer (Wilmington, USA) measurements were used to check RNA quality and quantity respectively. Samples diluted to final concentration of 1 µg/µl after DNaseI treatment (Fermentas). cDNA was synthesized from the mRNA using the cDNA synthesis system kit (Invitrogen) with minor modifications.

cDNA AFLP was carried out with minor modifications according to [31]**.** Double stranded cDNA (500 ng) was digested with *PstI* and *MseI* restriction enzymes at 37°C for 3 hours. Single stranded oligonucleotides used as adapter sequences; *Mse*I Adapter; 5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5', *Pst*I Adapter; 5'-CTCGTAGACTGCGTACATGCA-3' and 3'-TGTACGCAGTCTAC-5'. Digested cDNA was attached with these adapter fragments. Ligated products were pre-amplified under the conditions of 94ºC 30 sec, 56ºC 1 min, 72ºC 1 min, for 20 cycles by using pre-amplification primers *Pst*I: 5'-GACTGCGTACCAATTC-3', *Mse*I: 5'-GATGAGTCCTGAGTAA-3'. Pre-amplified products were diluted as 1:5 and screened with 40 different selective primer combinations (Table **1**). Samples were subjected to the following selective amplification thermocycler profile; [(94ºC, 60sec; 65ºC 60sec; 72ºC, 60sec);(94ºC, 60sec; 65-56ºC (decrease 1ºC each cycle), 60sec; 72ºC, 90sec) x 10], (94ºC, 60sec; 56ºC, 60sec; 72ºC, 60sec) x 22 cycle; +10°C(∞). Five microliters of AFLP products were heat-denatured and separated on 6% polyacrylamide gel in Sequi-Gen GT Sequencing Cell System (Bio-Rad) and run for 3.5 hrs under 200V at 50°C with 0.5X TBE electrophoresis buffer and immediately silver stained. TDFs presented only in the salt tolerant wheat (Alpu cv.) were discriminated to re-amplify under the same selective PCR conditions. Re-amplified products were run on 3% agarose gel for verifying the fragment size and TDFs were purified with PCR clean-up kit (Qiagen) according to the manufacturer's instructions.

Table 1: List of Corresponding Selective Primer Sequences used in cDNA AFLP Reactions

Name	Primer Sequence (5'-3')
P-GAC	GACTGCGTACATGCAGAC
P-TGG	GACTGCGTACATGCATGG
P-GTT	GACTGCGTACATGCAGTT
P-CCA	GACTGCGTACATGCACCA
M-ACC	GATGAGTCCTGAGTAAACC
M-ACG	GATGAGTCCTGAGTAAACG
M-CGA	GATGAGTCCTGAGTAACGA
M-CGT	GATGAGTCCTGAGTAACGT
M-CAA	GATGAGTCCTGAGTAACAA
M-CAG	GATGAGTCCTGAGTAACAG
M-CAT	GATGAGTCCTGAGTAACAT
M-CAC	GATGAGTCCTGAGTAACAC
M-CCA	GATGAGTCCTGAGTAACCA
M-CCT	GATGAGTCCTGAGTAACCT

Sequencing and Data Mining of Differentially Expressed Transcripts under Salt Stress

After fragment purification from agarose gel, TDFs were cloned into pGEM-T Easy vector (Promega) by following the manufacturer's instructions. Ampicillin containing selective LB (Difco) used as growth media for transformated DH5α competent cells with corresponding TDFs [32]**.** Selective growth media containing ampicillin, X-Gal (Sigma) and IPTG (Sigma) incubated at 37^{o}C overnight. Before plasmid DNA isolation, blue/white colony selection performed to screen the success of ligation and transformation process. Plasmid purification was done by using Plasmid DNA isolation miniprep kit (Qiagen) and the insert size was checked with colony PCR using the T7/SP6 primers. Five technical replicates used in colony PCR of each TDF. DNA sequencing performed

on the plasmid by using -47 sequencing primer (1.6 pmol/µl) and reactions were repeated for three times with puc18 control template that was provided by the GenomeLab DTCS Starter Kit (Beckman Coulter, S802018). Thus, accuracy of sequencing reaction and system (Beckman Coulter GeXP GenomeLab Genetic Analysis) checked out. Sequence similarities were analysed with Blastx program [33] which was defined in National Center for Biotechnology Information (NCBI) GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The threshold of positive match set to 25% identity and higher values. E-value cut-off was accepted as $=1e^{-5}$. A total of 14 *T. aestivum* specific TDFs were submitted to the dbEST NCBI, Bethesda, MD, USA. Corresponding GenBank accession numbers defined from GT066300 to GT066310 and GR972470, GR972471, GR972472. TDF numbers were condensed from 14 to 12 by using ClustalW sequence alignment tool. Among these, four TDFs were gave clear distinguishable peaks after multiplex qRT-PCR and their gene expression levels were assessed between bread and durum wheat leaf samples.

