ANALYSIS OF DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO DROUGHT STRESS IN WHEAT (*Triticum aestivum* L.) USING cDNA-SCoT

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Wheat (*Triticum aestivum* L.) is one of the world's most important food crops, contributing about one-fifth of human caloric intake (Shiferaw *et al*., 2013), providing about 20% of the calories consumed by mankind (FAO, 2011) and having an international trade volume greater than all other major food crops combined (Atchison *et al*., 2010). Wheat is an important crop in which to investigate tolerance to abiotic stress because it is grown in diverse environments and has great potential for adaptation to environmental conditions (Worland *et al*., 1994).

Wheat is the leading human food source, accounting for more than half of the world's total food consumption (Ergen and Budak, 2009) therefore; it is a major target for the development of cultivars that are high-yielding under water-limited conditions. For drought-related research and the improvement of modern crop varieties, plants exhibiting high drought resistance are the most suitable targets and the most promising sources of drought-related genes (Qin *et al*., 2016).

The global environmental changes will intensify the need to develop crops with tolerance to abiotic stresses, especially water deficits. Worldwide, drought and other abiotic stresses significantly reduce agricultural productivity, with losses estimated at 50% or more (Bray *et al*., 2000). Drought, the major stress factor limiting crop productivity worldwide (Araus *et al*., 2008), is expected to increase due to global climate change (Wheeler and Von Braun, 2013). Developing crop cultivars with improved drought tolerance is considered as sustainable and an economically viable approach to enhance crop productivity and ensure food security for the growing human population (Merchuk-Ovnat *et al*., 2016).

Plants are frequently challenged by adverse environmental conditions, including extreme temperatures, drought, and

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high salinity. Upon exposure to harmful environmental conditions, many related genes are induced (Cramer *et al*., 2011). Genetic diversity in crop species can be determined using morphological and agronomic characteristics as well as biochemical and DNA marker analysis (Liu, 1997). However, physiological and morphological traits are subjective and quantitative in practice, and they can be affected by environmental conditions and the experience of evaluators (Bolaric *et al*., 2005).

Different methods have been developed for the gene expression study in plants such as cDNA microarray, cDNA-Sequence-related amplified polymorphism (cDNA-SRAP), cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP), serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH), representational difference analysis (RDA), and mRNA differential display (DD) (Xu *et al*., 2011). All these markers have advantages and disadvantages based on the reproducibility of the results, available resources, technical expertise, and cost of development (Al-Qurainy *et al*., 2017).

A cDNA start codon-targeted (cDNA-SCoT) marker has been used for the study of gene expression in *Saccharum officinarum*, *Mangifera indica*, *Phoenix dactylifera* and *Dendrobium officinale* (Li *et al*., 2013). However, this marker has also been used for the assessment of genetic diversity in various plant species (Al-Qurainy *et al*., 2015).

In the present study, we isolate and characterize some of the key expressed sequence tags (ESTs) in response to drought stress in *Triticum aestivum* L. using cDNA-SCoT marker to determine the gene expression profiling and the function for each EST under drought stress.

MATERIALS AND METHODS

Plant material and drought experiment

Wheat seeds (*Triticum aestivum* L.) variety Giza 186 was used in this study and kindly was obtained from Field Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

Seeds of *Triticum aestivum* L. were germinated in greenhouse conditions under a 12-h light/12-h dark cycle at 22-24°C, relative humidity 80%, and watered day by day with tap water. Three weeks old wheat seedlings were subjected to drought stress according to Park *et al*. (2003) by removing the seedlings very carefully and dehydrated on Whatman 3-mm filter paper under controlled conditions. Wheat seedlings were sampled at different time points (C, 1 h and 6 h) with three biological replicas for each treatment. Control plants were well-watered and harvested at the same time as the stressed plants. All collected samples were immediately frozen in liquid nitrogen and stored at -80°C for future analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated from control and drought-stressed seedlings (1 h and 6 h) using TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Total RNA of each sample was measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Germany) to calculate the concentration; also each sample of total RNA was loaded into agarose gel to test the integrity of RNA. High quality of cDNA was prepared using Improm^{TM} Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

cDNA-SCoT-PCR Amplification and Detection

The PCR reaction was performed in a total volume of 25 µl using the SCoT primers (Table 1) for the study of expression profiling. These primers were selected from the literature according to Collard and Mackill (2009). The cDNA concentration was about 40 ng for PCR amplification with SCoT primer (30 pmol). The PCR amplification was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, USA). The cycling profile was 94°C for 5 min, 40 cycles at 94°C for 50 sec., 50°C for 50 sec., 72°C for 1 min, and a cycle of 72°C for 7 min, then held at 4°C. The amplified products were resolved on 1.5% agarose gel.

