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Identification of Differentially Expressed Genes by cDNA-AFLP Technique in Response to Drought Stress in *Triticum durum*

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Summary

Drought is the single largest abiotic stress factor leading to reduced crop yields. The identification of differentially expressed genes and the understanding of their functions in environmentally stressful conditions are essential to improve drought tolerance. Transcriptomics is a powerful approach for the global analysis of molecular mechanisms under abiotic stress. To identify genes that are important for drought tolerance, we analyzed mRNA populations from untreated and drought-stressed leaves of *Triticum durum* by cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique. Overall, 76 transcript-derived fragments corresponding to differentially induced transcripts were successfully sequenced. Most of the transcripts identified here, using basic local alignment search tool (BLAST) database, were genes belonging to different functional categories related to metabolism, energy, cellular biosynthesis, cell defense, signal transduction, transcription regulation, protein degradation and transport. The expression patterns of these genes were confirmed by quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) based on ten selected genes representing different patterns. These results could facilitate the understanding of cellular mechanisms involving groups of genes that act in coordination in response to stimuli of water deficit. The identification of novel stress-responsive genes will provide useful data that could help develop breeding strategies aimed at improving durum wheat tolerance to field stress.

Key words: cDNA-AFLP, drought stress, transcript-derived fragments, durum wheat, real-time PCR

Introduction

Durum wheat (*Triticum turgidum* L. var. *durum*) is one of the important staple food crops in the world. In Morocco, it is an economically and nutritionally important cereal crop and ranks third after barley and bread wheat (*1*). Durum wheat is traditionally grown under rainfed conditions in marginal environments of the semi-arid tropics. In these regions, water limitation is the most im-

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portant production constraint (*2*). Environmental stresses, such as water deficit, increased salinity of the soil and extreme temperature, are major factors limiting plant growth and productivity (*3*). Among such environmental stresses, drought is one of the greatest environmental constraints for agriculture worldwide (*4*). In response to various abiotic stresses, plants have developed different physiological and biochemical strategies to adapt to or tolerate stress conditions. The main physiological drought stress responses include stomatal closure, repression of cell growth and photosynthesis, and activation of respiration. At the biochemical level, many plants accumulate osmoprotectants such as sugars (sucrose, raffinose, trehalose), sugar alcohols (sorbitol and mannitol), amino acids (proline), and amines (glycine betaine and polyamines) (*5,6*). One of the main cellular events occurring during water deficit is extensive modification of gene expression resulting in a strict control of all the physiological and biochemical responses to the stress. The modification of gene expression is related to different pathways associated with stress perception, signal transduction, regulators and synthesis of a number of compounds (*7,8*). The identification and characterization of genes induced under abiotic stresses is a common approach to understanding the molecular mechanisms of stress tolerance in plants. In recent years, rapid advances in genomic technologies have led to an increasing understanding of global gene expression under water stress in plants (*9–11*). The products of the stress-inducible genes can be broadly classified into two groups. The first group includes functional proteins or proteins that probably function in stress tolerance. They are late embryogenesis-abundant (LEA) proteins, heat-shock proteins, osmoprotectant biosynthesis-related proteins, carbohydrate metabolism-related proteins, transporters, detoxification enzymes, antifreeze proteins, senescence-related genes, protease inhibitors and lipid-transfer proteins. The second group includes transcription factors, secondary messengers, phosphatases and kinases such as mitogen-activated protein kinases (MAPKs), and calcium-dependent protein kinases (CDPKs) (*12*) that regulate the expression of other genes in response to drought stress. Transcription factors are thought to be the major and most varied category because they act as direct or indirect regulators of drought-responsive gene expression (*13*). A wide range of techniques and strategies are being employed these days to identify genes involved in stress responses (*14*). Currently, several techniques, such as differential display reverse transcription-polymerase chain reaction (DDRT-PCR), serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH), cDNA-AFLP and cDNA microarray are available for transcriptomic analysis. Among these, cDNA-AFLP is an efficient, sensitive, and reproducible technology for the isolation of differentially expressed genes (*15,16*). In order to identify drought-responsive genes and to gain a better understanding of drought stress responses in durum wheat, genome-wide investigation of drought-responsive genes was conducted using cDNA-AFLP. Identification of the key genes that are differentially expressed in a whole-genome scale could help in developing resources for genetic improvement. The qRT-PCR analysis was also used to validate the expression patterns for some of the regulated genes. Here we report a number of transcript-derived fragments (TDFs) in durum wheat that were found to be activated or suppressed during the drought stress. The candidate genes can then be tested in further physiological studies and through breeding programs.