Evaluation of Expression Data by using Fluorescent-Based Multiplex Quantitative RT-PCR

Target specific primers (Table **2**) were designed by using eXpress Designer module of the eXpress Profiler software (Beckman Coulter). First strand cDNA synthesis was performed with reverse transcriptase, RT buffer (1X) supplied with the GeXP Start Kit (Beckman Coulter). Later, 2 µl gene-specific chimeric reverse primer mix (0.5µM) added to the reaction mixture. Thermal cycler reaction condition set to: 48ºC for 1 min; 37ºC for 5 min; 42ºC for 60 min; 95ºC for 5 min; hold at 4ºC. An aliquot (9.3 µl) of the first RT-PCR mixed with 4 µl PCR Buffer (5X) of Beckman Coulter GeXP Start Kit that was containing fluorescently-labeled universal forward primer. An unlabeled universal reverse primer, dNTP (10µM), 0.7 µl Thermo-Start DNA polymerase (5U/ µl) (ABgene), 4 µl $MgCl_2$ (25mM), and 2 µl of gene-specific forward chimeric primer plex (0.2 µM) added to get a final volume of 20 µl. PCR program was set to following conditions: 1 cycle of 95ºC for 10 min followed by 35 cycles of 94ºC 30 sec, 55ºC 30 sec, 68ºC 1 min; hold at 4ºC. Fluorescently labeled final PCR products separated via capillary electrophoresis using with the following conditions: capillary temperature at 50ºC, denaturation at 90ºC for 120 sec, injection for 30 sec at 2.0 kV, separation at 6.0 kV for 35 min. Output data were analyzed on both Fragment Analysis module and eXpress Analysis module of the eXpress Profiler software respectively. PCR reactions performed with two biological and two technical replicates and peak area calculations were done automotically by eXpress Analysis module. Peak heights were selected, correcting for preferential amplification of smaller fragments (normalization) was calculated based on the RFU (relative flourescense unit) intensity values of each peak. The relative gene expression level for each group was calculated by dividing mean average value of treatment to control. Following equations were used in the calculations. (i) control group gene expression rate= peak area value of control group gene / peak area value of control group actin gene; (ii) treatment group gene expression rate= peak area value of treatment group gene / peak area value of treatment group actin gene. Relative range of gene expression rate= (ii)/ (i).

Table 2: Primer Sequences of four Selected TDFs and Internal Housekeeping Control Gene (actin) used in Multiplex qRT-PCR Amplifications. Bold Letters Represent the Selective Part of the Primer and Italic Letters are Complementary Parts for the First Strand cDNA Amplification Primers

GenBank Accession Number	Primer Name	Product Length (bp)	Primer Sequence (5'-3')
GR972471	TDF2	186	F: *AGGTGACACTATAGAATA*TGAGAGACGAAAGCTAGGGG R: *GTACGACTCACTATAGGGA*ATTCTTGCGAACGTACTCCC
GT066302	TDF4	134	F: *AGGTGACACTATAGAATA*GCCTAAGATCAGGCCGAAAG R: *GTACGACTCACTATAGGG*ATTCGGCTAACCTAGCCTCCT
GT066305	TDF6	164	F: *AGGTGACACTATAGAATA*CCG GCGAGGAGCTTTAGTAG R: *GTACGACTCACTATAGGG*AGAATTTATGGTCGCGTTTTGA
GT066301	TDF11	122	F: *AGGTGACACTATAGAATA*GGGTGAGTCAGGGCCTAAG R: *GTACGACTCACTATAGGGA*TCCGTACCAACAAGGGGTAG
	Actin	145	F: *AGGTGACACTATAGAATA*CCCTCTATGCAAGTGGTCGT R: *GTACGACTCACTATAGGGA*GAAGAATGGCATGAGGAAGC

RESULTS

Quantitative and Functional Analysis of TDFs

By applying cDNA-AFLP transcript profiling with forty primer combinations, approximately 500 AFLP fragments were obtained from both control and salt applied groups of bread wheat genotypes after 8th hours of stress. Later, a low proportional decline (6%) occurred for the numbers of bread wheat specific fragments at 27th hours. An example of cDNA AFLP profile generated by *Pst*I+GTT/ *Mse*I+CGT primer pairs displayed in Figure **1**.