Isolation and re-amplification of cDNA fragments

The differentiated bands of interest (up regulated) were excised from the gels using a sterile razor blade; the DNA was purified from the agarose gels using Zymoclean™ Gel DNA Recovery Kit (The Epigenetics Company™, USA) according to the manufacturer's instructions. The re-amplification of differentiated bands was re-amplified by using the same set of corresponding primers and the same PCR conditions which using in amplification section. The PCR products for re-amplification were checked in a 2% agarose gel.

Sequencing of cDNA fragments

The re-amplified fragments were sequenced using ABI PRISM BigDye® terminator cycle sequencing ready reaction kit (Applied Biosystems, USA), in conjunction with ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, USA), at laboratory in South Korea (Macrogen Company). The nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 3730xl DNA Analyzer.

Sequence analysis

Analysis was performed using The Basic Local Alignment Search Tool (BLAST) algorithm program of National Center for Biotechnology Information (NCBI) database, USA (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Different expression patterns of cDNA-SCoT in response to drought stress

In order to isolate drought-responsive gene fragments from *Triticum aestivum* L. seedlings, cDNA-SCoT marker with 12 different primers was utilized. Different exposure times to drought (1 and 6 hrs) were chosen to detect genes differentially responding to drought treatment.

As a result of cDNA-SCoT marker analysis, a total of 17 fragments were clearly differentially expressed as shown in (Figs. 1 and 2) as an example. These fragments, varying in length from 263 to 837 bp, were excised from the gels, re-amplified by PCR and successfully sequenced.

Sequence analysis of cDNA-SCoT fragments

A specific nomenclature was adopted for cDNA-SCoT fragments. For example, cDNA Tad 1 refers to *Triticum aestivum* drought treated seedlings, amplified using primer number 1. Fragments number 1-1 to 1-3 were isolated from this primer, fragment number 2-4 was amplified using primer number 2, fragment number 3-5 was amplified using primer number 3, fragments number 4-6 to 4-8 were amplified using primer number 4, fragment number 5-9 was amplified using primer number 5, fragment number 6-10 was amplified using primer number 6, fragment number 7-11 was amplified using primer number 7, fragments number 8-12 to 8-13 were amplified using primer number 8, fragment number 9-14 was amplified using primer number 9, fragment number 10-15 was amplified using primer number 10, fragment number 11-16 was amplified using primer number 11 and lastly fragment number 12-17 were amplified using primer number 12

According to Table (2), it is possible to distinguish two fragment of the 17 ESTs related to drought stress: fragment Tad 1-1 showing significant homology with an EST with *Triticum aestivum* cDNA under drought stress with E-value 0.16 and fragment Tad 1-2 showing significant homology with an EST with *Cicer arietinum* cDNA under dehydration stress with E-value 0.018.

It is also possible to distinguish one fragment Tad 1-3 showing no significant sequence similarity to known sequences in the gene bank. This EST needs more investigation to confirm the relation to the abiotic stress process or may represent yet uncharacterized gene.

Fragments Tad 4-7, Tad 6-10, Tad 8-13, Tad 9-14 and Tad 11-16 showing homology with un-identifying clones with *Triticum aestivum* with E-value 2e-06, 3e-17, 6e-19, 9e-06 and 1.5 respectively, also, fragment Tad 12-17 showing homology with un-identifying clone with *Triticum aestivum* under heat stress with E-value 5e-05.

Fragment Tad 2-4 showing homology with Tetratricopeptide repeat-

containing protein kinase 1 (*Triticum aestivum*) with E-value 3.6, which up regulated after one hour from exposure to drought stress.

Receptor-like protein kinase 1 (RPK1) localizes to the plasma membrane and functions as a regulator of abscisic acid (ABA) signaling in *Arabidopsis*. Osakabe *et al*. (2010) investigated the effect of RPK1 disruption and overproduction upon plant responses to drought stress. Transgenic *Arabidopsis* overexpressing the RPK1 protein showed increased ABA sensitivity in their root growth and stomatal closure and also displayed less transpirational water loss. In contrast, a mutant lacking RPK1 function, *rpk1-1*, was found to be resistant to ABA during these processes and showed increased water loss. RPK1 overproduction in these transgenic plants thus increased their tolerance to drought stress. These results suggest that the overproduction of RPK1 enhances both the ABA and drought stress signaling pathways.

