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DIFFERENTIAL GENE EXPRESSION ANALYSIS IN *Vicia monantha* UNDER DROUGHT STRESS CONDITIONS

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In Egypt and many countries of WANA (West Africa, North Africa), broad bean or faba bean (*Vicia faba*), is considered the most important food legume; consumed green or dried, fresh or canned, as well as for animal feed (Bond *et al.*, 1985). Furthermore, feeding value of faba bean is high, and is considered in some areas to be superior to field peas or other legumes. It is one of the most important winter crops for human consumption in the Middle East (Duke, 1981).

Different wild *Vicia* species from the Northwest coastal region of Egypt have been identified (Atlas of Legume Plant of the North West of Egypt, 1993). *Vicia monantha* is a member of the family Fabaceae, belonging to the genus *Vicia*. It is a wild plant species that is native to the Northern African region, mainly Algeria and Egypt. It is characterized by its obvious withstanding to severe environmental conditions. Such wild plants are considered as an excellent source of stress-related genes awaiting their isolation and identification.

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982). Drought and salinity lead to inability of plants to acquire their water needs, resulting in loss of turgor and/or osmotic stress. At the molecular level, osmotic stress will trigger cascades of signals involving Ca^{++} and reactive oxygen molecules as primarily signals to activate pathways critical for plant survival under the stress conditions (Knight *et al*., 1997; Knight and Knight, 2001). At the cellular level, responses include metabolic adjustment to produce compatible solutes (Cherry, 1989), activation of transporters at the plasma and vacuolar membranes for ion sequestration or exclusion (Blumwald and Poole, 1985; Shi *et al.,* 2000) and activation of enzymes involved in detoxification of free radicals (Mittova *et al*., 2002; Bor *et al*., 2003; Mittova *et al*., 2004; Badawi *et al*., 2004). At the whole plant-level, responses include closure of plant stomatal apparatus coupled with an inhibition of vegetative growth and increase in root growth (Maggio *et al*., 2003).

Cloning and characterization of environmental stress-induced genes offer a chance for understanding the physiological responses of the plant cell to the environmental stresses. Moreover, it produces a source of genes for producing transgenic plants tolerant to abiotic stress. Differential display-polymerase chain reaction (DD- PCR) (Liang and Pardee, 1992) is a simple, powerful and sensitive method for the isolation of differentially expressed genes. It has been useful in characterizing and cloning of expressed sequence tags (EST) preferentially expressed in different tissues and/or under different conditions (Cushman and Bohnert, 2000; Martin-Laurent *et al.,* 1997; Roux and Perrot-Rechenmann, 1997; Visioli *et al*., 1997; Deleu *et al.,* 1999; Wei *et al*., 2000; Zhang *et al*., 2005; Yong *et al*., 2007; Yu *et al.,* 2006; Perumal Venkatachalam *et al*., 2009).

In this study, *Vicia monantha* was collected from Marsa Matrouh in the North coast of Egypt. In a previous study, the plant was subjected to salinity stress and a total of fifty three new EST was identified (Abd El-maksoud *et al*., 2009). In the current study, *Vicia monantha* was subjected to drought stress to isolate and characterize some of the key expressed sequence tags (ESTs) in response to drought stress using the differential - display technique.

MATERIALS AND METHODS

Plant material and drought experiment

Vicia monantha seeds were collected from the North coast of Egypt 13 km East of El-Dabah, Marsa Matrouh province. The seeds were stored under accession number 64211 at the International Center for Agricultural Research in the Dry Areas (ICARDA), Syria.

Seeds of *Vicia monantha* were germinated in greenhouse conditions at 22-24°C and 80% relative humidity, and watered day by day with tap water. After 6 weeks the plants were collected very carefully and dehydrated on Whatman 3-mm filter paper at room temperature and approximately 60% humidity under dim light according to Park *et al*. (2003). The degree of water stress was determined at different time points, i.e., at 0, 1, 4, 6 and 8 hours and the decrease in fresh weight was measured.