Specifically, differentially expressed 26 and 10 unique salt stress induced TDFs were observed only in salt tolerant genotype Alpu cv. at 8th and 27th hours of salt application respectively. After reamplification studies, 14 out of 36 salt stress induced reproducible fragments compared with proteins in public databases. According to Blastx analysis, some TDFs were found to be involved in different protein groups derived from *Zea*, *Oryza* and *Citrullus*. Whole sequenced transcripts were submitted to NCBI GenBank. Significantly, the highest match score was 100% with "Cytochrome P450 like TBP protein" for GT066307, while GT066308 was similar to the "NAD dependent-epimerase/dehydratase family protein" at level of 32% as represented in Table **3**. Consequently, 12 TDFs out of 14 showed homology to known expressed sequences and 2 TDFs (GT066305, GT066309) displayed no homology with any protein in the database.

Expression Patterns of Selected TDFs

AFLP derived comparative fragment analysis performed between control and treatment groups of all wheat genotypes. Salt stress induced TDFs extracted for sequencing and specific multiplex primer sets were designed for gene expression profiling. Beckman GeXP based multiplex PCR assay provided a high sensitivity and allowed us for rapid evaluation of gene expression with its internal gene control in the same reaction tube.

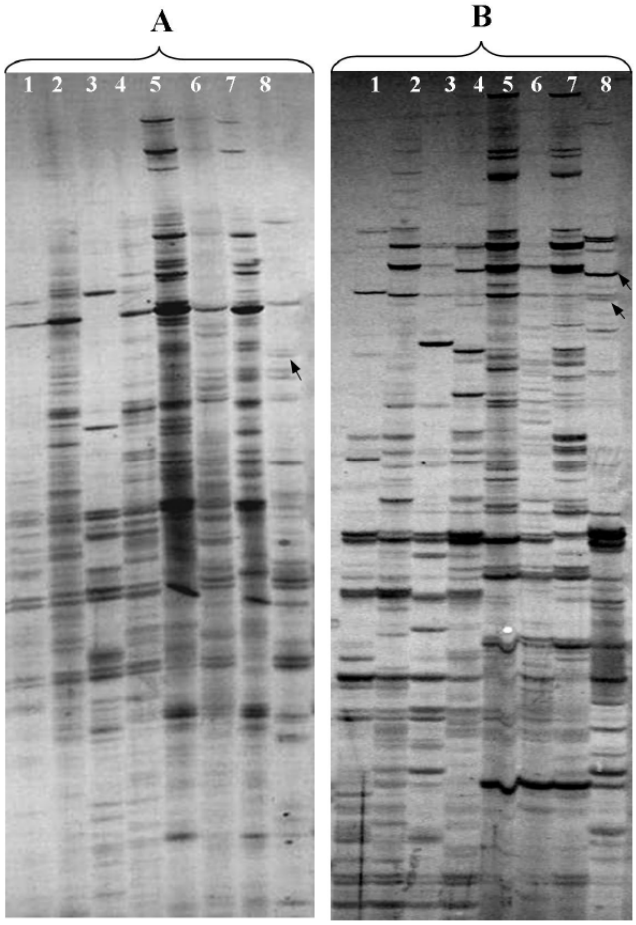

Figure 1: Silver stained polyacrylamide gel profile obtained from *Pst*I+GTT/ *Mse*I+CGT selective primer combination. a and b indicates the fragment profiles at 8th and 27th hours of salt stress respectively. TDFs tagged with black arrows. 1,3,5,7 represents the control groups of C1252, Meram, Es14 and Alpu respectively while 2,4,6,8 represents the treatment groups of same genotypes.