Fragment Tad 3-5 showing homology with an EST from *Oryza brachyantha* cytochrome P450 with E-value 2.5, which up regulated after one hour from exposure to drought stress.

In plants, fatty acids (FA) are subjected to various types of oxygenation reactions. Products include hydroxyacids, as well as hydroperoxides, epoxides, aldehydes, ketones and α, ω-diacids. Many of these reactions are catalysed by cytochrome P450s (P450s), which represent one of the important and largest superfamilies of proteins in plants. The existence of P450-type metabolizing FA enzymes in plants was established approximately four decades ago in studies on the biosynthesis of lipid polyesters. Biochemical investigations have highlighted two major characteristics of P450s acting on FAs: (a) they can be inhibited by FA analogues carrying an acetylenic function, and (b) they can be enhanced by biotic and abiotic stress at the transcriptional level. P450s catalyze numerous monooxygenation/ hydroxylation reactions in biochemical pathways; also, P450s are involved in a variety of metabolic pathways and participate in the homeostasis of phytohormones (Yan *et al*., 2016).

Fragment Tad 4-6 showing homology with an EST from *Oryza sativa* Japonica Group glycine-rich cell wall structural protein 2 with E-value 9e-32, which up regulated after one hour from exposure to drought stress.

In plants, glycine-rich proteins (GRPs) are characterized by the presence of semi-repetitive glycine-rich motifs. In general, those genes present developmentally regulated and tissue-specific expression pattern. In several plant genera, their expression is also modulated by biotic and abiotic factors (Sachetto-Martins *et al*., 2000). Recently, a reverse genetics approach was used to characterize a glycine-rich protein gene from *Arabidopsis* according to microarray results that could be involved in secondary cell wall formation.

The analysis indicated that this GRP is implicated in the maintenance of protoxylem structure (Yokoyama and Nishitani, 2006).

Fragment Tad 4-8 showing homology with an EST from *Elaeis guineensis* V-type proton ATPase subunit D with E-value 0.019, which up regulated after six hours from exposure to drought stress.

The vacuolar type H+-ATPase (V-type H+-ATPase) plays important roles in establishing an electrochemical H+-gradient across tonoplast, energizing Na+ sequestration into the central vacuole, and enhancing salt stress tolerance in plants (Zhang *et al*., 2014). V-type H+-ATPases not only maintain the dynamic balance of cytoplasm ion and cell metabolism as a kind of "dominate enzyme", but also respond to environmental factors through appropriately changing subunits expression and modulating enzyme activity. The structure of V-type H+-ATPases appears to be conserved across eukaryotes (Nishi and Forgac, 2002). However, most plant V-type H+-ATPases subunits are encoded by small multigene families, which have been detected in many plant genomes (Seidel, 2009).

Fragment Tad 5-9 showing homology with an EST from *Sesamum indicum* probably inactive leucine-rich repeat receptor-like protein kinase with E-value 0.24, which up regulated after six hours from exposure to drought stress.

The leucine rich repeat receptor like kinases (LRRK) constitute the largest subfamily of receptor like kinases (RLK), which play critical roles in plant development and stress responses (Shumayla *et al*., 2016). The LRRKs are involved in growth, development, and survival including organogenesis, morphogenesis, hormone signaling, and abiotic and biotic stress managing in plants (Diévart and Clark, 2003).

Fragment Tad 7-11 showing homology with an EST from *Triticum aestivum* plastid acetyl-CoA carboxylase (Acc-1) gene with E-value 0.52, which up regulated after one hour from exposure to drought stress.

Acetyl CoA carboxylase (ACC) is an enzyme involved in lipid biosynthesis, was uniquely identified in the tolerant genotype Peanut (COC041) and detectable only during water stress and is mediated cell wall strengthening under water stress (Kottapalli *et al*., 2009). In plants, ACC isozymes provide the malonyl CoA pools used for de novo fatty acid synthesis in plastids and mitochondria, and for fatty acid elongation, flavonoid (FL) and stilbene biosynthesis in the cytosol (Focke *et al*. 2003). From several studies, it is evident that the ACC reaction is the most important regulatory step, controlling metabolite flow in response to stress. From the water-deficit stress tolerance perspective, fatty acids are essential in membrane biogenesis, lipoic acid and cuticular wax synthesis and stress signalling (Zuther *et al*., 2004). Plastid- localized acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) of wheat (*Triticum aestivum*)

were cloned and sequenced by Gornicki *et al*. (1997). The sequences of the two genes, Acc-1,1 and Acc-1,2 are 89% identical. Their exon sequences are 98% identical. The amino acid sequence of the biotin carboxylase domain encoded by Acc-1,1 and Acc-1,2 is 93% identical with the maize plastid ACCase but only 80-84% identical with the cytosolic ACCases from other plants and from wheat. There is some experimental evidence suggesting that, in plants, ACCase activity controls metabolic flux through the fatty acid biosynthetic pathway and therefore may serve as an important regulation point of plant metabolism (Ohlrogge and Jaworski, 1997).

