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Identification and Functional Validation of Expressed Sequence Tags (ESTs) Preferentially Expressed in Response to Drought Stress in Durum Wheat

Ayman Diab[1,2*] • Ayman Amin[3] • Salwa Badr[4] • Jaime A. Teixeira da Silva[5] • Pham Thanh Van[6,7] • Bassem Abdelgawad[1] • Sami Adawy[1] • Reda Sammour[4]

[1] Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt
[2] Faculty of Biotechnology, October University of Modern Sciences and Arts (MSA), Egypt
[3] Department of Plant Physiology, Faculty of Agriculture, Cairo University, Giza, Egypt
[4] Department of Botany, Faculty of Science, Tanat University, Tanta, Egypt
[5] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[6] The United Graduate School of Agricultural Science, Ehime University, Matsuyama, Ehime 790-8566, Japan
[7] Vietnam Academy of Agricultural Sciences (VAAS), Institute of Agricultural Genetics (IAG), Pham Van Dong Street, Co Nhue, Tu Liem, Hanoi, Vietnam

Corresponding author: * **aimanalidiab@gmail.com**

ABSTRACT

Drought is a major planetary stress problem that places greater limits on cereal productivity. Crop species belonging to the tribe *Triticale* represent the main foodstuff sources for humans. Because durum wheat (*Triticum turgidum* L.) shows better adaptation to semi-arid climates than bread wheat, its genome are likely to contain a pool of genes that are expressed for wide environmental adaptability. To investigate and understand the genetic response of drought tolerance in durum wheat, differentially expressed sequence tags (dESTs) were detected, characterized and cloned from durum wheat under different regimes of drought stress and the functions of some of these dESTs were determined. In this study, genes involved in drought stress at early stages of growth were profiled by a differential display technique from Egyptian durum wheat (Sohag 3). One, three, six, and 10 hours of drought stress were applied to produce 15 expressed cDNA fragments, which highlighted the genes that were expressed under drought stress, and which might help to understand the plant's defense mechanism under drought conditions. This will also provide an opportunity to identify gene(s) for future use in transgenic research to enhance drought tolerance in wheat and other commercially important crops. The cloned dESTs could also be used as molecular markers for genetic and QTL mapping for wheat and other cereals under drought stress conditions.

Keywords: abiotic stress, molecular marker, EST, cloning, *Triticum turgidum*

INTRODUCTION

An analysis of major world crops shows that there is a large genetic potential for yield that is unrealized because of the need for better adaptation of plants to environments in which they are grown. Stresses can occur at any stage of plant growth and development, thus illustrating the dynamic nature of crop plants and their productivity. Genotypic selection for adaptation to such environments has already played an important role in agriculture, but the fundamental mechanisms are poorly understood. Environmental stresses come in many forms, yet the most prevalent effect of stresses on a plant is water deficiency. Drought is among the most widespread abiotic stresses that limits crop productivity (Cellier *et al*. 1998; Syed 2006; Amudha and Balasubramani 2011). Durum wheat (*Triticum turgidum* L. var. *durum*) is a commercially important tetraploid wheat species that originated in the Eastern Mediterranean and has been farmed in this region for the last 12,000 years (MacKey 2005). Durum wheat is considered to be an essential source of genetic improvement for drought stress tolerance because it is distributed in semi-desert areas and therefore it is adapted to this environmental stress (Maccaferri *et al*. 2003; Moragues *et al*. 2007). Durum wheat responds to drought stress through the expression of a specific set of genes that allows the plants to adapt to altered environmental conditions (Nouri *et al*. 2011). A large and increasing number of genes, transcripts and proteins have been correlatively implicated in stress response pathways, while their precise functions in either tolerance or sensitivity remain unclear (Bray *et al*. 2000). Extensive efforts have also been devoted to the characterization of genes induced or upregulated by drought (Close *et al*. 1989, 1993). Correlative evidence suggests a possible role for a number of these drought-induced genes in protecting cells from the harmful effects of dehydration (Xu *et al*. 1996; Close 1997; Bray *et al*. 2000). Large expressed sequence tag (EST) databases have been created for different developmental stages from the tissues and organs of plants exposed to a variety of environmental conditions (Zhang *et al*. 2001; Lazo *et al*. 2004; Chao *et al*. 2006; Diab *et al*. 2008; Neslihan and Hikmet 2009; Gadaleta 2010). The differentially ESTs isolated and identified in this work could help in molecular mapping of traits related to drought tolerance. If validated with accurate phenotyping and properly integrated in marker-assisted breeding programs, this approach will allow gene pyramiding and accelerate the development of drought-tolerant genotypes. An understanding of the genetic and physiological basis of drought tolerance would facilitate the development of improved crop management and breeding techniques and lead to improved yield in unfavorable environments.

MATERIALS AND METHODS

Plant materials and growth conditions

Durum wheat cv. 'Sohag 3' seeds were planted and grown under controlled conditions (28/25°C day/night, 120 μmol m^{-2} s^{-1} in a

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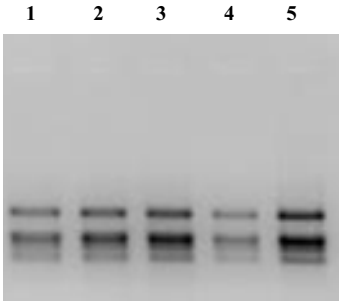

Fig. 1 RNA qualitative check for the control and drought treatments. Lanes: (1) control; (2, 3, 4, 5) 1, 3, 6 and 10 h of drought treatment, respectively.

16-h photoperiod) and stressed when 3 weeks of age. Drought stress was applied according to Oztur *et al*. (2002), by removing plants from soil and leaving them on paper towels under the same conditions indicated above. Leaf tissue was harvested after 1, 3, 6, and 10 h of stress, placed immediately in liquid nitrogen and stored at -80°C. Leaves from unstressed, control plants were harvested at the same times.

Total RNA isolation

Total RNA from control and drought-treated leaf tissues from all four time intervals was extracted using the TriPure Isolation Kit (Roche, Paris, France) according to the manufacturer's instructtions (**Fig. 1**). The isolated total RNA samples were screened using the RNA analyzer for qualitative and quantitative analysis.