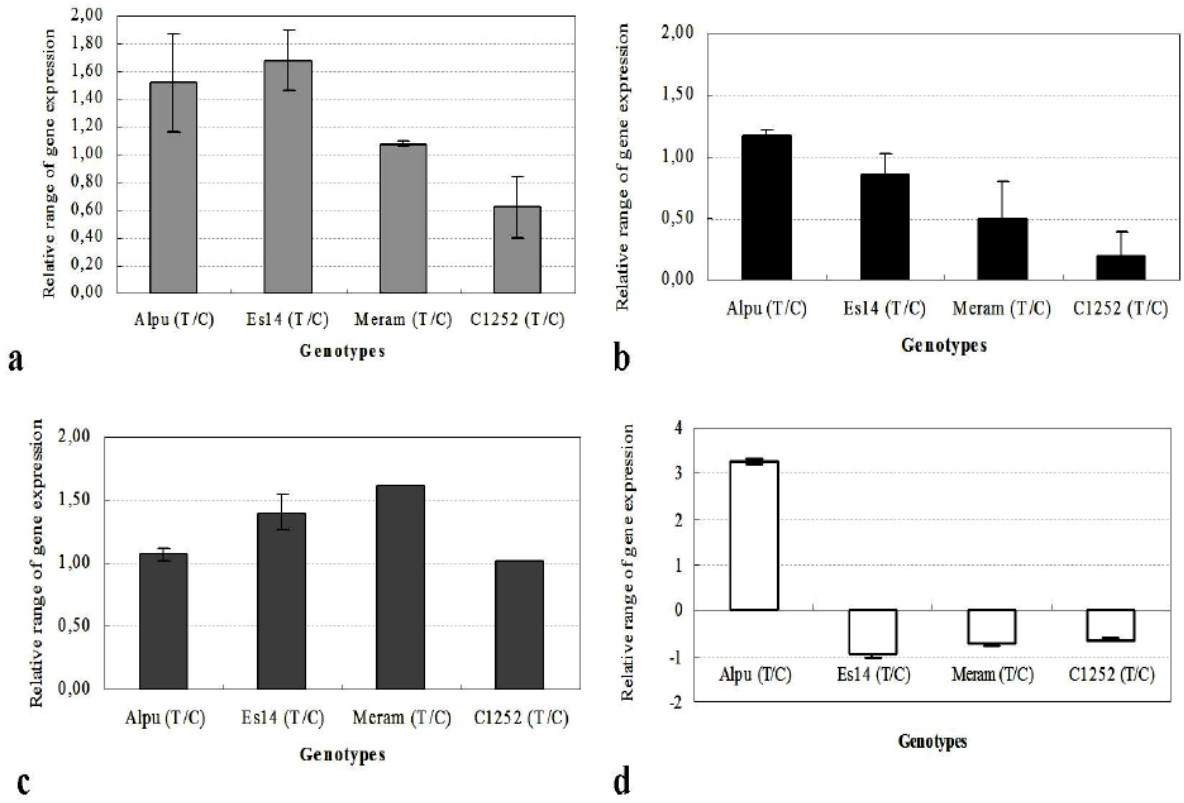

Figure 2: Bar graph display of relative range of expression level according to the estimation of T(salt treatment)/C(control) values. **a**- TDF2 (GR9724471), **b**- TDF4 (GT066302), **c**-TDF6 (GT066305) induced after 8 hours of salt stress and **d**-TDF11 (GT066301) induced after 27 hours of salt stress.

For gene expression comparison, fourteen TDFs selected according to their reproducible pattern and Blast information retrieval. Among them, 12 suitable TDFs evaluated for further gene expression analysis by multiplex qRT-PCR. Two TDFs (GT066303 and GT066306) could not be used due to the primer unavailability. Four primer pairs ensured perfect amplicons with expected fragment size. Their accession numbers were GR9724471, GT066302, GT066305 (induced after 8 hours of stress) and GT066301 (induced after 27 hours of stress). Other primer pairs exhibited either no amplification or very low amplified products.