Fragment Tad 8-12 showing homology with an EST from *Aegilops tauschii* subsp. tauschii target of MYB protein 1-like with E-value 4e-69, which up regulated after six hours from exposure to drought stress.

MYB (myeloblastosis) proteins constitute one of the largest transcription factor families in eukaryotes and are characterized by a highly conserved DNA-binding domain (DBD) known as the MYB domain (Rabinowicz *et al*., 1999). The MYB family is divided into four classes, including 1R-, R2R3-, 3R- and 4R-MYB proteins, according to the number of MYB domains (Dubos *et al*., 2010). The MYB family is one of the most abundant transcription factor families in plants. MYB proteins are involved in plant development, abiotic stress tolerance, hormone signal transduction and disease resistance (Peng *et al*., 2016). In general, most of the MYB proteins function as transcription factors and are characterized by the presence of variable numbers of N-terminus conserved MYB repeats (R), mainly associated with DNA-binding and protein-protein interactions. The variable C-terminal region is responsible for modulating the regulatory activity of the protein (Roy, 2016). Plants have developed elaborate and complex mechanisms to survive under abiotic stress conditions. Several line of evidences have implicated role of MYB protein in regulation of abiotic stress tolerance in plant genome (Yang *et al*., 2012).

Fragment Tad 10-15 showing homology with an EST from *Triticum turgidum* subsp. durum ethylene response factor 1 (ERF1) gene with E-value 2e-41, which up regulated after one hour from exposure to drought stress.

Ethylene is a gaseous phytohormone that plays vital roles in plant growth, development as well as response to abiotic and biotic stresses. Ethylene Response Factor1 (ERF1) is a key transcription factor for activating the ethylene signaling pathway by binding the GCC box and other cis-elements in the promoters of ethylene-responsive genes, which is an ideal candidate gene for improving plant tolerance of different abiotic stresses (Kavas *et al*., 2015). ERF1 is an upstream component in both jasmonate (JA) and ethylene (ET) signaling and is involved in pathogen resistance. Accumulating evidence suggests that ERF1 might be relat-

ed to the salt stress response through ethylene signaling. However, the specific role of ERF1 in abiotic stress and the molecular mechanism underlying the signaling cross talk still need to be elucidated. Also, ERF1 plays a positive role in salt, drought, and heat stress tolerance by stress-specific gene regulation, which integrates JA, ET and abscisic acid signals (Cheng *et al*., 2013).

Wu *et al*. (2013) used the cDNA-SCoT technique on sugarcane for the differential expression of gibberellin-induced genes for stalk elongation, which represented the up regulation and down regulation of genes. The cold resistance-related genes have been studied in sugarcane under cold stress using the cDNA-SCoT technique (Chen *et al*., 2010). The differentially expressed genes in sugarcane, induced by *Leifsonia xyli* subsp. *xyli*, were studied using the cDNA-SCoT technique (Chen *et al*., 2013). Also, Al-Qurainy *et al*. (2017) investigate the response of Khalas cultivar of *Phoenix dactylifera* under different salt stress concentrations (50, 100, and 150 mM NaCl), cDNA-SCoT marker showed a variation in different gene expressions profiling between treated and untreated plants under various NaCl concentrations.

So, based on our results can be reveal some important aspects associated closely with environmental tolerance (drought) in wheat which could be used for the elucidation of mechanisms underlying environmental stresses. Furthermore, even EST fragments with no apparent significant similarities can still be used to discover new genes related to the stress response mechanisms.

