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Full Length Research Paper

Complementary DNA-amplified fragment length polymorphism (AFLP-cDNA) analysis of differential gene expression from the xerophyte *Ammopiptanthus mongolicus* in response to cold, drought and cold together with drought

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In this study, we examine *Ammopiptanthus mongolicus*, the only desert ultra-broad-leaved evergreen shrub in Northwest China. During this study, *A. mongolicus* was exposed to drought 20% polyethylene glycol (PEG)), low temperature (4°C) and a combination of drought and low temperature (20% PEG and 4°C). Complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) technology was used to analyze differential transcriptional expression under the aforementioned abiotic stresses. We found that 9 of the studied expressed sequence tags (ESTs) are related to protein modification, 12 ESTs are involved in the control of transcription and translation, 9 ESTs are involved in metabolism, 11 ESTs are related to aging and defense genes, 1 EST plays a role in signal transduction, 3 ESTs are related to cell development, 3 ESTs may play a role in energy production and photosynthesis, 2 ESTs are related to protein transport and 49 ESTs are of unknown significance.

Key words: *Ammopiptanthus mongolicus*, cDNA-AFLP (Amplified fragment length polymorphism), differential gene expression.

INTRODUCTION

Environmental stress always affects plant growth, leading to changes in metabolic action. An example of this is the reversible inhibition of metabolism and growth experienced by a plant undergoing drought stress. Among the environmental stresses, drought and cold are the two key factors affecting plant growth and yield. In the past, scientists have studied a plant's resistance to stress at the molecular level, utilizing such techniques as gene comparison, expression regulation, signal transduction, etc. Generally, regulatory genes code for signal transduction proteins (Zhu, 2002; Sanders et al., 2002) and transcription factors (Mei et al., 1999). Functional genes include genes related to osmotic regulation, (McCue and Hanson 1992; Schwacke et al., 1999), defense (Oha et al., 2005; Li et al., 2001; Lim and Bowles, 2004), senescence (Buchanan-Wbllaston, 1997; Lee et al., 2001; Gepstein et al., 2003; Beers et al., 2000; Coupe et al., 2003; Eason et al., 2002; Cofeen and Wolpert, 2004), cell wall synthesis (Ahn et al., 2006) and photosynthesis (Dhingra et al., 2004; Houtza and Portis, 2003; Bartholomew et al., 1991; Hajela et al., 1990). Hence, gene expression analysis plays an important role in drought and cold resistance research. However, the mechanisms behind cold and drought resistance at cellular and molecular levels have been studied exclusively in modal plants, agricultural plants and plants of commercial value. Studies of gene expression

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Abbreviations: ESTs, Expressed sequence tags; **MS,** Murashige and Skoog; **PEG,** polyethylene glycol; **PCR,** polymerase chain reaction; **BLASTS,** Basic Local Alignment Search Tools; **TDFs,** transcript-derived fragments.

analysis in stress resistant and endangered plants under abiotic stress have received far less attention.

Ammopiptanthus mongolicus (Cheng. F) is the only super-xerophytic evergreen broadleaf shrub species growing in the desert region of northwest China. It possesses an extremely high tolerance to drought and cold and can survive winter tempe-ratures of less than -30°C (Liu et al., 1982). *A. mongolicus* therefore, provides an ideal opportunity to research stress resistance mechanisms. Until recently, studies on *A. mongolicus* have focused primarily on cold-inducible cDNA clones (Liu et al., 2005), *AmCIP* (Liu et al., 2006), *AmCBL1* gene expression (Chen et al., 2010) and *AmCBL1* gene promoter (Guo et al., 2010).