Under salt stress, relative range of gene expression for GR972471 increased as 1.52 and 1.68 fold in bread wheat cultivars, Alpu and ES14 respectively. In contrary, salt sensitive durum wheat sample C1252 displayed the lowest gene expression level for the same TDF as compared to other genotypes (Figure **2a**). This TDF (GR972471) also displayed 96% sequence similarity to *Oryza sativa* "unknown protein" (Table **3**). Similarly, analysis performed for TDF4 (GT066302) indicated that gene expression level of this transcript showed as 1.17 fold increment in Alpu and 0.85 fold in ES14 versus their control groups (Figure **2b**). Moreover, relative range of gene expression for TDF6 (GT066305) increased as 1.07 fold for Alpu, 1.40 fold for ES14, 1.62 fold for Meram and 1.01 fold for C1252 and its homology was not found in GenBank (Table **3**). However, GT066305 only detected in salt treated Alpu genotype, expression comparisons clearly demonstrated a noncorrelative pattern for this AFLP based fragment (Figure **2c**). In addition, it was also detected that gene expression level of TDF11 (GT066301) increased at 3.28 fold in Alpu genotype versus its control group at 27th hour of salt stress (Figure **2d**, Figure **3**). Gene expression of GT066301 apparently increased in Alpu as compared to other genotypes and similarity results indicated a close match with *Oryza* hypothetical protein at the level of 95% for this TDF (Table **3**).

In the current study, Alpu was displayed an up-regulated gene expression pattern that was evidently revealed in examined TDFs (TDF4 and TDF11) by qRT-PCR (Figure **2b**, **2d**) and, the remaining genotypes were varied in their gene expression. Results confirmed the time dependent profiles of hypothetical proteins in wheat and their gene

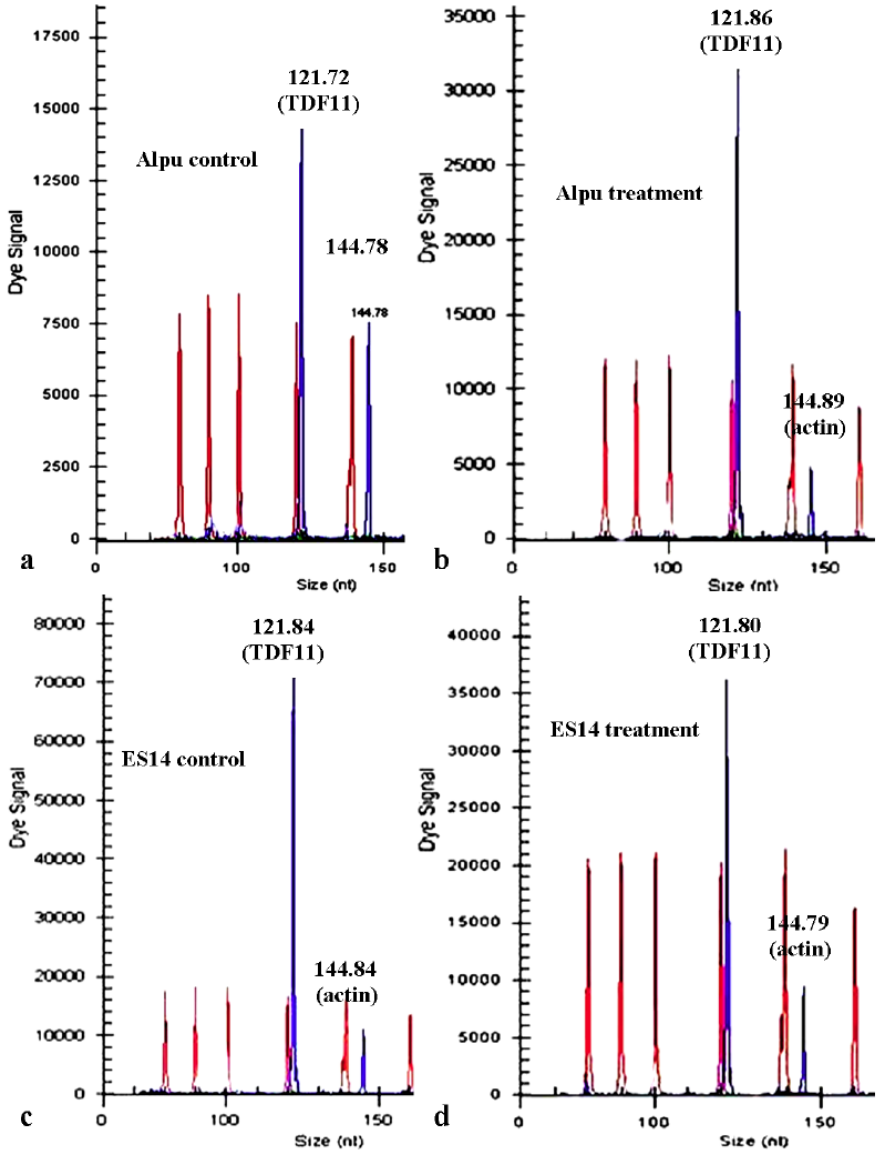

Figure 3: A comparative chromatogram overview of fluorescently labelled peak (rfuxmm^2)x10^3 of actin and TDF11 (GT066301) for Alpu (a-control, b-treatment) and ES14 (c-control; d-treatment) genotypes at 27th hours of salt stress. Red fluorescent dye being reserved for the size marker and targeted gene was in blue flourescence.