Materials and Methods

Plant material and drought treatment

The growth conditions and water stress experiment were already reported in our previous study (*17*). Leaves of durum wheat genotype 1804 were collected in our previous drought treatment (*17*). Briefly, the drought treatment was started at the flowering stage by withholding water, and pot soil was allowed to dry until it reached 45 % of available water content (AWC). The monitoring of AWC was performed by weighing the pots as reported (*17*). Plants were maintained at 45 % AWC for 10 days. Control and stressed samples were collected at four sampling times: 4 days (T4), 6 days (T6), 8 days (T8) and 10 days (T10) after the initiation of the stress treatment (45 % AWC). On the basis of the physiological and molecular results in our previously reported study (*17*), the condition T10 was used for cDNA-AFLP analysis. Under this condition, the relative water content was decreased to 70 % and the molecular analysis by RT-PCR showed that most of the studied genes peaked at ten days after water stress imposition, thus the leaves under this condition (T10) were collected for cDNA-AFLP experiment along with control samples.

RNA preparation and cDNA synthesis

Total RNA was extracted from leaves using the Spectrum Plant Total Kit (Sigma-Aldrich, St Louis, MO, USA) in accordance with the manufacturer's instructions. The RNA extracts were treated at 37 °C with Ambion® TURBO DNA-free™ DNase (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) to avoid possible DNA contamination. The concentration of RNA was determined by spectrophotometry using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was determined by running 2 μL of total RNA in a formamide denaturing gel. For cDNA synthesis, 20 μg of total RNA were used initially for first strand synthesis, followed by second strand synthesis using SuperScript double-stranded cDNA synthesis kit (Invitrogen, Life Technologies, Thermo Fisher Scientific) following the manufacturer's instructions.

cDNA-AFLP reaction

cDNA-AFLP was carried out using the AFLP small plant genomes kit (Applied Biosystems, Life Technologies) with minor modifications. About 500 ng of double-stranded cDNA were digested by EcoR1 and MseI restriction enzymes (Invitrogen). The digested products were ligated to EcoR1 and MseI adapters. The preselective amplification mixture was prepared by adding 3 μL of 10-fold diluted DNA from the restriction-ligation reaction, 1 μL of AFLP preselective primer pairs and 16 μL of AFLP® Amplification Core Mix (Applied Biosystems). The preselective amplification was carried out in a Veriti™ thermal cycler (Applied Biosystems) programmed at 72

°C for 2 min, followed by 20 cycles at 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min, with an incubation step at 60 °C for 30 min. The preselective amplification products were diluted 10-fold in $TE_{0.1}$ buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH=8.0). A volume of 3 μL of diluted preamplification products was reamplified with 64 primer combinations; 1 μL of each primer was used with 15 μL of AFLP® Amplification Core Mix. Selective amplification was carried out using a touchdown program in a Veriti™ thermal cycler (Applied Biosystems) programmed at 94 °C for 2 min, followed by 10 cycles at 94 °C for 20 s, 66 °C (–1 °C per cycle) for 30 s, 72 °C for 2 min, and 20 cycles at 94 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min with a subsequent hold for 30 min at 60 °C. For high-throughput analysis of differentially expressed fragments, the PCR products of the selective amplification were separated on a 6 % polyacrylamide gel.

Isolation, reamplification and sequencing of transcript-derived fragments

The polymorphic transcript-derived fragments (TDFs) based on their presence, absence or differential intensity were cut from the gel, with maximum care to avoid any contaminating fragment(s). DNA was purified using GenElute™ gel extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. Extracted target bands were used as template for reamplification using the same primers and program for selective amplification. The PCR products were resolved in a 2 % agarose gel, purified with ExoSAP-IT reagent (Affymetrix, Santa Clara, CA, USA) and directly sequenced using BigDye® Terminator v. 3.1 cycle sequencing kit (Applied Biosystems). Sequencing of the TDFs was carried out on an ABI 3130xl automated sequencer (Applied Biosystems).

Sequence analysis

The resultant sequences were analyzed for homologues using BLAST Network Service of National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). Each TDF sequence was compared against all sequences in the non-redundant databases using the BLASTX program (*18*), which compares translated nucleotide sequences with protein sequences.