In this study, complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) labeling technique was combined with an amplified restriction fragment polymorphism (AFLP)-derived technique to conduct RNA fingerprinting (cDNA-AFLP) (Pieter et al., 1995). This technique combines AFLP with mRNA differential display, offering many advantages. As a result, sequence information was not required in advance, we found this combination trustworthy, efficient and convenient. Furthermore, cDNA-AFLP labeling allowed us to study the dynamic expression of one gene under different conditions, while also affording us the chance to study the differential gene expression of many genes under the same conditions. In summary, this technique provides a reliable method for studying the expression patterns of genes involved in mechanisms of stress resistance (Xu et al., 2009; Wang et al., 2009; Polegri et al., 2010).

In this study, cDNA-AFLP labeling technique was employed to locate differentially expressed genes in *A. mongolicus* under different abiotic stresses. While conducting the analysis, a set of previously unknown expressed sequence tags (ESTs) was detected. We compared the behavior of *A. mongolicus* under drought and cold stress to detect, describe and understand plant responses. The results indicate that, different kinds of transcripts show different regulation capacity at different time points. This sort of research might provide information valuable to our understanding of the molecular mechanisms behind plant stress-resistance and may also provide the opportunity to develop stress resistant plants. In addition to these findings, a set of drought and cold-response genes which have not been reported, were identified.

MATERIALS AND METHODS

Plant material

A. mongolicus seeds were collected from Alashan, Inner Mongolia autonomous Region, China. Healthy seeds of *A. mongolicus* were surface sterilized for 30 s in 70% ethanol, followed by a 20 min submersion in 10% sodium hypochlorite. The seeds were rinsed thoroughly with distilled water and then, placed in 150 ml vessels. The vessels contained 50 ml of MS medium and 0.7% (w/v) agar, pH 5.8, which had been autoclaved at 121°C for 20 min. The seeds were incubated for 14 days at 25°C with a 16/8 h light/dark photoperiod. Two-week-old cotyledons were used for the subsequent experiments.

Stress treatment

The root systems of *A. mongolicus* seedlings were soaked in ½ Murashige and Skoog (MS) liquid medium for 24 h after being taken from the MS medium. Following this, we induced stress (Table 3). Those plants undergoing drought treatment were placed in ½ MS liquid medium containing 20% polyethylene glycol (PEG) at room temperature under a growth regime of 16/8 h light/dark. Those seedlings being exposed to low-temperature stress were incubated in 16/8 h light/dark at 4°C. The seedlings meant to experience a combination of drought and cold-stress were cultured on ½ MS liquid medium containing 20% PEG at 4°C in 16/8 h light/dark. Seedlings growing in ½ MS liquid medium without any induction were used as controls. Cotyledons samples from at least 6 individuals of *A. mongolicus,* taken at 6 h, were used for cDNA-AFLP analysis. Cotyledon samples from at least 6 individuals of *A. mongolicus,* taken at 6, 12, 24 and 48 h after each stress induction, were used for real-time PCR analysis. Samples were frozen in liquid nitrogen immediately after stress induction and stored at -80°C until use.

RNA preparation, cDNA synthesis and cDNA-AFLP analysis

Following a cetyltrimethylammonium bromide (CTAB) extraction method, total RNAs were extracted from the leaves of *A. mongolicus* that had been treated with drought, cold and a combination of drought and cold (Chang et al., 1993). RNA products were then, examined by agarose gel electrophoresis. $^{OD260}/_{OD280}$ and RNA concentration were detected and calculated by spectrophotometry.

M-MLV reverse transcriptase (NEB, England)) and Oligo(dT) primers were used during the first strand synthesis of our cDNA synthesis reaction. This cDNA was synthesized according to molecular clone (Sambrook and Russell). Double-stranded cDNA was synthesized according to a standard double-stranded cDNA synthesis protocol (Sambrook and Russell, 2001), using DNA polymerase I (*Escherichia coli*) and T4 DNA ligase (NEB, England). cDNA-AFLP analysis was performed according to Bachem (Bachem et al., 1998), using *EcoR* I and *Mse* I restriction enzymes. Polymerase chain reaction (PCR) products were separated on a 6% sequencing gel and visualized by silver staining. A clear cDNA-AFLP gel with no bands was obtained. For all primer combinations, a non-DNA sample was included to rule out the presence of any unwanted bands caused by primer dimers or contamination. Each cDNA-AFLP gel was run three times. For each replicate, the gel was run from the same cDNA sample but from a different amplification reaction. Only the reactions showing full reproducibility between replicates were used for data analysis. All of the procedures are presented in Table 1 and the cDNA-AFLP adaptors and primers are shown in Table 2.

