DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO SALT STRESS IN *Vicia monantha*

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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, particularly Algeria and Egypt. It is characterized by its noticeable withstanding of severe environmental conditions. Such wild plants are considered as an excellent source of stress-related genes awaiting their isolation and identification.

Some species of *Vicia*, such as *V. faba* (broad bean), *V. narbonensis* (narbon vetch), *V. villosa* (hairy vetch) and *V. sativa* (common vetch), represent economically important crops that are widely cultivated as a green manure cover, as well as grain and straw crops for animals and a soil binder throughout the North Temperate Zone of the new and old worlds.

Wild *Vicia* species, such as *V. cordata*, *V. nigra*, *V. narbonensis*, *V. sativa*, *V. monantha* and *V. villosa*, are of great interest to agronomists and plant breeders as crop plants in their own right and as possible sources of germplasm for cultivated *Vicia faba* (Atlas of legume plant of the North West of Egypt, 1993).

Plants are frequently exposed to stresses, which are usually defined as external factors exerting disadvantageous influences on them (Levitt, 1972). Water deficit, chilling and freezing, heat stress, salinity and oxygen deficiency are major stress factors restricting plant growth (Boyer, 1982; Salisbury and Ross, 1989). Some of which (such as temperature) can become stressful in a few minutes; others may take days to weeks (soil water) or even months (mineral nutrients) to become stressful. Salinity can affect any process in the plant life cycle, so that tolerance will involve a complex interplay of characters. Many researchers investigated details of the physiology and biochemistry of salt tolerance and also looked at methods to screen overall plant performance that could be used in breeding programs. In general, plants are relatively tolerant during germination but become more sensitive during emergence and early seedling up to later stages of growth (Azhar and McNeilly, 1989; Abdel-Tawab *et al.*, 1998). Drought and

salinity are becoming particularly widespread in many regions, and are expected to cause serious salinization of more than 50% of all arable lands by the year 2050. Drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano *et al*. 1999; Zhu, 2001).

Cloning and characterization of environmental stress-induced genes have greatly contributed to our understanding of the physiological responses of plant cells at the molecular level to different environmental factors. cDNA-amplified fragment length polymorphism (cDNA-AFLP) is an efficient, sensitive, and reproducible technology for the isolation of differentially expressed genes (Bachem *et al*., 1996). It requires no prior sequence information and is, therefore, a useful tool for the identification of novel genes (Ditt *et al*., 2001). cDNA-AFLP is widely available at a low cost for various plant species, even if there is little information at the molecular level (Breyne and Zabeau, 2001; Bei *et al*., 2006; El-Khishin, 2003; Ge *et al*., 2007; Niranjan *et al*., 2006; Roshandel, 2007). It is a polymerase chain reaction (PCR)-based technology like differential display (DD), but featuring higher reproducibility and availability. The most critical advantage is that cDNA-AFLP is based on linker-ligated PCR, whereas DD is based on arbitrarily primed PCR (Bachem *et al*., 1996; Pardee and McClelland, 1999; Shokry *et al*., 2007).

The objective of this study was to isolate and characterize some of the key expressed sequence tags (ESTs) in response to salt stress in *Vicia monantha* using cDNA-AFLP technique, clone and sequence the isolated ESTs and determine gene functions for each EST.

MATERIALS AND METHODS

Plant material and salinity experiment

Vicia monantha seeds, used in this study, were kindly obtained from the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria.

The seeds were manually scarified then soaked in sterile distilled water for seven days with shaking. Water was changed every day then seeds were germinated in sand soil in the greenhouse. Five seeds were planted in each pot with five replicates each. The seeds were irrigated with tap water every two days for four weeks. Then, seedlings were exposed to salt stress with 6000 ppm NaCl. Salt treated seedlings were collected after 1, 9 and 24 hrs then quickly frozen in liquid nitrogen. Control untreated seedlings were also collected, then, samples were stored at -80ºC for further use.

RNA extraction and cDNA synthesis

Total RNAs from about 500 mg of the frozen tissue from control and treated

seedlings were extracted using Tri Pure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany). RNA quality, integrity and quantity of each sample were determined by running 2 µl of total RNAs from each sample in a formamide denaturing gel alongside an RNA ladder (Invitrogen, Carlsbad, California, USA).