SUMMARY

A cDNA- start codon-targeted polymorphism (cDNA-SCoT) marker was employed to isolate and characterize differentially expressed sequence tags (ESTs) in response to drought stress in wheat (*Triticum aestivum* L.) at seedling stage, cultivar Giza 186. Three weeks old wheat seedlings were subjected to drought stress, the seedlings sampled at different time points (1 h and 6 h) after exposed to drought stress with three biological replicas for each treatment, and untreated seedlings were used as control. An array of 17 differentially expressed EST fragments in drought-stressed plants comparing to control was obtained, and characterized using the bioinformatics analysis. A cDNA-SCoT marker showed a variation in different gene expressions profiling between treated and untreated seedlings under drought stress.

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Table (1): List of SCoT primer sequences.

No.	Primer code	Primer sequence (5′-3′)
1	Scot-1	ACGACATGGCGACCACGC
2	Scot-2	ACGACATGGCGACCCACA
3	Scot-3	CAATGGCTACCACTAGCG
4	Scot-4	ACCATGGCTACCAGCGCG
5	Scot-5	CCATGGCTACCACCGGCA
6	Scot-6	CAACAATGGCTACCACGC
7	Scot-7	CCATGGCTACCACCGCAC
8	Scot-8	CAACAATGGCTACCACCA
9	Scot-9	AAGCAATGGCTACCACCA
10	Scot-10	ACGACATGGCGACCAACG
11	Scot-11	AACCATGGCTACCACCAC
12	Scot-12	CAACAATGGCTACCACGG

Table (2): Fragments blast analysis.

Primer Name	Fragment No.	No. of bases	Homology Accession No.	Homology	E- Value
Primer 1	Tad 1-1	521	BE604160.1	WHE1413-1416_I23_I23ZS Wheat drought stressed leaf cDNA library *Triticum aestivum* cDNA clone WHE1413-1416_I23_I23, mRNA sequence.	0.16
	Tad 1-2	525	GR410156.1	ICC4958_CD11_G05 ICC4958 dehydration stressed root cDNA library *Cicer arietinum* cDNA clone ICC4958_CD11_G05 5-, mRNA sequence.	0.018
	Tad 1-3	776		No significant similarity found.	
Primer 2	Tad 2-4	486	ABF50637.1	Tetratricopeptide repeat-containing protein kinase 1 [*Triticum aestivum*].	3.6
Primer 3	Tad 3-5	452	XM_006652607.2	PREDICTED: *Oryza brachyantha* cytochrome P450 704C1-like (LOC102706836), mRNA.	2.5
Primer 4	Tad 4-6	483	XM_015758926.1	PREDICTED: *Oryza sativa* Japonica Group glycine-rich cell wall structural protein 2 (LOC4348767), mRNA.	9e-32
	Tad 4-7	480	BE405207.1	WHE1211_C01_F01ZS Wheat etiolated seedling root cDNA library *Triticum aestivum* cDNA clone WHE1211_C01_F01, mRNA sequence.	2e-06
	Tad 4-8	496	XM_010938789.2	PREDICTED: *Elaeis guineensis* V-type proton ATPase subunit D (LOC105056563), transcript variant X1, mRNA.	0.019

Table (2): Cont'

Primer 5	Tad 5-9	508	XM_011076891.2	PREDICTED: *Sesamum indicum* probably inactive leucine-rich repeat receptor-like protein kinase At5g06940 (LOC105159717), mRNA.	0.24
Primer 6	Tad 6-10	560	CJ537901.1	CJ537901 Y.Ogihara unpublished cDNA library Wh_EMI *Triticum aestivum* cDNA clone rwhei3o03 3-, mRNA sequence.	3e-17
Primer 7	Tad 7-11	273	EU660901.1	*Triticum aestivum* clone BAC 198E19 plastid acetyl-CoA carbox-ylase (Acc-1) gene, complete cds; nuclear gene for plastid product.	0.52
Primer 8	Tad 8-12	515	XM_020335951.1	PREDICTED: *Aegilops tauschii* subsp. tauschii target of Myb protein 1-like (LOC109777297), mRNA.	4e-69
	Tad 8-13	349	CJ670593.1	CJ670593 Y.Ogihara unpublished cDNA library Wh_KMV *Triticum aestivum* cDNA clone whkv2p07 5-, mRNA sequence.	6e-19
Primer 9	Tad 9-14	492	CJ726093.1	CJ726093 Y. Ogihara unpublished cDNA library Wh_VSCB *Triticum aestivum* cDNA clone whvs12k20 5-, mRNA sequence.	9e-06
Primer 10	Tad 10-15	693	KJ689812.1	*Triticum turgidum* subsp. durum ethylene response factor 1 (ERF1) gene, complete cds.	2e-41
Primer 11	Tad 11-16	263	AK450060.1	*Triticum aestivum* mRNA, clone: tplb0034b20, cultivar Chinese Spring.	1.5
Primer 12	Tad 12-17	837	GD188641.1	Tau21-004-F05-A-043.g *Triticum aestivum* flower heat stress forward subtractive library *Triticum aestivum* cDNA clone Tau21-004-F05-A-043 3-, mRNA sequence.	5e-05