Real time PCR analysis

Leaf tissues in the stressed groups were sampled 6, 8 and 10 days after drought treatment, as well as in the control groups (*17*). The qPCR assays were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (*19*). Transcript abundance was assessed with three independent biological replicates and reaction was performed in duplicate. A mass of 1 μg of RNA treated with Ambion® TURBO DNA-free™ DNase (Life Technologies) was transcribed using the SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. To confirm the absence of genomic DNA contamination in RNA samples, we ran PCRs for the RNA samples without the reverse transcriptase enzymes (no RT controls) using CDC(a) (cell division control) specific primers (Table 1). Using Primer v. 3.0 program (SourceForge, Inc, Mountain View, CA, USA), specific primer pairs were designed on 10 TDFs chosen for the validation of cDNA-AFLP results (Table 1). The RT-PCR consisted of 12 μL of SensiMix NoRef with 0.4 μL of SYBR® Green I (Quantace, London, UK), 400 nM of each primer, 200 ng of cDNA and sterile distilled water to a total volume of 20 μL. The PCR mixtures were denatured at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. Melting curve analysis was performed to evaluate the presence of non-specific PCR products and primer dimers. The RT-PCR was carried out on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia). Target gene expressions were normalized relative to both the internal reference genes CDC(a) and ADP-ribosylation factor (ADP-RF(a)) (*20*). The suitability of these two genes was confirmed in our previous study (*17*). Relative expression levels for each of the 10 selected genes were computed based on the differences of cycle threshold (Ct) values between the two reference genes

Table 1. Primer sequences used for the validation of cDNA-AFLP analysis with qRT-PCR

Gene (GenBank accession number)	Forward	Reverse
Reference gene		
CDC(a) (Ta54227)	CAGCTGCTGACTGAGATGGA	ATGTCTGGCCTGTTGGTAGC
ADP-RF(a) (Ta2291)	TCTCATGGTTGGTCTCGATG	GGATGGTGGTGACGATCTCT
TDF (up-regulated in cDNA-AFLP)		
52 (JZ482440)	CGCTGTTCCGTAGACATGAA	GACGGTTGGGAGACCTTTCT
67 (JZ482441)	AACAGAGACCGAATCAAGCA	TGATAGCTTCAAGGATCAGATG
10 (JZ482435)	CGAATACGAACCGTGAAAGC	AAGAGCCGACATCGAAGGAT
92 (JZ482442)	ATGAAACAAAAGGCCCTCAA	CCAGTATTGCATCATTGGTGA
115 (JZ482436)	CTGGTGGCGTAAGACCATTT	CAAAATTGCAGTGTGGATGG
TDF (down-regulated in cDNA-AFLP)		
27 (JZ482428)	CACCTTCACCAGGCCTATTC	CGGCCCAATCTTTGAGTTTA
29 (JZ482429)	GACAAGTGCAGGACCGATTC	AGTTGGATGCCGACAAAATC
46 (JZ482430)	AGGTTACCGAACTCCCTGCT	ATCACATTCCGGAGGGTCTC
35 (JZ482438)	CTGTTTGTTGGCACCTCTGA	TCATGCTGGTGTTTGGTGAT
51 (JZ482431)	ATCGAGCAAAACACAGCACA	GAGAGGCTCGACGGAGTG

and the tested target genes (*21*). Statistical analyses were carried out using the *t*-test to compare mean values.

Results

Detection of differentially expressed transcripts

To isolate differentially expressed transcripts, we carried out cDNA-AFLP analysis on total RNA samples from leaves grown under normal and drought stress conditions. cDNA-AFLP analysis can reveal altered expression of any gene provided that it carries the restriction sites that have been chosen for analysis. Selective amplification with 64 primer combinations allowed the visualization of 3218 reproducibly detectable TDFs, 1216 of which were differentially expressed, corresponding to about 38 % of all visualized transcripts. Of the 1216 TDFs, 591 were up-regulated and 925 down-regulated. A total of 115 differentially expressed TDFs ranging in size from 300 to 600 bp were excised from the gel, reamplified and purified for direct sequencing, which yielded 76 cDNA fragments that gave rise to useable sequence data. Sequencing of several cDNAs failed, probably due to a mixture of the PCR products and these fragments were not further analyzed.

Functional classifications of differentially expressed TDFs

After sequencing 115 selected TDFs, reliable sequences were produced by 76 of them. Each sequence was identified by similarity search using the basic local alignment search tool (BLAST) program against the GenBank non-redundant (nr) public sequence database (NCBI). Sequences were classified into functional groups based on their homology with known proteins.