First and second cDNA strands were synthesized using Improm^{TM} Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

cDNA-AFLP analysis

AFLP analysis was performed using the AFLP™ Ligation/Preselective Amplification Module (Applied Biosystems, Foster City, California, USA). About 500 ng of double-stranded cDNA were used for cDNA-AFLP analysis according to Vos *et al*. (1995). The restriction enzymes used were *Mse*I and *Eco*RI.

The digested products were ligated to adapters with sequences as follows:
*Eco*RI forward adapter:
5'CTCGTAGACTGCGTACC3',
*Eco*RI reverse adapter:
5'AATTGGTACGCAGTCTAC3';
*Mse*I forward adapter:
5'GACGATGAGTCCTGAG3',
*Mse*I reverse adapter:
5'TACTCAGGACTCAT3'.

The ligated products were preamplified with the corresponding preselective primers (*Eco*RI: 5' CTCGTAGACTGCGTACCA 3', *Mse*I: 5' GACGATGAGTCCTGAGC 3').

Equal amounts of preamplified products were amplified with primers having selective nucleotides at the 3' end (*Eco*RI: AAC, ACA; *Mse*I: CAT, CTA, CTG, CTT). The five primer combinations tested were as follows: primer combination 1 (*Eco*RI- AAC x *Mse*I- CAT), primer combination 2 (*Eco*RI- AAC x *Mse*I- CTA), primer combination 3 (*Eco*RI- AAC x *Mse*I- CTG), primer combination 4 (*Eco*RI- AAC x *Mse*I- CTT) and primer combination 5 (*Eco*RI- ACA x *Mse*I- CAT).

Four µl of the AFLP products were heat-denatured and resolved in a 6% denaturing polyacrylamide sequencing gel (Sequi-Gen® unit, Bio-Rad Laboratories, Hercules, California, USA) run with 0.5X TBE electrophoresis buffer. The gels were silver stained using the silver sequence kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions. All the reactions for restriction digestion, adapter ligation, preamplification, and selective amplification were performed following the manufacturer's instructions.

Isolation and reamplification of cDNA fragments

The differentiated bands of interest were excised from the gels using a sterile razor blade, with maximum care to avoid contamination. Gel slices were incubated at 50 µl dd H_2O at 65°C for 30 min, and then left in room temperature for elution. Three µl of the aliquot were used for re-

amplification in a total volume of 25 ml, using the same set of corresponding selective primers. PCR conditions for the selective amplification were 94ºC for 2 min (1 cycle); 94ºC for 20 sec, 66ºC for 30 sec, 72ºC for 2 min (10 cycles) in which the annealing temperature decreases 1ºC per cycle (touchdown PCR); 94ºC for 20 sec, 56ºC for 30 sec, 72ºC for 2 min (20 cycles); followed by a final cycle of 60ºC for 30 min. The PCR products were resolved in a 2% agarose gel, and each single band was isolated and eluted separately. All PCR reactions were performed using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, USA).

Cloning and sequencing of cDNA fragments

The reamplified cDNA-AFLP fragments were cloned into pGEM®-T Easy Vector System (Promega, Madison, Wisconsin, USA) using *E. coli* (TOP10) competent cells strain according to Sambrook *et al.* (2001). After transformation, the cells were plated onto LB-agar plates containing ampicillin, X-gal, and IPTG and incubated overnight at 37°C. Competent cells transformed with pGEM-T easy vector were detected by blue/white colony screening.

DNA sequencing reactions were performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, California, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the GeneAmp® PCR System 9700 instrument, and the reaction was conducted in a total volume of 20 µl, containing 8 µl of terminator ready reaction mix, 100-500 ng of PCR product, and 2 pmol of M13 universal forward primer (provided with the kit). 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 nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 310 Genetic Analyzer. The data were provided as fluorometric scans from which the sequence was assembled using the sequence analysis software.

Sequence analysis

Nucleotide sequences were analyzed for homology to nucleotide sequences in GenBank non-redundant databases using the BLAST program (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Identification of salinity-regulated transcripts in Vicia monantha

In order to isolate salt-responsive gene fragments from *Vicia monantha* seedlings, cDNA-AFLP technique with five different primer combinations was utilized. Different exposure times to salinity (1, 9 and 24 hrs) were chosen to detect genes differentially responding to 6000 ppm of salt treatment.