Reverse transcription of mRNA (cDNA)

Reverse transcription reactions were performed on all treatment and control samples using the M-MLV reverse transcription kit (Promega, Madison, USA) according to the manufacturer's instructions. The reverse transcription reactions were performed on the total RNA samples of all treated and control plants using the Oligo dT primer to target the polyA tail at the 3′ end of stress-related genes. Then, differential display polymerase chain reaction (PCR) was carried out on the cDNA obtained using an anchor primer (T11G) and arbitrary primer (AP1), attempting to hit the open reading frames (ORFs) at the 5′ end of these genes. cDNA products were qualitatively examined on a 1% agarose TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0) gel.

Differential display polymerase chain reaction (DD-PCR)

PCR was carried out using *Taq* DNA polymerase (Fermentas, Vilnius, LIthuania) in the reverse transcriptase reactions of all treatments and control using an arbitrary primer (AP_1: 5′-AAG CTT GAT TGC C-3′) and an anchored primer ($H_{11}G$: 5′-TTT TTT TTT TTG-3′) in a GeneAmp® PCR System 9700 device under the following conditions: 1X PCR buffer supplied with 1.5 mM $MgCl_2$, 2.5 mM of dNTPs, 0.1 µM of forward and reverse primers and 0.2 U of *Taq* DNA. PCR products were qualitatively examined on a 1% agarose TE buffer gel.

Differential display of amplified cDNA on sequencing gel using silver staining technique

DD-PCR amplified fragments were separated on a 6% denaturing polyacrylamide gel (Amin 2002) using a vertical Sequi-Gen® Sequencing Cell (Bio-Rad, Irvine, USA) and bands were developed using the SILVER SEQUENCE™ DNA staining kit (Promega) according to the manufacturer's instructions.

Isolation of DD-fragments from the gel and re-amplification

The differentially expressed bands were excised from the gel using a sterile scalpel blade. Gel slices were soaked in 50 µl dH_2O in a 0.2-ml Eppendorf tube. The tube was inserted in a heat block at 98-100°C with the cap tightly closed for 15 min. The tube was left at room temperature for 1 h. Then tubes were centrifuged for 2 min to collect condensation and pellet the gel debris. The supernatant was transferred to a new Eppendorf tube. The re-amplification PCR reactions were carried out under same conditions as mentioned in the DD-PCR section.

Cloning of DD-fragments

The pGEM-T easy vector system kit (Promega) was used to clone the DD-fragments according to the manufacturer's instructions. White colonies were examined using PCR. The universal primers used were M13 forward (5′-GTT TTC CCA GTC ACG AC-3′) and M13 reverse (5′-CAG GAA ACA GCT ATG-3′) primers.

Sequencing of the DD-fragment insertion

The automated DNA sequencing reaction was performed using an ABI PRISM Rhodamine Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, USA) in conjunction with a Perkin-Elmer ABI PRISM 310 Genetic Analyzer (Applied Biosystems Division).

Identification of the EST using the NCBI database

Sequence data were analyzed for their identity using PLASTN search for homology with the sequence of a gene already recorded in the database of the NCBI (National Center for Biotechnology Information). BLAST N is the alignment for nucleotides using the BLAST tool in the GenBank (http://www.ncbi.nlm.nih.gov) (Altschul *et al*. 1997). Only alignment hits associated with high E-values were considered to be highly similar.

RESULTS

Differential display of amplified cDNA on sequencing gel and silver staining

The sequencing gel was stained with silver stain in order to visualize differential display (DD) fragments. A total of 15 fragments were found to be differentially displayed due to

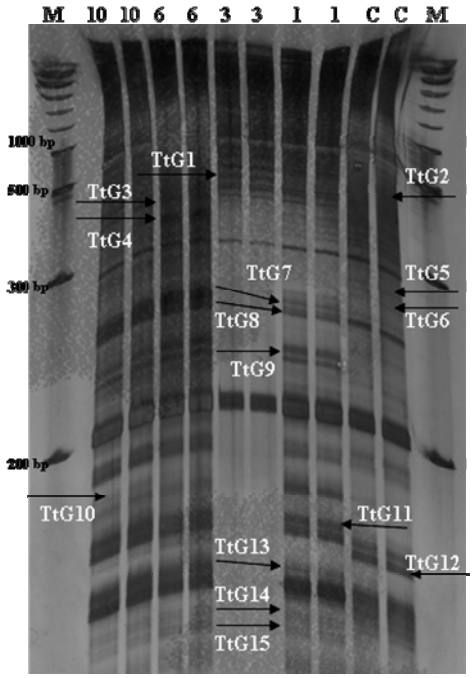

Fig. 2 Polyacrylamide gel showing the differential display fragments under control and stress conditions. Arrows indicate the 15 differentially expressed fragments on a duplicate basis. Lanes: (1) 100 bp DNA ladder marker; (2,3) control, (4,5), (6,7), (8,9) and (10,11) represent 1, 3, 6 and 10 h of drought treatment, respectively.

Table 1 Sequences and BLASTN analysis of the 15 DD-fragments.