RNA extraction and cDNA synthesis

Total RNAs were extracted from 700 mg of the harvested untreated and drought-treated seedlings according to the procedure of Chomczynski (1993), using the TriPure isolation reagent (Roche Molecular Biochemicals, Germany). First and second cDNA strands were synthesized using Improm™ Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

Differential display analysis

Differential display was carried out according to Liang and Pardee (1992) with some modifications, where PCR amplification of cDNA was carried out by GenHunterRNAimage kit according to the manufacturer's instructions with the anchor primers (T11G- 5'- TTT TTT TTT TTG -3', T11A- 5'- TTT TTT TTT TTA-3) in combination with the arbitrary primer (AP5- 5'- AAG CTT AGT AGG C -3'). Supertherm gold DNA polymerase Taq (Hoffmann-La-Roche) was used for amplification. Separation of amplified fragments was carried out on 6% denaturing polyacrylamide gels using Sequi-Gen® Sequencing Cell (Bio-Rad Laboratories, Hercules, California, USA). The gels were silver stained using the silver sequence kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions.

Isolation and reamplification of cDNA fragments

Sterile scalpel blades were used to cut the desired bands from the gel. Gel slices were incubated in 50 µl dd H_2O at 65°C for 30 min, and then left at room temperature for elution. Three µl of the aliquot were used for re-amplification in a total volume of 25 µl, using the same set of corresponding primers. The reactions of PCR and the re-PCR for the selected DD fragments were carried out in a GeneAmp® PCR System 9700 instrument, programmed for 94°C for 1 min (1 cycle); 94°C for 30 sec, 38°C for 2 min, 72°C for 30 sec (40 cycles); 72°C for 5 min (1 cycle), then held at 4°C.

Cloning and sequencing of cDNA fragments

The reamplified DD fragments were transformed into pGEM-T Easy vector system (Promega, Madison, WS, USA) using *E. coli* DH5α competent cells strain according to Sambrook *et al*. (2001). After transformation, all cells were spread on LB plates containing ampicilin with IPTG and X-Gal and, incubated overnight at 37°C. Competent cells transformed with pGEM-T easy vector were detected by blue/white colony screening.

The DNA sequence was determined by automated DNA sequencing method. The automated DNA sequencing reactions were performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the GeneAmp® PCR System 9700 instrument. The cycle sequencing program was set at 96°C for 2 min (1 cycle); (96°C for 10 sec, 50°C for 10 sec, and 60°C for 4 min, repeated for 25 cycles); 60°C for 2 min (1 cycle), with rapid thermal ramping. The data were provided as fluorometric scans from which the sequence was assembled using the sequence analysis software (Sequencher version 4.1.4).

Sequence analysis

Sequences were analyzed using Blast programs of the National Center for Biotechlogy (NCBI), USA [www.ncbi.nlm.nih.gov/Blast].

RESULTS AND DISCUSSION

Expression pattern of DD-cDNA transcripts

Differential display technique was carried out, to isolate novel EST, responsible for drought tolerance in *Vicia monantha* seedlings. *Vicia monantha* was selected as it can grow in severe environment in the North coast of Egypt. Drought was induced by harvesting six-weeks plants and placing them on filter paper as described by Gao *et al*. (2008). The degree of water stress was determined at different time points, at 0, 1, 4, 6 and 8 hours. The decrease in fresh weight of *Vicia monantha* seedlings at 1, 4, 6 and 8 hours, was 30%, 42%, 50%, and 56%, respectively.

Differential display-PCR was performed according to Gao *et al*. (2008). The amplified products were analyzed on 6% urea polyacrylamide sequencing gels (Fig. 1). A number of 25 fragments differentially expressed due to drought treatment were successfully identified. The isolated ESTs were named Vmd1 to Vmd25. The ESTs were submitted to GenBank under accession number from AB602400 to AB602424.