expression regulation under salt stress. Salt stress induced TDFs not only exhibited a different gene expression levels among bread wheat genotypes but also confirmed in durum wheat genotypes at different tolerance levels.

DISCUSSION

Wheat is a polyploid crop and sequencing of its whole genome has been nearly completed [34]. Verification of stress related genome regions, either up-regulated or down-regulated pattern, can help us to identify and select the most applicable individuals and populations during stress tolerant crop breeding. In addition, time dependent expression of stress-responsive/inducible genes have been enhanced by the identification of new gene regions related to tolerance [35, 36, 37]. In the frame of the omic technology applications, transcript profiling is one of the feasible technics [38]. In this study, transcript profiling approach have been performed to screen differentially expressed cDNA AFLP derived fragments in bread and durum wheat genomes under short term salt stress. Significantly, multiplex qRT-PCR analysis showed that relative range of AFLP derived TDF (GT066301) gene expression was up regulated as 3.28 fold under salt stress for only in salt tolerant genotype Alpu and a sharp decrease was occurred for the rest of the genotypes at 27th hours of stress. Considerable variations were identified between contrasting bread

Table 3: Blastx Homologies of cDNA-AFLP Fragments in NCBI GenBank

Accession Number	Blast homology	% Max* Identity	E- value**	Expression Pattern	Length (bp)
GR972470	similar to MGC53016 protein *Strongylocentrotus purpuratus*	37	2.4	8th	229
GR972471	Unknown protein (*Oryza sativa* Japonica Group) (AAV44205)	96	2e-26	8 th	252
GR972472	GK16754 (*Drosophila willistoni*) gene product from transcript	44	9.3	8 th	219
GT066302	Hypothetical protein (*Oryza sativa*) (Japonica cultivar-group) (AAT76998)	95	1e-06	8 th	129
GT066303	Hypothetical protein (*Oryza sativa*) (japonica cultivar-group) (AAT76998.1)	96	4e-06	8 th	99
GT066304	Probable cytochrome P450 monooxygenase – from maize (T02955)	93	7e-16	8 th	189
GT066305	No homology	-	-	8 th	189
GT066306	Probable cytochrome P450 monooxygenase – from maize (T02955)	93	8e-9	8 th	126
GT066307	Cytochrome P450 like-TBP *Citrullus lanatus* (BAD26579)	100	1e-15	8 th	149
GT066308	NAD-dependent epimerase/dehydratase family protein (*Desulfovibrio esulfuricans* subsp. desulfuricans str. G20) (ABB36834)	32	5.4	8 th	164
GT066309	No homology	-	-	8 th	166
GT066300	Chlorophyll a-b binding protein (*Physcomitrella patens*)	83	7e-08	27 th	245
GT066301	hypothetical protein (*Oryza sativa* Japonica Group) (AAT76998.1)	93	2e-09	27 th	161
GT066310	hypothetical protein CHLREDRAFT-155068 (*Chlamydomonas reinhardtii*)	95	2e-07	27 th	178

*Threshold of a positive match for %max identity>25%, **E-value cut-off=$1e^{-5}$

and durum wheat genotypes in respect to gene expression levels and cDNA AFLP patterns. Previously, same differences was reported related to salt stress among *Triticale* members [39]. Alterations at genome level consistent with the obtained data and suggested that many of the gene expression changes were found to be associated with polyploidy [40].