A: TtG1			
Query	191	ATG–AAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGG	239
Subject	97	ATGCAAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCATGG	146
B: TtG2			
Query	103	TAGTTTCATACCTG–TCTAGGTAAAGCGAACGTCAAGCGTGCGTAGAGTTGTATC–GATG	160
Subject	3	TAGTTTC–TACTTGTTCTAGGTAAAGCGAACGTCAAGCGTGCGTAGAGTCGTATCAG–TG	60
Query	161	GTCGATAGAACTTGAGGGAA–ATTTTTTTCTACCTTTAGCTTCTCATTGGGTCCAACACT	219
Subject	61	GCCGATAGGACTTGAGAGAGTATTTGTT–CTACCTTTAGCTCCTCGTTGGGTTCGACACT	119
Query	220	CCTACTTANCGAAAGAGGCTACAATTGATCCCCTATACTTGTGGGCAATCAAG	272
Subject	120	CTTACTTATCTAAATAGGCTACAACT–ATCCCGTATACTTGCGGGTTATCAAG	171
C: TtG3			
Query	167	AATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTGAGT	226
Subject	377	AATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTGAGT	436
Query	227	CGTATTAGAGCTTGGCGTAATCAT	250
Subject	437	CGTATTAGAGCTTGGCGTAATCAT	460
D: TtG4			
Query	63	CTATAATGAACAATTCCCAC–TAGTATATGTAAGTGACAACATA–GGAGACTCTCTATCA	120
Subject	392	CTATATTGAA–AATTCCCACNTAGTATATGAAAATGACAACATAG GGAGACTCTCCAT–A	335
Query	121	AGAAGATCATGGTGCTNCTTGGGAGCACAAGCGTGGTAAAAGGATAGTAGTATTGCCCCT	180
Subject	334	TGAAAAACATGGTGCTACTTTGAAGCACAAGTGTAGT–ATAGGATACTAACAATGCCCCT	276
Query	181	TCTCT–CTT–TTTCTCTCAGTTAGTGTTT–TTGTTT–TTTTTC–TCTTTT–TTGGGCCTT	234
Subject	275	ACTCTATTTGTTTAT–TTTTTTTCTTTTTCTTTTTTCTTTTTCNTTTTTTCTTTTTCTTT	217
Query	235	CTCTTTTTTTGTCCTCTTTTT–T–T—C–ATCCGGAGTCTCATCCCGACTTGTGGGGCAA	289
Subject	216	TTCTTTTTTTAT–ATTTTTTTCTCTCACTATCCGGAGTCTCATCCCGACTTGTGGGGCAA	158
Query	290	TC	291
Subject	157	TC	156
E: TtG5			
Query	62	CCTAGGGCAATTTGAGGAAGCCCATCATTGGAATATACAAGCCAAGTTCTATAATGAACA	121
Subject	404	CCTAGGGC–ATTTGAGGAAGCCCATTGTTGGAATATACAAGCCAAGTTCTATAATGAAAA	346
Query	122	ATTCCCACTAGTATATGAAAGTGACAACATAGGAGACTCTCTATCA–AGAAGATCATGGT	180
Subject	345	ATTCCCACTATTATATGAAAGTGACAAAATAAGAGACTCTCTATCATA–AAGATCATGGT	287
Query	181	GCTACTTGGGAGCACAAGCGTGGTAAAA–GGATAGTAGTATTGCCCCTTCTCTCT–TTTT	238
Subject	286	GCTACTTTGAAAGCACGAGTGTGGAAAAAAGGATAGTAGCATTGTCCCTTTTATTTCTTTT	227
Query	239	CTCT	242
Subject	226	CTCT	223
F: TtG6			
Query	198	GGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTGAGTCATATTAGAGC	257
Subject	23	GGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTGAGTCATATTAGAGC	82
Query	258	TTGGCGTAA	266
Subject	83	TTGGCGTAA	91
G: TtG7			
Query	173	GCTT–AATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAG	231
Subject	2	GCTTC AATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAG	61
Query	232	TGAGTCGTATTAGAGCTTGGCGTAATCAT	260
Subject	62	TGAGTCGTATTAAAGCTTGGCGTAATCAT	90
H: TtG8			
Query	167	GCTT–AATCGGATCCCGGGCCCGTCGACTGCAG–AGGCCTGNATGCAAGCTTTCCCTATA	224
Subject	96	GCTTC AATCGGATCCCGGGCCCGTCGACTGCAGCAGGCCTGCATGCAAGCTTTCCCTATA	155
Query	225	GTGAGTCGTATTAGAGCTTGGCGTAATCATG	255
Subject	156	GTGAGTCGTATTAGAGCTTGGCGTAATCATG	186
I: TtG9			
Query	145	CCGGGCCGTCGACTGCAGATGCAAGCTTTCCCTATAGTGAGTCGTATTAGA	204
Subject	124	CCGGGCCGTCGACTGCAGATGCAAGCTTTCTTTATAGTGAGTCGTATTAGA	183
Query	205	GCTTGGCGTAATCATG	220
Subject	184	GCTTGGCGTAATCATG	199
J: TtG10			
Query	131	AGTCGTATTAGAGCTTGGCGTAATCATG	158
Subject	93	AGTCGTATTAGAGCTTGGCGTAATCATG	66
K: TtG11			
Query	96	CTTAATCGGATCCCGGGCCCGTCGACTGGCCTGC	134
Subject	88	CTTAATCGGATCCCGGGCCCGTCGACTGGCCTGC	126
L: TtG12			
Query	37	ACACCAAAAATACACTTGGCCGGATTAAGCATCATCTTATAGACCCGGAGATTATCAAAA	96
Subject	65	ACACCAAAGACACACTTGGCCGGGTTAAGCATCATCTTATAGACCCGGAGATTATCAAAA	124
Query	97	GTTTCC–TTCAAATCAGCTATCAATGTCTC–TGCTTTCATA GG–TTTCACCACGATATCA	153
Subject	125	GTTTCCCTT–AAATCATCTATCAGGGTTTCCT–CTTTGAT–GGA TTTAACCACAATGTCG	181
Query	154	TCCACATAAGCATGAACATTGTGCCC	179
Subject	182	TCCACATAAGCGTGAACATTGCGCCC	207
M: TtG13			
Query	74	ATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCNTGCAAGCTTTCCCTATAGTAGATC	133
Subject	386	ATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTAGATC	445
Query	134	GTATTAGAGCTTGGCGTAATCATG	157
Subject	446	GTATTAGAGCTTGGCGTAATCATG	469

Table 1 (Cont.)