As a result of cDNA-AFLP analysis, a total of 184 fragments were clearly differentially expressed, all of which were longer than 80 bp in length (Fig. 1). In a few cases different cDNA fragments belonged to the same transcript; and in addition, sequencing failed for several fragments even after cloning. These fragments were discarded. Fifty three fragments, varying in length from 80 to 285 bp, were excised from the gels, re-amplified by PCR, cloned and successfully sequenced.

Sequence analysis of cDNA-AFLP fragments

The sequence comparison of the 53 cloned fragments against sequences in the NCBI database revealed that 30% of the fragments had no significant sequence similarity, while 70% of the fragments showed homology to genes with known functions, i.e., 13% showed high similarity to previously identified salinity-related genes in other plant species, 9% showed high similarity to drought-related genes, while 48% showed high similarity to cold-related genes (Fig. 2). Sequences of the fifty-three clones have been submitted to the NCBI database and accession numbers are presented in Table (1) along with other fragment characteristics.

A specific nomenclature was adopted for cDNA-AFLP fragments. For example, cDNA Vms1 refers to *Vicia monantha* salt treated seedlings, amplified using combination number 1. Fragments number 1-1 to 1-8 were isolated from this combination, fragments number 2-9 to 2-24 were amplified using combination number 2, fragments number 3-25 to 3-41 were amplified using combination number 3, fragments number 4-42 to 4-45 were amplified using combination number 4 and lastly fragments number 5-46 to 5-53 were amplified using combination number 5.

Table (1) shows that genes were either up- or down-regulated by salt treatment. Based on the type of regulation of the obtained cDNA-AFLP fragments (C, 1, 9 and 24 hrs), the expression profile was divided into: (I) Up-regulated patterns, which either were expressed due to stress, or gradually increased as time of treatment increased, (II) Up- & down-regulated patterns, (III) Down- & up-regulated patterns and (IV) Down-regulated patterns, which were either unexpressed or gradually showed decreased expression as time of treatment increased.

According to Table (1), it is possible to distinguish seven of the 53 ESTs related to salinity according to the following: two (Vms3-32 and Vms3- 38) which were up regulated; four (Vms2-12, Vms2-16, Vms3-25 and Vms3-40) were up & down regulated; one (Vms2-24) was down regulated.

Fragments Vms2-12, Vms2-16, Vms2-24 & Vms3-38 showed significant homology with ESTs of the halophyte *Salicornia bigelovii* cDNA under salt stress (200 mM NaCl) with E-values 6e-13, 1e-10, 2e-10 & 3e-12, respectively. Fragments Vms3-25, Vms3-32 and Vms3-40 showed significant homology with *Lolium temulentum* cDNA under salt

stress (500 mM NaCl) with E-values 2e-07, 8e-13 & 1e-08, respectively.

Twenty five of the 53 selected ESTs were responsive to cold, according to the following: five (Vms2-10, Vms2-15, Vms2-22, Vms3-34 and Vms5- 47) were up regulated; ten (Vms1- 3, Vms2-19, Vms2-20, Vms2-11, Vms3- 26, Vms3-28, Vms3-29, Vms3- 35, Vms4-42 and Vms5- 46) were up & down regulated; eight (Vms1-4, Vms1-7, Vms2-13, Vms2-23, Vms3-27, Vms3-36, Vms3-41 and Vms4-45) were down & up regulated; two (Vms1-5 and Vms3-30) were down regulated.

Fragment Vms1-3 showed significant homology with an EST from the *Oryza sativa* Japonica group, cDNA clone similar to an unknown protein under cold stress germination. Fragments Vms1-4, Vms2-13 & Vms2-15 showed significant sequence homology to cold-responsive cDNA *Medicago sativa* subsp. falcata cDNA with E-value 2e-16, 2e-13 & 3e-11, respectively. Fragments Vms1-5, Vms1-7, Vms2-11, Vms2-20, Vms3-28, Vms3-30, Vms3-35, Vms3-41, Vms4-45 & Vms5-46 showed significant sequence similarity with ESTs from *Brassica napus* cold acclimation-light cDNA with E-values 3e-11, 2e-12, 7e-12, 1e-09, 1e-14, 6e-13, 1e-10, 4e-11, 9e-07 & 8e-07, respectively. Fragments Vms2-10, Vms2-19, Vms2-22, Vms3-26, Vms3-27, Vms3-29, Vms3-34 & Vms4-42 showed significant homology with ESTs from *Physcomitrella patens* cDNA under cold acclimation with E-value 4e-14, 5e-14, 6e-14, 2e-17, 4e-14, 4e-14, 5e-14 & 3e-22, respectively.