The sequence comparison of the 76 TDFs against the nr database revealed that 62 % (47 TDFs) of them had homology with genes with known functions, whereas for 23.7 % (18 TDFs) there were not hits and 14.5 % (11 TDFs) had homology with proteins with unknown function (Fig. 1). The TDFs with known or putative function were submitted to the NCBI database and are presented in Table 2 with GenBank Accession numbers. The up- and down-regulated genes are also categorized into these functional groups (Table 2). Fig. 1 shows the percentages of durum wheat genes assigned to different functional categories. Approximately 17.1 % of TDFs are involved in transcription regulation, and a further 13.15 % in signal transduction. Other relevant groups of differentially expressed TDFs include metabolism (5.26 %), energy metabolism (5.26 %), transport (9.21 %), protein degradation (5.26 %), cellular biosynthesis (3.94 %), and cell defense (2.63 %).

Validation of expression patterns by qRT-PCR analysis

To validate the reliability of the cDNA-AFLP for detection of differentially expressed genes and verification of the expression patterns observed in the cDNA-AFLP analysis, qRT-PCR was carried out for ten TDFs belonging to different functional categories, five up-regulated (TDFs 52, 67, 10, 92 and 115) and 5 down-regulated (TDFs 27, 29, 46, 35 and 51) transcripts. These selected TDFs were studied during three time-points (6, 8 and 10 days after stress application). Since contamination with DNA would affect the results of RT-PCR, the samples with no RT controls were tested with CDC-specific primers and no PCR products were obtained, indicating that all RNAs were free of genomic DNA (data not shown). The absence of non-specific PCR products and primer dimer artifacts was checked by melting curves for each gene; a sole, symmetric and sharp curve indicated that only one product was accumulated. Relative quantitative method was used to describe expression patterns of selected genes. Fold changes in gene expression were normalized to ADP and CDC reference genes and relative to the untreated controls. Significance differences between means within days at $p<0.05$ and $p<0.01$ were found. Twofold up-regulation and 0.5-fold down-regulation were considered as significant. Expression profiles of the 10 TDFs in wheat leaves after drought treatment are shown in Fig. 2.

The comparison between the cDNA-AFLP analysis and the qPCR results showed that eight of the ten examined TDFs had the same expression profiles. The remaining two transcripts (TDFs 35 and 46) showed differential expression patterns, and their expression was considered unchanged in RT-PCR analysis (Fig. 2). These results indicate that the original cDNA-AFLP pattern was validated in 80 % of the cases, so the general approach is a reliable method for identifying up-regulated and down-regulated genes in durum wheat leaves under drought stress.

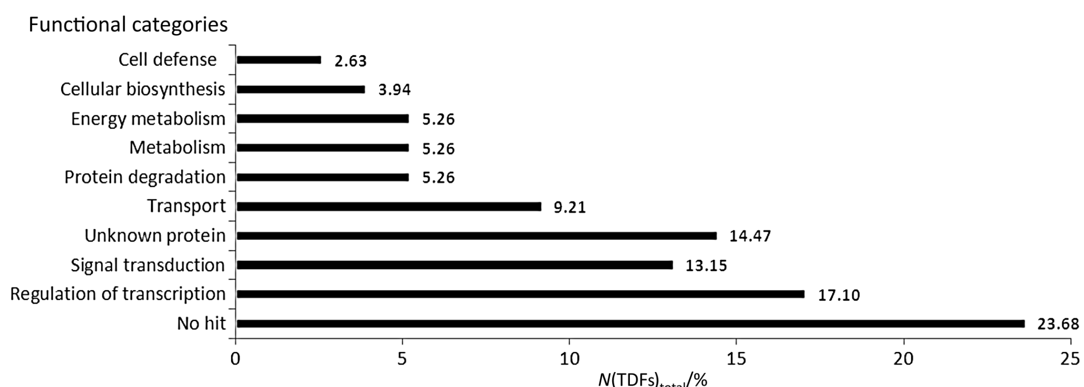

Fig. 1. Classification of differentially accumulated transcript-derived fragments (TDFs) after drought stress. A total of 76 TDFs were classified based on the BLASTX homology search