N: TtG14			
Query	15	CCTGCATGCAAGCTTTCCCTATAGTGAGTCGTA–TTAGAGCTTGGCGTAATCATGG	69
Subject	513	CCTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTTAGAGCTTGGCGTAATCATGG	568
O: TtG15			
Query	22	AAGACCTAGGGCANTTTGAGGAAGCCCNTCNTTGNAATACTACNAAC–CCAAGTTCTATA	80
Subject	203	AAGACCTAGGGAAATTTGAGGAATCCCATCGTTGGAATA–TAC—ACA CCAAGTTCTATA	259
Query	81	ATGAACAATTCCCACTAG	98
Subject	260	ATGAACAATTCCCACTAG	277

Shaded codons indicate dissimilarity; N indicates unidentified sequence due to sequencing error. (**A**) TtG1 fragment shows significant homology with EST from AhDS275 groundnut drought stressed subtracted cDNA library for *Arachis hypogaea* cDNA similar to zinc finger FYVE domain containing protein 28, mRNA sequence with accession number EC268672.1. Score = 86.1 bits (46), expect = 1e-21, identities = 49/50 (98%), gaps = 1/50 (2%), strand = plus/plus. (**B**) TtG2 fragment shows significant homology with EST from TRR-184 TR7RS *Triticum turgidum* subsp. *dicoccoides* cDNA, mRNA sequence after 5 weeks of germination under drought stress with accession number FL580859.1. Score = 165 bits (89), expect = 1e-45, identities = 146/173 (84%), gaps = 7/173 (4%), strand = plus/plus. (**C**) TtG3 fragment shows significant homology with EST from ala29 high-salt treated *Aeluropus lagopoides* library, *A. lagopoides* cDNA clone APX similar to *Zea mays* cytosolic ascorbate peroxidase APx1, mRNA sequence with accession number GT734398.1. Score = 156 bits (84), expect = 2e-42, identities = 84/84 (100%), gaps = 0/84 (0%), strand = plus/plus. (**D**) TtG4 fragment shows significant homology with EST from wpi2s.pk001.d13 wpi2s *Triticum aestivum* cDNA clone wpi2s.pk001.d13 5-end, mRNA sequence with accession number CA737380.1 after 48 h of infection with *Septoria tritici* strain. Score = 128 bits (69), expect = 3e-34, identities = 187/242 (77%), gaps = 18/242 (7%), strand = plus/minus. (**E**) TtG5 fragment shows significant homology with EST from 20-day-old AJ615235 *Triticum turgidum* subsp. *durum* etiolated seedling *Triticum turgidum* subsp. durum cDNA clone 11346R, mRNA sequence with accession number AJ615235.1. Score = 219 bits (118), expect = 2e-61, identities = 163/184 (89%), gaps = 5/184 (3%), strand = plus/minus. (**F**) TtG6 fragment shows significant homology with EST from FDR-173 finger millet drought stress cDNA library, *Eleusine coracana* cDNA similar to nodulation protein nolXb, mRNA sequence with accession number EB643416.1. Score = 128 bits (69), expect = 4e-35, identities = 69/69 (100%), gaps = 0/69 (0%), strand = plus/plus. (**G**) TtG7 fragment shows significant homology with EST from AhDS10 groundnut drought stressed subtracted cDNA library *Arachis hypogaea* cDNA similar to AhDS transacting *trans* protein ICP0, mRNA sequence with accession number EC268409.1. Score = 152 bits (82), expect = 4e-42, identities = 87/89 (98%), gaps = 1/89 (1%), strand = plus/plus. (**H**) TtG8 fragment shows significant homology with FMSS-301 finger millet salt stress cDNA library *Eleusine coracana* cDNA similar to transcription factor JUN-D, mRNA sequence with accession number EB739737.1. Score = 152 bits (82), expect = 3e-41, identities = 88/91 (97%), gaps = 2/91 (2%), strand = plus/plus. (**I**) TtG9 fragment shows significant homology with EST from MDS 78 mulberry S-13 dehydration stress subtracted cDNA library, *Morus indica* cDNA similar to ATP phosphoribosyltransferase regulatory subunit, mRNA sequence with accession number EL773143.1. Score = 130 bits (70), expect = 2e-35, identities = 74/76 (97%), gaps = 0/76 (0%), strand = plus/plus. (**J**) TtG10 fragment shows significant homology with EST from AhDS27 groundnut drought stressed subtracted cDNA library *Arachis hypogaea* cDNA similar to sucrose nonfermenting protein 2-like 1, mRNA sequence with accession number EC268425.1. Score = 52.8 bits (28), expect = 3e-12, identities = 28/28 (100%), gaps = 0/28 (0%), strand = plus/minus. (**K**) TtG11 fragment shows significant homology with EST from AhDSa241 groundnut drought stressed subtracted sdapter ligation cDNA library, *Arachis hypogaea* cDNA similar to tyrosine 3-monooxygenase, mRNA sequence with accession number EC366515.1. Score = 73.1 bits (39), expect = 3e-18, identities = 39/39 (100%), gaps = 0/39 (0%), strand = plus/plus. (**L**) TtG12 fragment shows significant homology with EST from AL816106 j:122 *Triticum aestivum* cDNA clone G06_j122_plate_14, 14 days post anthesis. mRNA sequence with accession number AL816106.1. Score = 148 bits (80), expect = 1e-40, identities = 125/146 (86%), gaps = 4/146 (4%), strand = plus/plus. (**M**) TtG13 fragment shows significant homology with EST from EBCG1181 fingermillet, Fermentas *Eleusine coracana* cDNA similar to MAPKKK, mRNA sequence with accession number EV473053.1. Score = 152 bits (82), expect = 2e-41, identities = 83/84 (899%), gaps = 0/84 (0%), strand = plus/plus. (**N**) TtG14 fragment shows significant homology with EST from EST00018 wheat PI137739 induced cDNA library *Triticum aestivum* cDNA clone ABO00020 3-, mRNA sequence 5 days post-aphid infection with accession number BU808666.1. Score = 97.1 bits (52), expect = 6e-25, identities = 55/56 (98%), gaps = 1/56 (2%), strand = plus/plus. (**O**) TtG15 fragment shows significant homology with EST from MS22 mango fruit tissue express library *Mangifera indica* cDNA, mRNA sequence with accession number CD002004.1 which represent partial catalase gene. Score = 93.5 bits (50), expect = 2e-24, identities = 68/78 (87%), gaps = 4/78 (5%), strand = plus/plus.