Cold acclimation improves freezing tolerance in plants. In higher plants, many advances have been made toward identifying the signaling and regulatory pathways that direct the low-temperature stress response.

Fragments Vms2-23 & Vms5-47 showed significant homology with *Poncirus trifoliata* cDNA clone S3 similar to auxin and ethylene responsive GH3-like protein under cold stress.

The GH3 multigene family in *Arabidopsis* is involved in plant growth and development (Terol *et al*., 2006). It is composed of 19 members that possess adenylation activity on different substrates such as IAA, JA or SA, while the rice GH3 family has 13 members. The GH3 gene group was first described as an early auxin-responsive gene (Hagen and Guilfoyle, 1985). Although the exact function of the GH3 protein in cold stress is not clear, it suggests that the protein plays a role in the cold response and warrants more investigation (Liu *et al*., 2005).

Fragment Vms3-36 showed significant homology with *Poncirus trifoliata* cDNA clone S134 similar to phosphoenolpyruvate carboxykinase under cold stress. Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme in the gluconeogenic pathway by which pyruvate is converted back into Glc (Glucose). The role of this enzyme has been emphasized in photosynthesis of C4

plants and more recently in senescing cotyledons of cucumber, a C3 species (Kim and Smith, 1994). In *B. napus* cold-acclimated seedlings, the increase of PEPCK may contribute to the accumulation of Glc, although there are many other ways for Glc to accumulate at low temperature.

Five of the 53 ESTs were related to drought according to the following: one (Vms1-1) was up regulated; two (Vms2-9 and Vms2-17) were up & down regulated; two (Vms2-21, Vms5-50) were down & up regulated.

Fragments Vms2-9 & Vms2-17 showed significant sequence homology with ESTs from *Saccharum officinarum* cDNA under drought stress with E-values 3e-10 & 3e-11, respectively. Fragment Vms5-50 showed significant sequence homology with an EST from Groundnut under drought stress with E-value 4e-23. Fragments Vms1-1 & Vms2-21 showed significant homology with *Cucumis sativus* C-repeat/DRE binding factor 1 (cbf1) with E-values 2e-06 & 1e-06, respectively.

The dehydration responsive element binding proteins (DREB) are important transcription factors that induce a set of abiotic stress-related genes and impart stress endurance to plants. The DREB transcription factors could be dichotomized as DREB1 and DREB2, which are involved in two separate signal transduction pathways under low temperature and dehydration, respectively. They belong to the ERF (ethylene responsive element binding factors) family of transcription factors (Riechmann *et al*., 2000; McGrath *et al*., 2008) since then; DREB genes have been isolated from a wide variety of plants.

Other studies showed that transgenic *Arabidopsis* plants over-expressing CBF1 (CRT/DRE binding factor) showed elevated expression of target COR (Cold Responsive) transcripts and were more tolerant to freezing stress than their non-transgenic counterparts as determined by electrolyte leakage and whole plant assays (Jaglo-Ottosen *et al*., 1998). Similarly, transgenic *Arabidopsis* plants over-expressing DREB1 a had enhanced drought and salinity resistance (Kasuga *et al*., 1999).

Also, tomato plants over-expressing the Arabidopsis-CBF1/DREB1B gene showed elevated tolerance to drought (Hseih *et al*., 2002a,b) and salinity (Lee *et al*., 2003); and strawberry plants expressing the *Arabidopsis* CBF1/DREB1B gene had less membrane damage in response to freezing temperatures than their non-transgenic counterparts (Owens *et al*., 2002).

It is also possible to distinguish 16 ESTs with no significant sequence similarity to known sequences in the gene bank. Expression was as follows: two (Vms3-37 and Vms3-39) were up regulated; four (Vms1-2, Vms3-31, Vms5-49 and Vms5-51) were up & down regulated; six (Vms1-6, Vms2-14, Vms2-18, Vms3-33, Vms4-43 and Vms4-44) were down & up regulated; four (Vms1-8, Vms5-48,

Vms5-52 and Vms5-53) were down regulated. These ESTs need more investigation to confirm their relation to the abiotic stress process or they may represent yet uncharacterized genes.