Sequence analysis of DD- fragments

The isolated expressed sequence tags were classified according to their time of expression (Table 1). In response to the drought stress, five cDNA fragments (Vmd1, Vmd4, Vmd7, Vmd12 and Vmd15) were induced only at one hour after the drought stress. Three cDNA fragments (Vmd2, Vmd9, and Vmd25) were induced only at four hours after the drought stress. Two cDNA fragments (Vmd11 and Vmd24) were induced only at six hours after the drought stress. Eight cDNA fragments (Vmd5, Vmd10, Vmd13, Vmd14, Vmd17, Vmd20, Vmd22 and Vmd23) were induced only at eight hours after the drought stress. Vmd19 was expressed only in the control one and four hours of the induced stress. Vmd16 was expressed in the control, four hours and six hours of the drought stress. Vmd3 was expressed at four hour and six hours of the drought stress. Vmd21 was expressed at one, four and six hour of the induced stress. Two fragments (Vmd6 and Vmd8) were expressed at the control only and showed no expression due to drought stress. On the other hand, one fragment (Vmd18) was showed no expression in the control and expressed due to the induced drought stress at all time points.

The isolated fragments were analyzed using Blast programs of the National Center for Biotechnology (NCBI) (Table 2). Scanning of Vmd2, Vmd3, Vmd8, Vmd13, Vmd17, Vmd19, Vmd21, Vmd22, Vmd23 and Vmd24 cDNA fragments in the gene bank showed no significant homology in BlastN.

The Vmd5 showed significant homology with expressed sequence tags of *Medicago truncatula* cDNA. The Vmd6 showed significant homology with expressed sequence tags of *Medicago truncatula* cDNA under fungus infection.

Two cDNA fragments, Vmd10 and Vmd 11, showed similarity with *Lotus japonicus* cDNA clone (LjFL3-033-AB04.r0). The Vmd14 showed significant homology with expressed sequence tags of *Pisum sativum* clone no. (MERIZ0012I19.B). The Vmd 25 showed significant homology with expressed sequence tags from *Populus trichocarpa* cDNA clone (WS01211).

After analysis by BlastN alignment, Vmd1 and Vmd12 showed significant homology with expressed sequence tags from drought stressed Cowpea. The Vmd20 showed significant homology with expressed sequence tags from *Brassica napus* root under drought stress.

The Vmd4, Vmd16 and Vmd18 showed significant homology with expressed sequence tags from *Beta vulgaris* under salt stress (El-Zohairy *et al*., 2009). The isolated EST was identified in our genomics laboratory at AGERI, from *Beta vulgaris* seedlings under 250 mM NaCl stress.

The components of drought and salt stress cross talk with each other as both these stresses ultimately result in dehydration of the cell and osmotic imbalance. Virtually every aspect of plants physiology as well as cellular metabolism is affected by salt and drought stress. Drought and salt signaling encompasses three important parameters: (1) reinstating osmotic as well as ionic equilibrium of the cell to maintain

Cellular homeostasis under the condition of stress, (2) control as well as repair of stress damage by detoxification signaling, (3) signaling to coordinate cell division to meet the requirements of the plant under stress (Liu and Zhu, 1998).

Vmd 9 showed significant homology with expressed sequence tags of *Brassica napus* cold acclimation.

The primary environmental factors responsible for triggering increased tolerance against freezing, is the phenomenon known as 'cold acclimation.' It is the process where certain plants increase their freezing tolerance upon prior exposure to low non-freezing temperatures. Cold acclimation results in protection and stabilization of the integrity of cellular membranes, enhancement of the antioxidative mechanisms, increased intercellular sugar levels as well as accumulation of other cryoprotectants including polyamines that protect the intracellular proteins by inducing the genes encoding molecular chaperones. All these modifications help the plant to withstand and surpass the severe dehydration associated with freezing stress. (Mahajan and Tuteja, 2005).

The Vmd7 showed significant homology with Expressed sequence tags from Groundnut drought stressed similar to serine/threonine-protein kinase. The Vmd7 was expressed only after one hour of drought stress. The first proteins expressed due to stress in plants are the signal perception and transduction proteins.