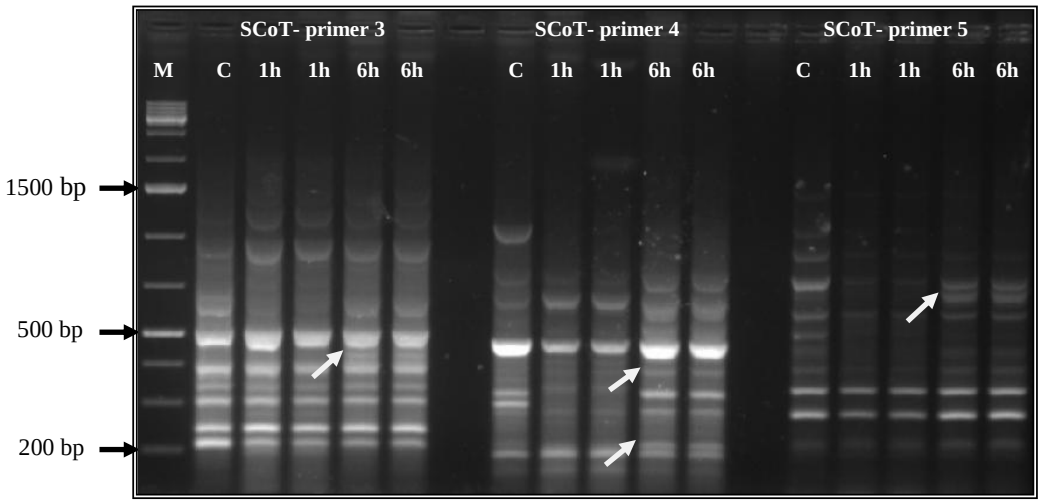

Fig. (1): Agarose gel electrophoresis (1.5%) of the amplified cDNA-SCoT marker profiling generated from *Triticum aestivum* seedlings using different SCoT primers. Lane C, control; Lane 1 h, 1 hour and Lane 6 h, 6 hours after drought treatment. Arrows indicate the differentially expressed products that were isolated for further investigation. M is 1 Kb plus DNA Ladder marker (Fermentas, Germany).

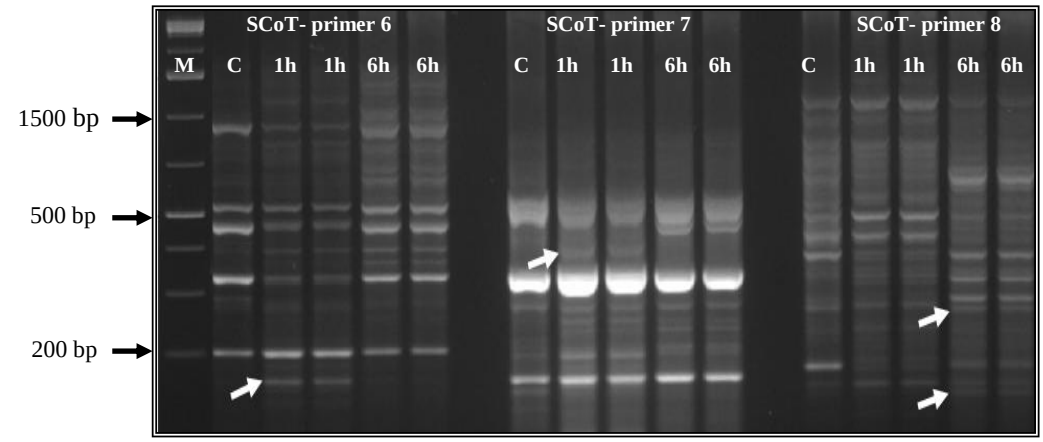

Fig. (2): Agarose gel electrophoresis (1.5%) of the amplified cDNA-SCoT marker profiling generated from *Triticum aestivum* seedlings using different SCoT primers. Lane C, control; Lane 1 h, 1 hour and Lane 6 h, 6 hours after drought treatment. Arrows indicate the differentially expressed products that were isolated for further investigation. M is 1 Kb plus DNA Ladder marker (Fermentas, Germany).