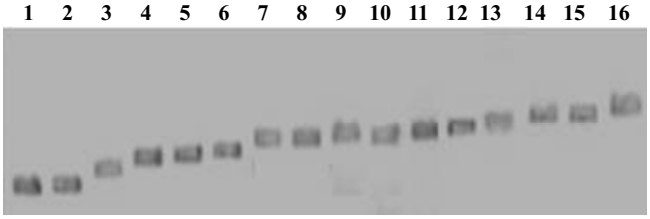

Fig. 3 Agarose gel of PCR products for the differentially displayed fragments re-amplified with T11G and AP1 primers. Lanes 1-15 represent the amplified fragments TtG15, TtG14, TtG13, TtG12, TtG11, TtG10, TtG9, TtG8, TtG7, TtG6, TtG5, TtG4, TtG3, TtG2 and TtG1, respectively.

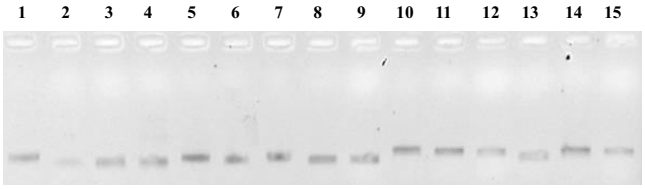

Fig. 4 Agarose gel of PCR products using different white clones resulted from the transformation of the re-amplification products. Lanes 1-15 represent the amplified fragments TtG10, TtG13, TtG14, TtG15, TtG7, TtG8, TtG9, TtG11, TtG12, TtG1, TtG2, TtG3, TtG6, TtG4 and TtG5, respectively.

drought treatment and/or the length of the treatment. In order to facilitate the subsequent dealing with DD-fragments, a specific nomenclature was adopted based on *Triticum turgidum* (Tt), the primers used in the amplification, and the band number on the gel (**Fig. 2**). For example, DD-fragment TtG1 refers to band number 1 amplified using anchor primer T11G.

Isolation of DD-fragments and re-amplification

The obtained fragments were scored (**Table 1**) depending on their pattern of expression across the four drought treatment time intervals (control, 1, 3, 6 and 10 h). Bands of interest were separately excised from the gel and cDNA products were purified from the gel for subsequent molecular analysis. Selected cDNAs were used in PCR reactions to re-amplify the products using the same primer set and PCR conditions except for the concentration of dNTPs (all 12 ρmole/µl). The PCR products were identified by electrophoresis on a 1.2% agarose gel (**Fig. 3**).

Screening and confirmation of colonies

Screening revealed two types of colonies, namely white (with recombinant plasmids) and blue (negative without plasmids). Only white colonies were used for the verification of the recombinant plasmid DD-fragments (**Fig. 4**).

DD-fragments sequences and sequence analysis

The sequences and BLASTN analysis of the 15 DD-fragments are shown in Table 1A-O. There are 4 major categories of genes encompassing 47% of the gene products that are related to drought, 13% for salt stress, 20% for biotic infection while 20% were considered to be unassigned or unknown with no similarity to any know sequence.

DISCUSSION

Egyptian durum wheat (var. 'Sohag 3'), presumably containing genes of resistance to drought (Diab *et al*. 2007), was profiled for gene expression under different drought stress periods. Upon sequencing and quality check performance of the differentially expressed sequences, 15 dESTs were identified. The 15 clones were classified into four different functional categories based on their putative function predicted from the BLAST output.

The TtG1 fragment, which appeared in all drought treat-

Table 2 Expression patterns of the differentially displayed amplified cDNA fragments.

DD-fragment		Treatments				
Size	**Name**	**Control**	**1 h**	**3 h**	**6 h**	**10 h**
400 bp	TtG1	-	++	++	++	++
360 bp	TtG2	++	-	-	-	-
350 bp	TtG3	-	-	-	++	++
320 bp	TtG4	-	-	-	++	++
290 bp	TtG5	++	-	-	-	-
270 bp	TtG6	++	-	-	-	-
260 bp	TtG7	+	++	n	++++	++++
255 bp	TtG8	+	++	n	++++	++++
220 bp	TtG9	-	++++	n	++	+
190 bp	TtG10	-	-	-	++	++
182 bp	TtG11	-	++	n	++	++
180 bp	TtG12	++	-	-	-	-
177 bp	TtG13	-	++	n	++++	++++
175 bp	TtG14	-	++	n	++	++
169 bp	TtG15	-	++	n	++	++

(+) Expression level of DD-fragment, (-) Absence of DD-fragment, (n) pure amplification

ments but not in the control plants (**Fig. 2; Table 2**), was similar to the Zinc finger FYVE domain-containing protein (**Table 1A**) which is present in a range of eukaryotic proteins involved in membrane trafficking and phosphoinositide metabolism (Stenmark *et al*. 1996). Geetha *et al*. (2009) reported that several zinc finger protein transcripts are preferentially induced under drought in peanut (*Arachis hypogaea*).

The TtG2 fragment, which disappeared after 1 h of drought treatment (**Fig. 2; Table 2**), showed significant homology with an EST from *Triticum turgidum* cDNA (accession number FL580859.1). This sequence was isolated after 5 weeks of germination under drought stress and is classified as unknown gene function (**Table 1B**). Ergen and Budak (2009) identified a similar sequence (84%) using suppression-subtractive hybridization from contrasting wild emmer wheat in response to drought stress. The results obtained in this study suggest that this fragment might have a down regulation effect on durum wheat under drought stress. This fragment might be of interest if the function is known, although a further study is needed to determine the function of this fragment.

The TtG3 fragment appeared in the 6 and 10 h drought treatments but did not appear in any other treatment nor in the control plants (**Fig. 2; Table 2**). This fragment showed significant homology with *Zea mays* cytosolic Ascorbate Peroxidase (APX) APX1, whose mRNA sequence has accession number GT734398.1 (**Table 1C**). APXs are widespread but are mainly found in green algae and higher plants (Marcel *et al*. 2010). APX catalyzes the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor. Previously, APX was thought to play the most essential role in protecting cells in higher plants. Enhanced expression of APX in plants has been demonstrated during different stress conditions (Sarvajeet and Narendra 2010). Koussevitzky *et al*. (2008) suggested that cytosolic APX1 plays a key role in protecting plants against a combination of drought and heat stresses. These findings support our result, especially that the TtG3 fragment was found only after 6 and 10 h of drought stress, which indicates that this expressed sequence has a vital role in protecting durum wheat against severe drought stress.