The Fifty three isolated and characterized cDNA fragments were deposited in the Genbank as a result of screening for salt-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 and transformation of 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, cDNA-amplified fragment length polymorphism (cDNA-AFLP) was used to analyze differentially expressed genes in *Vicia monantha* under salt stress. Four weeks old seedlings were treated with 6000 ppm sodium chloride (NaCl) for 1, 9 and 24 hrs, and untreated seedlings were used as control. Fifty-three differentially expressed cDNA-AFLP fragments were selected. No significant sequence similarity was found for sixteen of the fragments, while the rest of the fragments showed significant sequence homology with salt, drought and cold stress- responsive genes, as well as transcriptional factors and expression regulators. The results suggest that a multitude of processes are implicated in the salinity stress response.

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Table (1): Description of cDNA-AFLP fragment sequences as compared to database sequences and expression patterns of differentially expressed fragments.

Fragment No.	Length (bp)	Accession No.	Regulation	Differential Expression/time				Homology	E- Value
				C	1	9	24		
Salinity									
Vms3-32	254	EB70 9950.1	Up	-	-	+	+	S7SLT_C45 *Lolium temulentum* salt stressed subtraction library *Lolium temulentum* cDNA, mRNA sequence.	8e-13
Vms3-38	254	DY52 9915.1		-	+	+	+	Sb185 *Salicornia bigelovii* Torr. cDNA library based on suppression subtractive hybridization *Salicornia bigelovii* cDNA, mRNA sequence.	3e-12
Vms2-12	190	DY52 9903.1	Up & down	+	++	+	-	Sb173 *Salicornia bigelovii* Torr. cDNA library based on suppression subtractive hybridization *Salicornia bigelovii* cDNA, mRNA sequence.	6e-13
Vms2-16	211	DY52 9903.1		-	+	-	+	Sb173 *Salicornia bigelovii* Torr. cDNA library based on suppression subtractive hybridization *Salicornia bigelovii* cDNA, mRNA sequence.	1e-10
Vms3-25	175	EB70 9950.1		-	-	+	-	S7SLT_C45 *Lolium temulentum* salt stressed subtraction library *Lolium temulentum* cDNA, mRNA sequence.	2e-07
Vms3-40	183	EB70 9950.1		-	-	+	-	S7SLT_C45 *Lolium temulentum* salt stressed subtraction library *Lolium temulentum* cDNA, mRNA sequence.	1e-08
Vms2-24	285	DY52 9903.1	Down	++	+	+	-	Sb173 *Salicornia bigelovii* Torr. cDNA library based on suppression subtractive hybridization *Salicornia bigelovii* cDNA, mRNA sequence.	2e-10
Cold									
Vms2-10	178	EE295 956.1	Up	-	-	+	++	EST105 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	4e-14
Vms2-15	192	EL610 423.1		-	-	-	+	mfcorj6h12 *Medicago falcata* SSH Library of cold-responsive cDNA *Medicago sativa* subsp. falcata cDNA, mRNA sequence.	3e-11
Vms2-22	244	EE295 956.1		-	-	-	+	EST105 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	6e-14

Vms3-34	215	EE295956.1		-	-	+	++	EST105 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	5e-14
Vms5-47	232	EF377536.1		-	-	+	+	EF377536 *Poncirus trifoliata* leaves 1 year old plants *Poncirus trifoliata* cDNA clone S3 similar to auxin and ethylene responsive GH3-like protein, mRNA sequence.	7e-23
Vms1-3	81	CX056239.1		-	+	-	+	AG-CTC1 Rice cold stress germination cDNA-AFLP *Oryza sativa* Japonica Group cDNA clone AG-CTC1 5' similar to Unknown protein, contains RNA recognition motif [*O. sativa*], mRNA sequence.	2e-07
Vms2-19	214	EE295956.1		-	-	+	-	EST105 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	5e-14
Vms2-20	211	EV203710.1		-	+	-	-	0104863 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	1e-09
Vms2-11	183	EV203710.1		+	+++	-	-	0104863 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	7e-12
Vms3-26	178	EE296094.1	Up & down	-	+	-	-	EST023 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	2e-17
Vms3-28	188	EV203787.1		-	+	-	-	0104940 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	1e-14
Vms3-29	197	EE296094.1		-	+	+	-	EST023 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	4e-14
Vms3-35	224	EV203664.1		-	+	+	-	0104817 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	1e-10
Vms4-42	248	EE296094.1		-	-	+	-	EST023 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	3e-22
Vms5-46	199	EV203624.1		-	+	-	-	0104777 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	8e-07
Vms1-4	146	EL610409.1	Down & up	+	-	++	++	mfcorj6g8 *Medicago falcata* SSH Library of cold-responsive cDNA *Medicago sativa* subsp. falcata cDNA, mRNA sequence.	2e-16