Plants react to external stimuli by initiating signaling cascade which activate the expression of appropriate responses. The signaling pathways comprise a network of protein-protein reactions and signaling molecules (for example, ROS, Ca2+ etc.).

Protein kinases such as serine/threonine-protein kinase play an important role in the signaling cascade pathway, by which the plant can regulate cellular processes in response to osmotic stress.

The mitogen-activated protein kinases (MAPK) are widely expressed serine/threonine kinases that mediate signals for the regulation of important cellular function cascades. They are composed of three functionally linked protein kinases. MAP kinase activation is regulated by upstream dual-specificity kinases, which are known as MAPK kinases (MAPKKs; also known as MKKs). The activation of MAPKKs is regulated by other upstream kinases, known as MAPKK kinases (MAPKKKs; also known as MKKKs) and requires the phosphorylation of conserved threonine and tyrosine residues in the so-called TEY (Thr, Glu, Tyr) activation loop by a specific MAPK kinase (MAPKK) (Triesmann, 1996).

In Arabidopsis, AtMEKK1 (a MAPKinase-kinase-kinase) and AtMPK3 (a MAPKinase) are activated by dehydration, touch and cold (Mizoguchi *et al.*, 1996). Jonak *et al.* (1996) reported the role of AtMPK3 during dehydration, which is transcriptionally upregulated upon drought stress. In Arabidopsis, AtMPK4 and AtMPK6 are post-translationally activated by cold, osmotic stress, and wounding (Ichimura *et al.*, 2000). Rentel *et al.* (2004) confirmed the role of OXI1, a serine/threonine kinase homologue identified as a downstream component to the AtMPK3 and AtMPK6.

The twenty five isolated and characterized cDNA fragments were deposited in the Genbank as a result of screening for drought-related genes in *Vicia monantha*. Success in isolation of these fragments opens the door to several future aspects, like: isolation of full-length genes which have important roles to help plants survive under severe stress conditions, cDNA fragments with no significant similarities or cDNA fragments with unknown function can be used to discover new genes related to the stress response mechanisms. Moreover, transfer of the isolated genes to important crops will increase the tolerance of these plants to stress. This will help enhance our national program for land reclamation, by means of increasing our cultivated area with abiotic tolerant cultivars.

SUMMARY

In this study, the Differential display reverse transcription (DDRT) technique was used to analyze differentially expressed genes in *Vicia monantha* under drought stress. Six weeks old seedlings were dehydrated for 1, 4, 6 and 8 hrs, and untreated seedlings were used as control. Twenty five differentially expressed fragments were identified and characterized. The fragments were classified according to their time of expression after the drought stress. The significance of the function of the identified differentially expressed genes was discussed in relation to their possible roles as stress genes. Ten fragments showed no significant homology with any database sequences in the GenBank. Results of the database sequence alignment identified two fragments revealing significant homology with expressed sequence tags from drought stressed Cowpea, three fragments showed significant homology with *Beta vulgaris* under salt stress. One fragment had significant homology with Expressed sequence tags of *Brassica napus* under cold stress. One fragment had significant homology with Expressed sequence tags from *Brassica napus* root under drought stress. More importantly, a fragment had significant homology with Expressed sequence tags from Groundnut drought stressed similar to serine/threonine-protein kinase. These results implicate that several pathways are involved in the plant's response to drought stress which still needs to be elucidated further.

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Table (1): Expression patterns of the differentially displayed amplified cDNA fragments.