The TtG4 fragment started to appear after 6-10 h of drought but did not appear in any other treatment nor in the control plants (**Fig. 2; Table 2**). This fragment showed significant homology with an EST from *T. aestivum* cDNA clone wpi2s.pk001.d13 5-end, whose mRNA sequence has accession number CA737380.1 after 48 h of infection with a *Septoria tritici* strain (**Table 1D**). These results suggest that the TtG4 fragment obtained in this work is expressed under drought stress, an abiotic stress, and has a similar sequence to an EST expressed after the infection with *Septoria tritici*, a biotic stress. Plants have evolved a wide range of mechanisms to cope with biotic and abiotic stresses. To date, the molecular mechanisms that are involved in each stress has been revealed comparatively independently, and so our understanding of convergence points between biotic and abiotic stress signaling pathways remain rudimentary. However, recent studies have revealed several molecules, including transcription factors and kinases, as promising candidates for common players that are involved in crosstalk between stress signaling pathways (des Marais and Juenger 2010). Emerging evidence suggests that hormone signaling pathways regulated by abscisic acid, salicylic acid, jasmonic acid and ethylene play key roles in the crosstalk between biotic and abiotic stress signaling (Fujita *et al*. 2006).

The TtG5 fragment appeared in all drought treatments (**Fig. 2; Table 2**). This fragment showed significant homology with and EST from AJ615235 *T. turgidum* cDNA clone 11346R, whose mRNA sequence has accession number AJ615235.1 but is classified as having an unknown gene function (**Table 1E**). The fragments that showed an unknown gene function could be used as markers for genetic mapping and correlation studies with QTL analysis under drought stress to predict and assign a tentative function for these fragments. However, more research is needed to identify the functions of those ESTs and to validate their role.

The TtG6 fragment showed low expression with after drought treatment for 1 h (**Fig. 2**). It showed significant homology with finger millet (*Eleusine coracana*) drought stress similar to Nodulation protein nolXb from this legume, and has an mRNA sequence with accession number EB643416.1 (**Table 1F**). Nodule formation in the *Rhizobium*–legume symbiosis is a multi-step process resulting from a complex interaction between the two partners (Stacey *et al*. 2006). Perception of *Rhizobium*-induced nodulation (Nod) factor by the host plant leads to a cascade of responses, which include root hair swelling, calcium oscillation, root hair curling, bacterial colonisation, infection thread differentiation and nodule primordial development (Kinkema *et al*. 2006).

The TtG7 fragment appeared in all drought treatments (**Fig. 2; Table 2**). This fragment showed significant similarity to groundnut drought stressed subtracted cDNA library *Arachis hypogaea* cDNA similar to AhDS transacting *trans* protein ICP0 with accession number EC268409.1 (**Table 1G**). Govind *et al.* (2009) identified key transcription factor-related genes (AhDS) differentially expressed under different water deficit conditions from a relatively drought-tolerant crop, peanut. It is presumed that the genes expressed during the course of gradual stress in tolerant species are responsible for altering the cellular metabolism, leading to adaptation under severe stress. Among the well known examples are genes encoding proteins related to protection of cellular structures and denaturation of proteins and enzymes. These results suggest that this fragment is involved in altering the cellular metabolism in a way to protect plants from water deficiency. However, more research is needed to identify functions for those ESTs and to validate their role.

The TtG8 fragment appeared after 1 h of drought treatment, and continued to appear in all drought treatments (**Fig. 2; Table 2**). This fragment showed significant homology with FMSS-301 finger millet salt stress cDNA library for *Eleusine coracana*, having a cDNA similar to transcription factor JUN-D, and whose mRNA sequence has accession number EB739737.1 (**Table 1H**). The transcription factor JUN-D is reported to have no *trans*-activation potential and is known for its negative regulatory function in cellular proliferation (Meixner *et al*. 2004). The results suggested that this fragment (TtG8) has a role in protecting plants against salt and drought stress. Salt and drought stress are related and some plants have the same defense mechanism of signal transduction that consists of ionic and osmotic homeostasis signaling pathways (Jian 2002).

The TtG9 fragment was expressed in all drought treat-

ments (**Fig. 2; Table 2**). The fragment showed significant homology to ATP-phosphoribosyltransferase (ATP-PRT) regulatory subunit, whose mRNA sequence has accession number EL773143.1 (**Table 1I**). ATP-PRT is a complex allosterically regulated enzyme, which controls the flow of intermediates through the histidine biosynthetic pathway. It is the first enzyme of this biosynthetic pathway and catalyses the condensation of ATP and 5-phosphoribosyl-a-1-pyrophosphate to form phosphoribosyl-ATP (Mousdale and Coggins 1991). This fragment appears to have an essential role in drought tolerance and is a very promising EST for drought tolerance in durum wheat.

The TtG10 fragment showed over expression after 1 h of drought treatment (**Fig. 2; Table 2**). This fragment showed significant homology with groundnut drought-stressed sequence similar to sucrose non-fermenting protein (SNF1), whose mRNA sequence has accession number EC268425.1 (**Table 1J**). SNF1 is a family of protein kinases that play diverse roles ranging from energy metabolism to transcriptional control in stress and energy signaling (Baena-González *et al*. 2007), hormone signaling (Shukla and Matto 2008), ABA signaling (Chae *et al*. 2007), abiotic stress response (Umezawa *et al*. 2004), and plant defense against pathogens and insects (Schwachtje *et al*. 2006). This result suggests that this fragment (TtG10) has a multifunctional role in plants. It is a very promising EST, especially for transgenic research to develop plants resistance to abiotic and biotic stresses.

The TtG11 fragment appeared in the control plants and all drought treatments and showed over expression pattern in all drought treatments (**Fig. 2; Table 2**). This fragment showed significant homology to tyrosine 3-monooxygenase, an mRNA sequence with accession number EC366515.1 (**Table 1K**). Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to DOPA, which is the initial and rate-limiting step in the biosynthesis of dopamine, norepinephrine, and epinephrine. The activity of TH can be regulated by two mechanisms: short-term direct regulation of enzyme activity and medium- to long-term regulation of gene expression (Fujisawa and Okuno 2005).