In this work, the results of Blastx analysis based on sequence alignment indicated that two TDFs (GT066305, GT066309) showed no homology and four stress responsive transcripts (GT066302, GT066303, GT066301, GT066310) were predicted as 'hypothetical'. Major alterations in transcriptional activities were noted to accompany the response of plants against to salt stress. As pointed out by [41] and [42], hypothetical and putative proteins which include genes encoding proteins with uncharacterized domains have been relevant to salinity. Relationship between compatible solutes and salt stress tolerance has been revealed alternative parameters for selection of tolerant plants. In this respect, proline is one of the compatible solutes and its accumulation is increased under salt stress in transgenic *Arabidopsis* after transferring *Triticum aestivum* salt-related hypothetical protein *(TaSRHP)* [43]. Genes induced under salt stress has been investigated in some other grasses and a novel sheepgrass gene named as "LcSAIN1" showed close similarity with hypothetical proteins from wheat, barley and other closely related crops. In transgenic *Arabidopsis* and rice, an overexpression pattern of LcSAIN1 also caused an increase in the amount of transcription factors, compatible solutes which enable plants more tolerant during salt stress [44]**.** Studies resulted the hypothetical proteins after salt treatement in different plants such as rice [45]. In a recent example

of comparative assessment of *Thellungiella halophila* proteins, one fifth of the total salt stress induced proteins have been identified as hypothetical [46]**.** In *Sorghum*, [47] Ngara observed 22 hypothetical protein induction after salt stress application in moderately salt tolerant plant. In another study three protein spots that were matched with hypothetical proteins have been found in halophytic plant *Nitraria sphaerocarpa* after 150mM NaCl treatment [48]. Not only for plants but also for bacteria, hypothetical proteins have crucial roles on management of stress tolerance and ensuring adaptation mechanisms under saline environments. In wheat, hypothetical HPS-like protein coding gene proved tolerance against to salt stress by decreasing carbonhydrate amount and closing stomatal aperture [49]. In addition, overexpression of *Triticum aestivum* salt related hypothetical protein (TaSRHP) caused an enhanced resistance under saline conditions in *Arabidopsis* [43]. This type of increment in hypothetical protein gene expression might be assumed to be a paralel correlation with stress tolerance for wheat and might be permitted to the predictions of potential role for this protein. Like many other discovered genes, GT066301 might be used as a candidate transcript for salt stress tolerance screening in addition the sodium and potassium excluders which was investigated by [50, 51].

As it was listed in Table **3**, TDFs assigned to three protein groups; photosynthesis, oxidative mechanism and unclassified proteins. Several functional classes known to be appeared during salt stress has been reported by [52]. As an example, GT066300 were detected at 27th hours of salt stress and matched with chlorophyll a/b-binding protein from photosynthesis metabolism. In the present work, there were also induction of two TDFs GT066304, GT066306 at 8th hours of salt stress and they were found to be sequence similarity to maize probable cytochrome P450 monooxygenase protein with 93% identity (Table **3**). Cytochrome P450 monooxygenase plays a central role in plant oxidative metabolism [53]. Based on gene expression profiles, a short list of candidate salt-tolerance genes reported in wheat and cytochrome P450 monooxygenase gene expression detected as an up-regulated pattern in leaf tissues of salt tolerant wheat germplasm lines after 300mM NaCl application during 42 days [23]. In the present work, cytochrome P450 gene expressed occurred more earlier and at lower concentration of NaCl (150mM) in salt tolerant wheat genotype (Alpu cv.). Clearly, cytochrome P450 expression was induced by the both concentrations of 150mM and 300mM NaCl stress in terms of hour and day dependent periods respectively.

Detailed work on cDNA clones/ESTs reported from salt-stressed libraries showed that transcripts upregulated in salt stress belong to a variety of functional classes such as RNA metabolism, transcription, signaling, translational machinery, transport proteins, osmoprotectants, ROS scavengers, cell death and ageing, photosynthesis, general metabolism, protein transport/turnover, other stress proteins, and several unclassified proteins [23, 54]. Genes identified, isolated and cloned by such approaches are needed to be functionally-characterized. So, data mining of the transcript profiling can supply a systematic strategy for functional analysis and it may reveal the relationship between effective genes in salt tolerance and wheat genome [55].

As a defence mechanism, plants demonstrate clear differences in the amount of some protein groups including putative novel genes and genes with unknown function. In the sum, identification of several genes in response to salt stress could be helped to clarify the fine networks underlying salt tolerance in plants. Consequently, this study revealed the time dependent gene induction of hypothetical proteins that might be considered as salt stress responsive determinants in wheat. Thus, regulation of hypothetical proteins under stress conditions may enable an alternative protein type for classifying stress tolerant plants.

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