Vms1-7	211	EV203603.1		-	+	-	++	0104756 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	2e-12
Vms2-13	191	EL610407.1		+	-	-	++	mfcorj6g6 *Medicago falcata* SSH Library of cold-responsive cDNA *Medicago sativa* subsp. falcata cDNA, mRNA sequence.	2e-13
Vms2-23	202	EF377536.1		+	-	+	+	EF377536 *Poncirus trifoliata* leaves 1 year old plants *Poncirus trifoliata* cDNA clone S3 similar to auxin and ethylene responsive GH3-like protein, mRNA sequence.	2e-18
Vms3-27	182	EE295956.1		+	-	+	-	EST105 P. patens Differentially Express Library *Physcomitrella patens* cDNA, mRNA sequence.	4e-14
Vms3-36	238	EF377537.1		+	-	+	-	EF377537 *Poncirus trifoliata* leaves 1 year old plants *Poncirus trifoliata* cDNA clone S134 similar to phosphoenolpyruvat carboxykinase, mRNA sequence.	4e-15
Vms3-41	269	EV203664.1		+++	+	-	++	0104817 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	4e-11
Vms4-45	218	EV203710.1		+	-	++	++	0104863 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	9e-07
Vms1-5	198	EV203711.1	Down	+	+	-	-	0104864 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	3e-11
Vms3-30	199	EV203710.1		+	+	-	-	0104863 *Brassica napus* Cold acclimation - light *Brassica napus* cDNA , mRNA sequence.	6e-13
Drought									
Vms1-1	167	DQ776899.1	Up	-	-	+	+	*Cucumis sativus* C-repeat/DRE binding factor 1 (cbf1) mRNA, complete cds.	2e-06
Vms2-9	180	ES697508.1	Up & down	-	+	+	-	DrSHF 60 *Saccharum officinarum* Expressed sequence tags from the Forward SSH library, mRNA sequence.	3e-10
Vms2-17	213	ES697508.1		-	++	-	+	DrSHF 60 *Saccharum officinarum* Expressed sequence tags from the Forward SSH library, mRNA sequence.	3e-11
Vms2-21	230	DQ776899.1	Down & up	+	-	-	+	*Cucumis sativus* C-repeat/DRE binding factor 1 (cbf1) mRNA, complete cds.	1e-06
Vms5-50	134	DT044297.1		+	-	+	+	GDI_B__27 Groundnut (K-134) drought stressed subtracted cDNA library *Arachis hypogaea* cDNA, mRNA sequence.	4e-23

Non-significant									
Vms3-37	162		Up	-	-	+	+	No significant similarity found.	
Vms3-39	155			-	-	-	+	No significant similarity found.	
Vms1-2	103		Up & down	-	+	+	-	No significant similarity found.	
Vms3-31	116			-	+	+	-	No significant similarity found.	
Vms5-49	131			-	-	+	-	No significant similarity found.	
Vms5-51	135			-	+	-	+	No significant similarity found.	
Vms1-6	122		Down & up	+	+	-	+	No significant similarity found.	
Vms2-14	113			+	+	-	++	No significant similarity found.	
Vms2-18	130			+	-	-	+	No significant similarity found.	
Vms3-33	126			+	+	-	+	No significant similarity found.	
Vms4-43	118			+	-	-	+	No significant similarity found.	
Vms4-44	142			+	-	+	++	No significant similarity found.	
Vms1- 8	155		Down	+	+	-	-	No significant similarity found.	
Vms5-48	208			+	+	-	-	No significant similarity found.	
Vms5-52	245			++	+	-	-	No significant similarity found.	
Vms5-53	184			++	-	-	-	No significant similarity found.	