The TtG12 fragment showed over expression in all drought treatments (**Fig. 2; Table 2**). This fragment showed significant homology with *Triticum aestivum* cDNA clone G06_j122_plate_14, whose mRNA sequence of 14 days post anthesis was classified as having an unknown gene function with accession number AL816106.1 (**Table 1L**). This fragment might be very interesting, especially for QTL mapping and correlation studies to determine a tentative function. However, reverse genetic and transgenic approaches could also help to determine the function of this fragment and in explaining why this fragment is expressed under drought stress.

The TtG13 fragment showed over expression after 1 h of drought stress (**Fig. 2; Table 2**). This fragment showed significant homology to MAPK kinase, whose mRNA sequence has accession number EV473053.1 (**Table 1M**). In plants, MAPK pathways are involved in the regulation of development, growth, programmed cell death and in responses to a diversity of environmental stimuli including cold, heat, reactive oxygen species, UV, drought and pathogen attack (Colcombet and Hirt 2008). Via a phosphorylation mechanism, these cascades are minimally composed of a MAPKKK (MAPK kinase kinase), a MAPKK (MAPK kinase) and a MAPK, link upstream receptors to downstream targets. In previous studies the best-characterized MAPKs were MPK3, MPK4 and MPK6. While MPK4 negatively regulates biotic stress signalling (Pitzschke *et al*. 2009), MPK3 and MPK6 are key players in stomatal development and stomatal dynamics (Yoo *et al*. 2008).

In a pattern of low expression pattern after one hour of drought treatment, the TtG14 fragment was significantly low in its expression (**Fig. 2; Table 2**). This fragment showed significant homology with a cDNA library from *Triticum aestivum* (clone ABO000203). This sequence was isolated after 5 days of post aphid infection and classified as unknown gene function with accession number BU808666.1 (**Table 1N**). This fragment has unknown roles in durum wheat subjected to drought stress. More studies are needed to determine the role and function of this EST under drought stress.

The TtG15 fragment appeared in all drought treatments but did not appear in the control plants (**Fig. 2; Table 2**). This fragment showed significant homology with an EST from mango (*Mangifera indica*) fruit tissue express library cDNA, whose mRNA sequence has accession number CD002004.1, which represents partial catalase (CAT) gene (**Table 1O**). CAT is an iron porphyrin enzyme that catalyzes the dismutation of H_2O_2:H_2O_2 to water and dioxygen and serves as an efficient scavenger of reactive oxygen species (ROS), preventing cellular damage (Munkhbaatar *et al*. 2010). As a part of the antioxidant response system, CAT plays a role in maintaining the redox homeostasis of the cell as one of the antioxidant defense genes that respond to environmental as well as physiological oxidative stress (Munkhbaatar *et al*. 2010). It is clear that this fragment has a very significant role in protecting durum wheat against drought stress.

In summary, our study suggests that the data mining of EST databases is a feasible way to generate informative molecular markers for genetic studies, which would offer an opportunity to identify new PCR-based EST-SSR markers for durum wheat and other related species. These sequences may provide an estimate of diversity in the expressed portion of the genome and may be useful for comparative mapping, for tagging important genes for drought response, and for integrating a physical and functional genome for additional map-based cloning of important genes. This study might also help in understanding the mechanism of defense in durum wheat against drought stress.

REFERENCES

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402

Amin A (2002) Markers for quantitative trait analysis and bulk segregant analysis of salt tolerance in wheat (*Triticum aestivum* L.). PhD thesis, University of East Anglia, UK, 240 pp

Amudha J, Balasubramani G (2011) Recent molecular advances to combat abiotic stress tolerance in crop plants. *Biotechnology and Molecular Biology Reviews* **6 (2)**, 31-58

Baena-González E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. *Nature* **42**, 448-938

Bray EA, Bailey SJ, Weretilnyk E (2000) Responses to abiotic stresses. In: Gruissem W, Buchannan B, Jones R (Eds) *Biochemistry and Molecular Biology of Plants*, American Society of Plant Physiologists, Rockville, MD, pp 1158-1249

Cellier F, Conéjéro G, Breitler JC, Casse F (1998) Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. *Plant Physiology* **116**, 319-328

Chao S, Lazo GR, You F, Crossman CC, Hummel DD, Lui N, Laudencia-Chingcuanco D, Anderson JA, Close TJ, Dubcovsky J, Gill BS, Gill KS, Gustafson JP, Kianian SF, Lapitan NLV, Nguyen HT, Sorrells ME, McGuire PE, Qualset CO, Anderson OD (2006) Use of a large-scale Triticeae expressed sequence tag resource to reveal gene expression profiles in hexaploid wheat (*Triticum aestivum* L.). *Genome* **49**, 531-544

Chae MJ, Lee JS, Nam MH, Cho K, Hong JY, Yi SA, Su SC, Yoon IS (2007) A rice dehydration-inducible SNF1-related 2 phosphorylates an abscisic acid responsive factor and associates with ABA signaling. *Plant Molecular Biology* **63**, 151-169

Close TJ (1997) Dehydrins: A commonalty in the response of plants to dehydration and low temperature. *Physiologia Plantarum* **100**, 291-296

Close TJ, Fenton RD, Moonan F (1993) A view of plant dehydrins using antibodies specific to the carboxy-terminal peptide. *Plant Molecular Biology* **23**, 279-286

Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Molecular Biology* **13**, 95-108

Colcombet J, Hirt H (2008) *Arabidopsis* MAPKs. A complex signaling network involved in multiple biological processes. *Biochemistry Journal* **413**, 217-226

des Marais DL, Juenger TE (2010) Pleiotropy, plasticity, and the evolution of plant biotic stress tolerance. *Annals of the New York Academy of Sciences*

1206, 56-79

Diab AA, Ageez A, Abdelgawad BA, Salem TZ (2007) Cloning, sequence analysis and *in-silico* mapping of a drought-inducible gene coding for *S*-adenosylmethionine decarboxylase from durum wheat. *World Applied Sciences Journal* **2 (4)**, 333-340

Diab AA, Ozturk NZ, Benscher D, Nachit MM, Sorrells ME (2008) Drought-inducible genes and differentially expressed sequence tags associated with components of drought tolerance in durum wheat. *Scientific Research and Essays* **3**, 9-26