Table 2. Differentially expressed TDFs in response to drought stress

TDF	Accession number	Sequence similarity (number of TDFs)	GenBank hit	BLASTX score	Expression
		Protein degradation			
TDF 10	JZ482435	putative senescence associated protein (3)	AAR25995.1	2.00E-44	Up
TDF 26	JZ482471	cysteine proteinase-3-like	XP_004243707.1	0.006	Up
		Cell defense			
TDF 2	JZ482458	Bax inhibitor-1-like protein	Q94A20.1	1.00E-20	Up
TDF 57	JZ482467	disease resistance protein RGA2	EMT24070.1	6.00E-05	Down
		Cellular biosynthesis			
TDF 24	JZ482427	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, chloroplastic-like	ABV02021.1	1.00E-20	Down
TDF 35	JZ482438	sterol C-14 reductase	CAP06396.1	1.00E-75	Up
TDF 6	JZ482464	diacylglycerol kinase	EMS63785.1	3.00E-17	Up
		Metabolism			
TDF 67	JZ482441	β-D-xylosidase-7-like	XP_003570630.1	1.00E-09	Up
TDF 11	JZ482447	AAA+ ATPase domain	XP_002463440.1	4.00E-74	Up
TDF 3	JZ482459	adipocyte plasma membrane-associated protein	EMS48382.1	2.00E-52	Up
TDF 12	JZ482468	putative allyl alcohol dehydrogenase	BAJ87887.1	4.00E-150	Up
		Energy metabolism			
TDF 115	JZ482436	proteasome subunit alpha type-2	EMT28480.1	1.00E-84	Up
TDF 2F	JZ482458	mitochondrial protein, putative	XP_003588355.1	6.00E-38	Up
TDF 39	JZ482449	NADH-ubiquinone oxidoreductase Fe-S protein, putative	XP_002535141.1	2.00E-14	Up
TDF 63	JZ482462	cytochrome P450	EMS67451.1	1.00E-10	Up
		Transport			
TDF 31	JZ482437	coatomer subunit beta-2-like isoform 2	XP_003567369.1	3.00E-33	Up
TDF 92	JZ482442	cytochrome c oxidase biogenesis protein Cmc1-like (2)	BAK05311.1	7.00E-57	Down
TDF 62	JZ482451	ureide permease	EMS65551.1	4.00E-08	Up
TDF 94	JZ482454	kinesin-like protein KIF2C	EMT23830.1	8.00E-06	Up
TDF 112	JZ482455	ATPase, F1 complex, gamma subunit	BAJ87277.1	5.00E-08	Up
TDF 83	JZ482465	aquaporin PIP1-2	EMS49467.1	0.007	Down
		Signal transduction			
TDF 64	JZ482463	serine/threonine-protein kinase GSO2	EMS56608.1	3.00E-91	Up
TDF 103	JZ482432	vesicle-associated membrane protein-associated	EMT12988.1	3.00E-82	Up
TDF 108	JZ482433	protein kinase domain	NP_001060011.2	0.83	Up
TDF 45	JZ482439	two-component response regulator-like PRR95 (2)	EMT20128.1	6.00E-92	Up
TDF 104	JZ482445	protein kinase, catalytic domain (2)	XP_002468389.1	4.00E-13	Up
TDF 113	JZ482446	putative receptor-like protein kinase (3)	EMT14014.1	1.00E-92	Up
		Regulation of transcription			
TDF 109	JZ482434	elongator complex protein 3	EMT15243.1	1.00E-26	Up
TDF 27	JZ482428	DEAD box ATP-dependent RNA helicase 37	EMT03384.1	3.00E-13	Up
TDF 29	JZ482429	RNA polymerase beta chain	NP_114251.1	3.00E-64	Down
TDF 46	JZ482430	putative gag/pol polyprotein (2)	AAQ56328.1	7.00E-16	Down
TDF 1F	JZ482443	retrotransposon gag protein (2)	CAH66707.1	1.00E-16	Down
TDF 52	JZ482440	putative histone acetyltransferase ELP3	ADB92637.1	8.00E-80	Up
TDF 51	JZ482431	retrotransposon protein, putative, Ty3-gypsy subclass (4)	AAX92815.1	6.00E-15	Down
TDF 23	JZ482457	DEAD box ATP-dependent RNA helicase 40	EMS48902.1	1.00E-04	Up

Up=up-regulated, Down=down-regulated
The numbers in the brackets represent the frequency of sequenced TDFs