Stage specific ESTs	ESTs (Accession No.)
ESTs expressed at 1h of treatment.	Vmd1 (AB602400),Vmd4(AB602413), Vmd7(AB602406),Vmd12(AB602411) and Vmd15(AB602414)
ESTs expressed at 4h of treatment.	Vmd2 (AB602401), Vmd9 (AB602408)and Vmd25 (AB602424)
ESTs expressed at 6h of treatment.	Vmd11 (AB602410) and Vmd24 (AB602423)
ESTs expressed at 8h of treatment.	Vmd5 (AB602404), Vmd10 (AB602409), Vmd13 (AB602412), Vmd14 (AB602413), Vmd17 (AB602416), Vmd20 (AB602419), Vmd22 (AB602421) and Vmd23 (AB602412)
ESTs expressed at control, 1h and 4h of drought treatment	Vmd19 (AB602418)
ESTs expressed at control, 4h and 6h of treatment.	Vmd16 (AB602415)
ESTs expressed at 4h and 6h of treatment.	Vmd3 (AB602402)
ESTs expressed at 1h, 4h and 6h of treatment.	Vmd21 (AB602420)
ESTs expressed at control.	Vmd6 (AB602405), Vmd8 (AB602407)
ESTs expressed drought treatment.	Vmd18 (AB602417)

Table (2): Description of DD- fragment sequences as compared to database sequences & expression patterns of differentially expressed fragments.

Fragment No.	Length (bp)	Accession No.	Homology	E-Value	Max. Ident.
Vmd1	301	AB602400	Expressed sequence tags from drought stressed Cowpea .(FF556244.1)	$3e^{-32}$	87%
Vmd4	318	AB602403	Expressed sequence tags from *Beta vulgaris* under salt stress (AB552774.1)	$4e^{-126}$	92%
Vmd7	230	AB602406	Expressed sequence tags from Groundnut drought stressed similar to serine/threonine-protein kinase(EC366477.1	0.063	100%
Vmd9	277	AB602408	Expressed sequence tags of *Brassica napus* cold acclimation - dark Brassica napuscDNA (EV193291.1)	0.9	78%
Vmd12	275	AB602411	Expressed sequence tags from drought stressed Cowpea (FF542625.1)	3.3	85%
Vmd15	288	AB602414	Expressed sequence tags from a halophyte *Suaeda salsa* similar to hypothetical 59.1kD protein (BE231361.1.)	3.5	93%
Vmd16	303	AB602415	Expressed sequence tags from *Beta vulgaris* under salt stress (AB552774.1)	$1e^{-152}$	100%
Vmd18	320	AB602417	Expressed sequence tags from *Beta vulgaris* under salt stress (AB552774.1)	$1e^{-121}$	91%
Vmd20	334	AB602419	Expressed sequence tags of *Brassica napus* Root - drought *Brassica napus* cDNA (EV220099.1)	1.2	81%
Vmd5	116	AB602404	Expressed sequence tags of *Medicago truncatula* cDNA (EX529531.1).	$3e^{-28}$	85%
Vmd6	214	AB602405	Expressed sequence tags of *Medicago truncatula* cDNA under fungus infection	0.2	96%
Vmd10	270	AB602409	Expressed sequence tags of *Lotus japonicus* cDNA (FS355391.1)	0.9	100%
Vmd11	270	AB602410	Expressed sequence tags of *Lotus japonicus* cDNA (FS355391.1)	0.9	100%
Vmd14	284	AB602413	Expressed sequence tags from *Pisum sativum*(FG537974.1)	$2e^{-10}$	70%
Vmd25	432	AB602424	Expressed sequence tags from *Populus trichocarpa* cDNA	$3e^{-16}$	95%
Vmd2	265	AB602401	No significant homology.	-	-
Vmd3	290	AB602402	No significant homology.	-	-
Vmd8	219	AB602407	No significant homology.		
Vmd13	278	AB602412	No significant homology.	-	-
Vmd17	313	AB602416	No significant homology.	-	-
Vmd19	329	AB602418	No significant homology.	-	-
Vmd21	340	AB602420	No significant homology.		
Vmd22	344	AB602421	No significant homology.	-	-
Vmd23	400	AB602422	No significant homology.	-	-
Vmd24	417	AB602423	No significant homology.	-	-