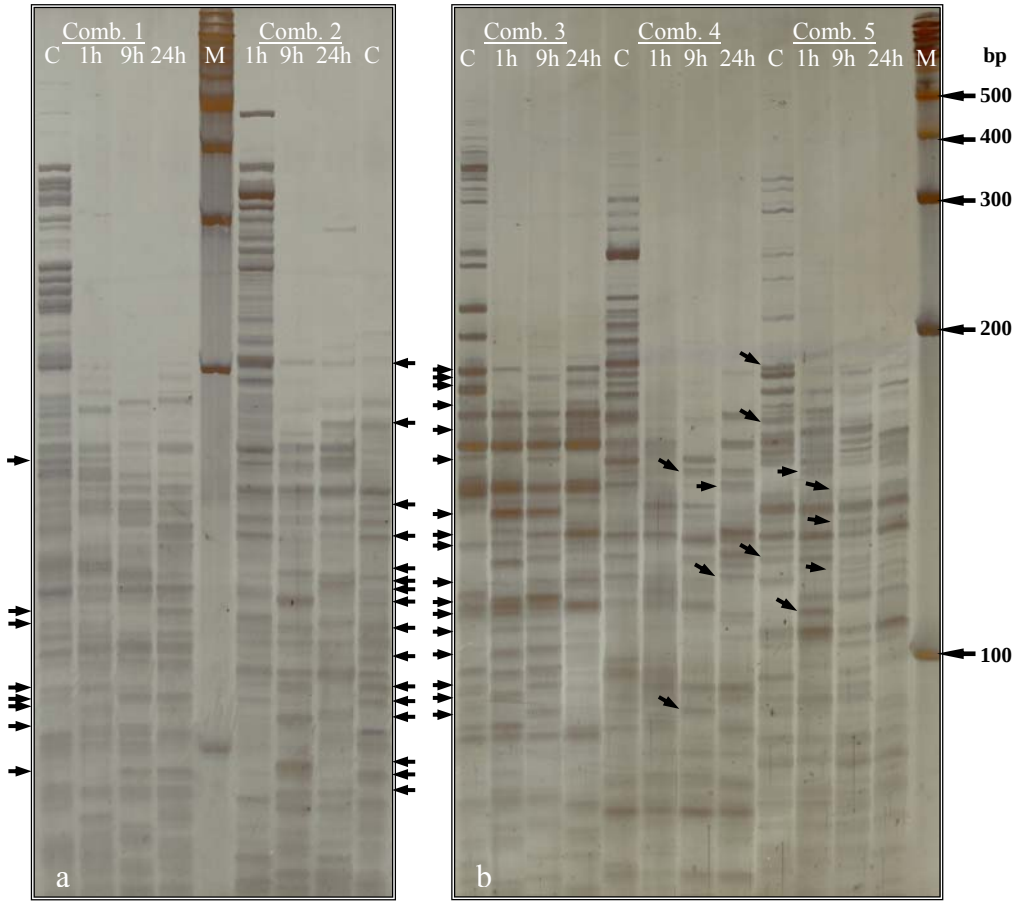

Fig. (1): Representative profile of a silver-stained cDNA-AFLP polyacrylamide gel (6%) showing the differential expression of the genes under different stages of salt stress in *Vicia monantha*. C = control, and stress (1h, 9h and 24h). M = 100 bp DNA ladder Gene RulerTM 100 bp DNA Ladder Marker (Fermentas, Germany). Arrows indicate the differentially expressed products that were isolated for further investigation. (1a) using primer combinations 1 and 2, (1b) using primer combinations 3, 4 and 5.

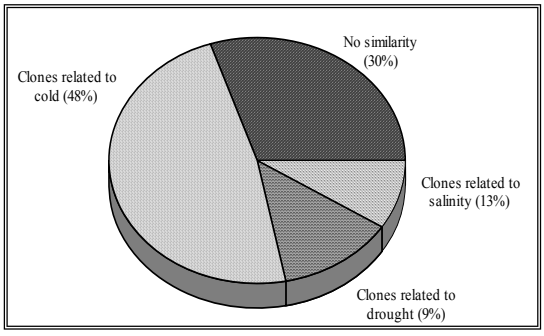

Fig. (2): Distribution of the cDNA-AFLP fragments based on their homology.