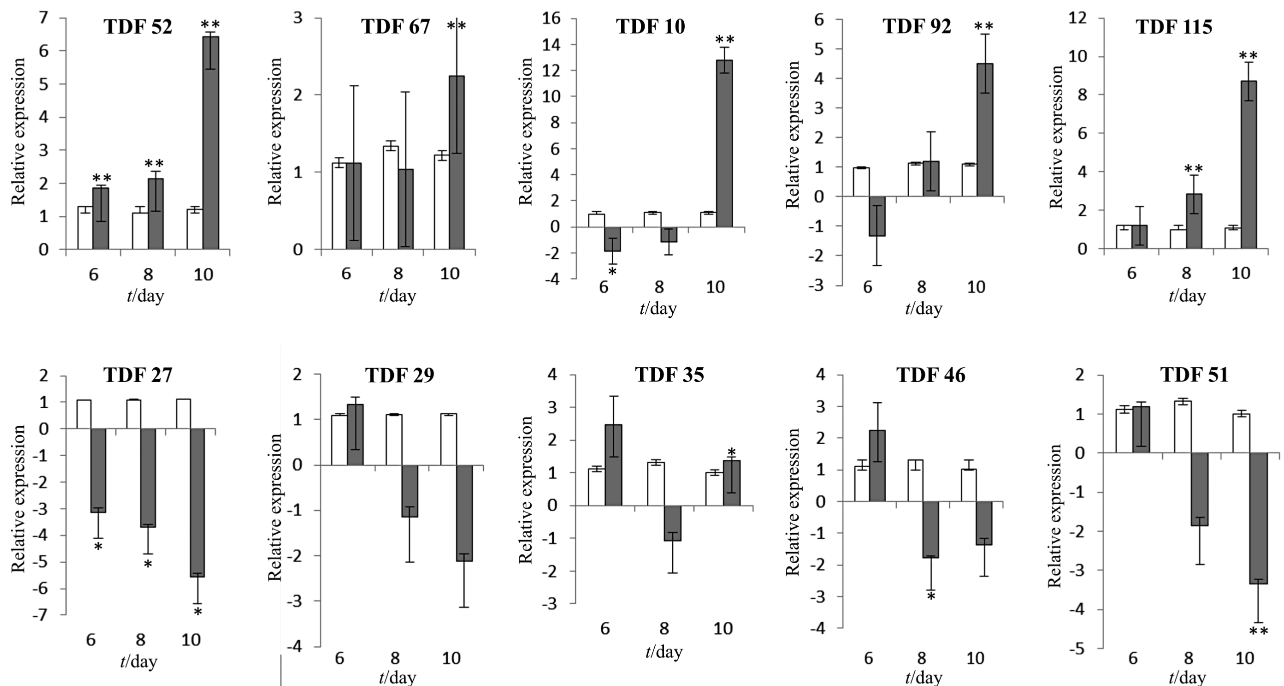

Fig. 2. Validation of expression patterns of selected genes from cDNA-AFLP using real time quantitative PCR. Data represent number of fold change of gene expression in stressed *vs.* control samples. The transcript levels of each gene were monitored in *Triticum durum* leaves 6, 8 and 10 days after the water stress treatment was applied. Relative gene quantification was calculated by Pfaffl method. All data were normalized to the internal reference genes *CDC* and *ADP*. The fold change of the control samples (white bars) was approx. equal to 1. The mean expression value was calculated for every TDF with three independent biological replicates and error bars show standard deviations. Statistical analyses were carried out using the *t*-test to compare the mean values. Up-regulation by 2-fold and down-regulation by 0.5-fold were considered as significant. *significant difference at $p<0.05$; **highly significant difference at $p<0.01$; without asterisk=no significant difference observed

Discussion

Plants induce expression of a number of genes in response to water limitation. The early response at the cellular level results partly from cell damage, and corresponds partly to adaptive processes that initiate changes in the metabolism and structure of the cell that allows it to function under low water potential (*22*). To identify stress-specific genes, it would be more rewarding to isolate the differentially expressed genes providing a clear picture of the transcriptome under stress from a relatively drought-tolerant crop. A wide range of techniques and strategies are being employed these days to identify genes involved in stress responses (*14*). Although DNA microarrays are currently the standard tool for genome-wide expression analysis, their application also is limited to organisms for which the complete genome sequence or large collections of known transcript sequences are available (*23,24*). Next generation sequencing, such as RNAseq are also valuable tools for transcriptional studies, especially when working with organisms that do not have completely sequenced genomes. Here, we applied AFLP-based transcript profiling method that allows genome-wide expression analysis. cDNA-AFLP has a low cost and does not require sequence information and expensive or sophisticated equipment. Using this technique, we detected 3218 TDFs with 64 primer combinations of which 1216 were differentially expressed. We were able to obtain 76 reliable sequences that were allocated to eight differential functional categories: transcription regulation, signal transduction, metabolism, energy metabolism, transport, protein degradation, cellular biosynthesis, cell defense, and 11 sequences with an unknown function. Our analysis identified many genes that had previously been implicated in plant stress responses, as well as novel genes. Transcriptome analysis has revealed seven TDFs with signal transduction functions, including serine/threonine protein kinase, vesicle-associated membrane protein-associated and two-component response regulator-like protein kinase. All these genes were up-regulated, indicating that they are essential in plant defense, as previously reported (*25,26*). Protein kinases are important components in signal transduction system initiated by biotic and abiotic stresses in plants. They are known to play a central role in signalling during pathogen recognition and the subsequent activation of plant defense mechanisms (*27*). In wheat, several members of protein kinases proved to be involved in the responses to abiotic stresses such as drought, salt, cold and H_2O_2, and plant hormones such as abscisic acid and gibberellins (*28,29*). The protein kinases include CDPKs, which play an important role in hyperosmotic stress response (*30*) and MAPKs, essential to both biotic and abiotic stresses (*31,32*). These proteins compose two functional groups due to their distinct functions in mediations of abiotic stress signals.