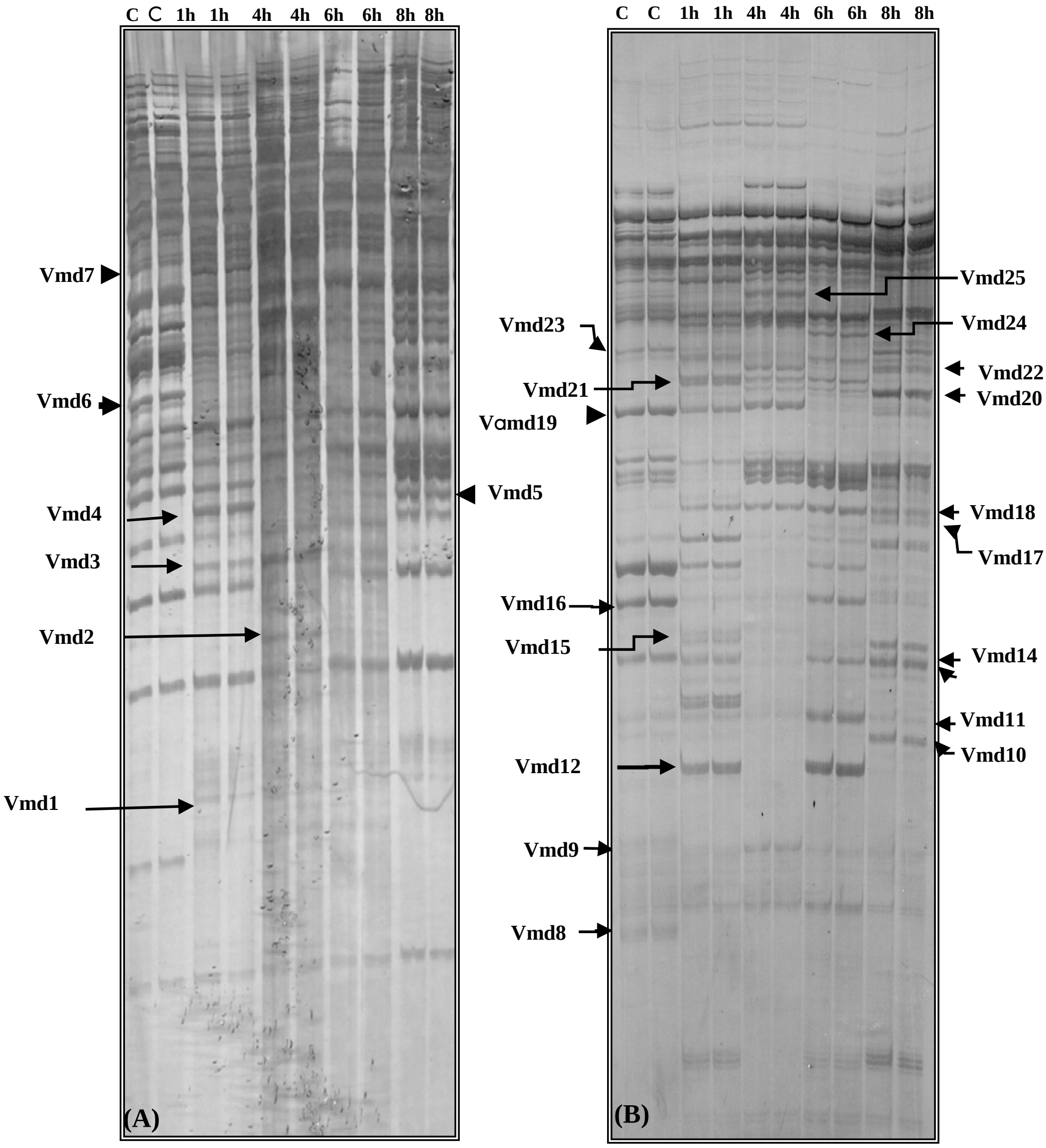

Fig. (1): DD-polyacrylamide gels of shoot cDNAs under control(c) and stress (1, 4, 6 and 8 h) conditions utilizing different primer combinations, A) T11A with AP5 and B) T11G with AP5. Arrows indicate a number of differentially expressed bands on a duplicate basis.