Ergen NZ, Budak H (2009) Sequencing over 13000 expressed sequence tags from six subtractive cDNA libraries of wild and modern wheats following slow drought stress. *Plant Cell and Environment* **32**, 220-236

Fujisawa H, Okuno S (2005) Regulatory mechanism of tyrosine hydroxylase activity. *Biochemical and Biophysical Research Communications* **338**, 271-276

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi SK, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: A current view from the points of convergence in the stress signaling networks. *Current Opinion in Plant Biology* **9 (4)**, 436-442

Gadaleta A, Mastrangelo AM, Russo MA, Giove SL, D'onofrio O, Mango T, Cellini F, Blanco A, Cattivelli L, Cifarelli RA (2010) Development and characterization of EST-derived SSRs from a 'totipotent' cDNA library of durum wheat. *Plant Breeding* **129 (6)**, 715-717

Govind G, Harshavardhan V, Thamme G, Kalaiarasi PJ, Iyer DR, Muthappa SK, Nese S, Makarla UK (2009) Identification and functional validation of a unique set of drought induced genes preferentially expressed in response to gradual water stress in peanut. *Molecular Genetic and Genomics* **281**, 591-605

Jian KZ (2002) Salt and drought stress signal transduction. *Annual Review of Plant Biology* **53**, 247-273

Kinkema M, Scott PT, Gresshoff PM (2006) Legume nodulation. Successful symbiosis through short- and long-distance signaling. *Functional Plant Biology* **33**, 707-721

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R (2008) ASCORBATE PEROXIDASE 1 plays a key role in the response of *Arabidopsis thaliana* to stress combination. *Journal of Biological Chemistry* **283**, 34197-34203

Lazo GR, Chao S, Hummel DD, Edwards H, Crossman CC, Lui N (2004) Development of an expressed sequence tag (EST) resource for wheat (*Triticum aestivum* L.), EST generation, unigene analysis, probe selection, and bioinformatics for a 16,000-locus bin-delineated map. *Genetics* **168**, 585-593

MacKey J (2005) Wheat, its concept, evolution and taxonomy. In: Royo C, Nachit MM, Di Fonzo N, Araus JL, Pfeiffer WH, Slafer GA (Eds) *Durum Wheat Breeding: Current Approaches and Future Strategies*, Food Products Press, New York, pp 3-61

MacCaferri M, Sanguineti MC, Donini P, Tuberosa R (2003) Microsatellite analysis reveals a progressive widening of the genetic basis in the elite durum wheat germplasm. *Theoretical and Applied Genetics* **107**, 783-797

Marcel Z, Furtmüller P, Obinger C (2010) Evolution of structure and function of class I peroxidases. *Archives of Biochemistry and Biophysics* **500**, 45-57

Meixner A, Karreth F, Kenner L, Wagner EF (2004) *JunD* regulates lymphocyte proliferation and T helper cell cytokine expression. *EMBO Journal* **23**, 1325-1335

Moragues M, Moralejo MA, Sorrells ME, Royo C (2007) Dispersal of durum wheat landraces across the Mediterranean basin assessed by AFLPs and microsatellites. *Genetic Resources and Crop Evolution* **54**, 1133-1144

Mousdale DM, Coggins JR (1991) Amino acid synthesis. In: Berger P, Sandmann G (Eds) *Target Sites for Herbicide Action*, Plenum Press, New York, pp 29-56

Munkhbaatar P, Ho THD, Myung KK, Pulla RK, Yang D (2010) Isolation of a novel catalase (*Cat1*) gene from *Panax ginseng* and analysis of the response of this gene to various stresses. *Plant Physiology and Biochemistry* **48**, 451-460

Neslihan ZE, Hikmet B (2009) Sequencing over 13000 expressed sequence tags from six subtractive cDNA libraries of wild and modern wheats following slow drought stress. *Plant, Cell and Environment* **32 (3)**, 220-236

Nouri A, Etminan A, Teixeira da Silva JA, Mohammadi R (2011) Assessment of yield, yield related traits and drought tolerance of durum wheat genotypes (*Triticum turjidum* var. *durum* Desf.). *Australian Journal of Crop Science* **5 (1)**, 8-16

Oztur ZN, Talamé V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology* **48**, 551-573

Pitzschke A, Adam S, Heribert H (2009) MAPK cascade signaling networks in plant defense. *Current Opinion in Plant Biology* **12**, 421-426

Sarvajeet SG, Narendra T (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* **8**, 10-16

Syed SH (2006) Molecular breeding for abiotic stress tolerance: Drought perspective. *Proceedings of the Pakistan Academy of Sciences* **43 (3)**, 189-210

Schwachtje J, Minchin PE, Jahnke S, van Dongen J, Schittko U, Baldwin IT (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proceedings of the National Academy of Sciences USA* **103**, 12935-12940

Shukla V, Matto AK (2008) Sucrose non-fermenting 1-related protein kinase 2 (SnRK2): A family of protein kinases involved in hyperosmotic stress signaling. *Physiology and Molecular Biology of Plants* **14**, 91-100

Stacey G, Libault M, Brechenmacher L, Wan J, May GD (2006) Genetics and functional genomics of legume nodulation. *Current Opinion in Plant Biology* **9**, 110-121

Stenmark H, Aasland R, Toh BH, D'Arrigo A (1996) Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *Journal of Biological Chemistry* **271**, 24048-24054.

Umezawa T, Yoshida R, Maruyama M, Yamaguchi-Shinozaki K, Shinozaki K (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress responsive gene expression in *Arabidopsis thaliana. Proceedings of the National Academy of Sciences USA* **101**, 17306-17311

Xu D, Duan X, Wang B, Hong B, Ho THD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiology* **110**, 249-257

Yoo SD, Cho YH, Tena G, Xiong Y, Sheen J (2008) Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4 signaling. *Nature* **451**, 789-795

Zhang L, Ma XL, Zhang Q, Ma CL, Wang PP, Sun YF, Zhao YX, Zhang H (2001) Expressed sequence tags from a NaCl-treated *Suaeda salsa* cDNA library. *Gene* **267**, 193-200