Additionally, TDFs 113, 91 and 13 exhibited sequence homology with putative receptor protein kinase that has been found to be involved in the plant defense and adaptation response (*33*) and has also been suggested to be involved in abiotic stress response. Our finding confirms

the latter observation and is in agreement with other transcriptomics analyses (*34–36*).

A significant outcome of this study was the identification of many transcription regulatory genes among the stress-inducible genes, suggesting that various transcriptional regulatory mechanisms function in durum wheat under the drought stress. Stress gene induction occurs primarily at the level of transcription, and regulating the temporal and spatial expression patterns of specific stress genes is an important part of the plant stress response (*37*). We found that TDFs 23 and 27 exhibited sequence homology with DEAD box RNA helicase. RNA helicases have been implicated in every step of RNA metabolism, including nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay, and organellar gene expression (*38,39*). The DEAD box RNA helicases compose the largest subfamily of RNA helicases. DEAD box RNA helicases had been reported to play an important role during development and stress responses in various organisms (*40–42*). Rice *OsBIRH1* encoding DEAD box RNA helicase was shown to function in defense responses against pathogen and oxidative stresses (*40*). The *Arabidopsis LOS4* gene, which encodes a DEAD box RNA helicase protein, has been demonstrated to be crucial for expression of cold-responsive genes and for chilling and freezing tolerance (*41*). Stress response suppressor 1 (STRS1) and STRS2 encoding DEAD box RNA helicases were shown to function as negative regulators of ABA-dependent and ABA-independent signalling networks (*42*). In our research, the DEAD box RNA helicases were up-regulated under drought stress, which might indicate their importance in drought response. To our knowledge there has been no report of the up-regulation of this transcript under drought stress.

According to the sequence analysis, TDF 112 was predicted to encode ATPase F1 complex gamma subunit. ATPase was suggested to control stomatal apertures in guard cells (*43*). On the other hand, TDF 83 was homologous to plasma membrane intrinsic protein aquaporin PIP1-2, a significant component in cellular water transport, which is enriched in zones of rapid cell division and expansion (*44,45*). Its expression was generally down-regulated upon drought stress. The aquaporin PIP has been found to function in water transport through the plasma membrane in many plant species (*46–49*). The down-regulation of the aquaporin TDF 83 in wheat leaves under drought stress may be due to an attempt to avoid the possible loss of water. Conversely, in a different study, two aquaporins were overexpressed under abiotic stress (*50*). In rice seminal roots, the identified aquaporin showed an inducible expression pattern that parallelled that of root length (*35*). Many different aquaporins are known to respond to dehydration by up- or down-regulation or show no response at all (*51*). Similar behaviour characterized aquaporin homologues in wild emmer wheat genotypes where some members were strongly up-regulated in both genotypes, in both tissues, and at all times, whereas others were mostly down-regulated (*52*).

In addition, TDF 2 is matched to Bax-inhibitor-1 protein (BI-1), whose involvement in plant defense response is well documented. BI-1 is one of the most intensively characterized cell death suppressors conserved between plants and mammals (*53,54*). In plants, BI-1 is induced by a variety of stress stimuli such as pathogen attack, oxidative stress and heat stress, and its overexpression suppresses cell death activation (*53,55*). Subsequently, plant BI-1 genes from rice and *Arabidopsis* were isolated and shown to be an evolutionary conserved protein that, when overexpressed in yeast and plant cells, suppresses cell death induced by mammalian Bax (*56,57*). In fact, numerous studies by transgenic approaches have revealed that overexpression of plant BI-1 resulted in attenuation of cell death induced by biotic (pathogens) and abiotic stresses (*58–60*). Similar to the results of gene expression of maize leaves under drought stress using RNA-seq (*61*), the expression of BI-1 TDF 2 was induced in our study, thus indicating the importance of this protein as a survival factor in plants for stress tolerance and plant defense.

Other sequences identified in this study appear to be quite interesting because they are homologous to the genes coding for proteins involved in energy metabolism and metabolism categories, including cytochrome P450 (TDF 63) and alcohol dehydrogenase (TDF 12), respectively. Gene expression analysis has revealed that most cytochrome P450 genes are strictly regulated in response to phytohormones, pathogens, UV damage, heavy metal toxicity, mechanical injury, drought, high salinity and low temperatures (*62*). Therefore, cytochrome P450 genes encode for a superfamily of enzymes in plants that seem to be involved in response to biotic and abiotic stresses (*62,63*). The cytochrome P450 production was significantly increased under salinity stress in *Arabidopsis* (*64*). A previous study using cDNA microarray screening revealed that five genes encoding cytochrome P450 family proteins were found to be highly drought induced (*65*). Our finding is in agreement with the latter observation. The up-regulation of this gene might indicate important functions in the cellular response to drought stress in durum wheat leaves.

TDF 12, alcohol dehydrogenase (ADH), is one of the anaerobic proteins that catalyze the reduction of pyruvate to ethanol, resulting in continuous NAD^+ regeneration. ADH activity is considered essential for the survival of plants during anaerobic conditions (*66*). Transcriptional activation of the *Adh* gene has also been noticed in response to several environmental stresses including saline conditions (*67,68*). In our experiment, ADH was induced under drought stress treatment. Consistent with the drought-stress response at the mRNA level, drought and temperature stresses induced the expression of the alcohol dehydrogenase gene in *Arabidopsis* (*69,70*). It has also been demonstrated that *Arabidopsis* mutants with defective *Adh* expression showed defective responses to cold and osmotic stresses (*70*). On the other hand, TDF 10 had sequence homology with senescence-associated genes (*SAGs*). Leaf senescence is the sequence of degradative processes leading to the remobilization of nutrients and eventual leaf death. The senescence process is highly regulated, involving photosynthetic decline, protein degradation, lipid peroxidation, and chlorophyll degradation (*71*). The senescence syndrome is controlled in part by internal factors such as hormones and by external factors

such as photoperiod, abiotic stresses and pathogens, all of which influence gene expression (*72*). Several dozen genes up-regulated during senescence, designated as senescence-associated genes, have been identified in various species (*72–74*). In our cDNA-AFLP data, the *SAG* was up-regulated in durum wheat leaves under drought condition, which probably indicates the senescence of leaves. The *SAG*-encoded proteins are likely to participate in macromolecule degradation, detoxification of oxidative metabolites, induction of defense mechanisms, and signalling and regulatory events (*75*).

Another interesting group of genes identified in this study includes those genes that do not share any significant identity with sequences annotated in the public databases. They might correspond to genes not yet characterized in any plant species. Several studies have reported the identification of new transcripts using the same approach in other plants (*34,76–78*). The characterization of genes not yet described in databases should be considered because it may provide new information about the plant stress response. Techniques such as overexpression or silencing may confirm their role and determine their functions under drought stress in durum wheat.

Conclusions

We conclude that cDNA-AFLP technique applied here to investigate drought-responsive genes from durum wheat resulted in identifying several TDFs under water stress. More than 1200 TDFs with altered patterns of gene expressions were observed. Annotation of the TDFs predicted that most of them encoded proteins involved in multiple functional groups including transcription regulation, signal transduction, metabolism, transport, protein degradation, and cell defense. These findings add new information to the broad picture of stress-activated plant genes and facilitate the understanding of cellular mechanisms involving groups of gene products that act in coordination in response to water stress. On the other hand, since little information on wheat genome is available and large discrepancies between genomes of model plants (*Arabidopsis* and rice) and wheat genome exist, the enrichment of the wheat expressed sequence tag (EST) information is highly required. This study provides a large amount of information concerning groups of genes regulated under drought stress in durum wheat, which will hopefully serve as a basis to further elucidate the biology of durum wheat response to water deprivation. As a result of this study, we have also found a number of new genes involved in durum wheat drought response. The role and characterization of these genes should be elucidated to provide useful data that could help the development of breeding strategies aimed at improving durum wheat tolerance to drought stress.

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