Real time PCR analysis

To verify the feasibility and effectiveness of polyacrylamide gel electrophoresis and the trend of expression changes in differential expression fragments, four EST fragments (EST013, EST021, EST029and EST083) were selected for real time PCR analysis. The *Actin* gene of *A. mongolicus* was chosen as a reference gene for this analysis. Treatments of *A. mongolicus* are shown in Table 3.

Total RNAs prepared from leaf tissue were transcribed as described

Table 1. cDNA-AFLP analysis procedure

Step	Reaction system	Procedure
Enzyme cut	cDNA 10 µl, 10×NEB Buffer 23 µl 100×BSA (100 µg·ml-1) 0.3 µl EcoR I (20 U·µl-1) 1 µl, Mse I (10 U·µl-1) 1 µl, H_2O 14.7 µl	37°C, 4.5 h; 65°C,10 min
Ligation	Product (last step) 10 µl, EcoR I adaptor (50 pmol·µl-1) 1.5 µl, Mse I adaptor (50 pmol·µl-1) 1.5 µl, T4 ligase (5 U·µl-1) 0.5 µl, 10×T4 DNA ligase buffer 2 µl, H_2O 4.5 µl	16°C, overnight
Pre-amplification	Product (last step) 10 µl, 10×PCR buffer 5 µl, EcoR I pre-amplification primer (20 µM) 1.5 µl, Mse I pre-amplification primer (20 µM) 1.5 µl, ExTaq (5 U·µl-1) 0.25 µl, dNTP (10mmol·L–1) 4 µl, H_2O 27.75 µl	94°C, 5 min; 94°C, 50 s; 52°C, 45 s; 72°C, 50 s; 31 cycles; 72°C, 10 min
Selectivity amplification	solution of 20 X dilution product of pre-amplification 2 µl, 10×PCR buffer 2 µl, EcoR I selectivity amplification primer (10 µM) 1 µl, Mse I selectivity amplification primer (10 µM) 1 µl, 10 mmol·L-1 dNTP 1.5 µl, Taq (5 U·µl-1) 0.2 µl, H_2O 12.4 µl	94°C, 3 min; 94°C, 30 s; 65°C 30 s; 72°C, 1 min; Tm decease 0.7°C every cycle, 12 cycles; 94°C, 30 s; 56°C, 30 s; 72°C, 1 min; 31 cycles; 72°C, 5 min
Polyacrylamide gel electrophoresis	selectivity amplification product, 6 µl Loading buffer (carboxamide 20 ml, 0.5 mol·L^{-1} EDT(pH 8.0400 µl, bromophenol blue 20 mg, xylene cyanine 20 mg)	100W, 50°C, preelectrophoresis; 95°C, 5 min; put on ice; spotting; electrophoresis 2 to 3 h; silver staining
Recovery	Cut the gel of differential fragment, H_2O 10 µl,	100°C , 15 min; 4°C, 4 h
Amplification	Last step product, Eoo、Moo	9°C 1 min; 94°C, 30 s; 56°C, 45 s; 2°C, 1 min; 35 cycles; 72°C, 10 min; electrophoresis

The re-amplified cDNAs were sub-cloned into the pMD18-T vector and sequenced.

earlier. Each reaction contained 2 µl of SYBR Green I (ABI), 2 µl of cDNA sample and 0.2 µl (20 mM) of gene-specific primers, in a final volume of 20 µl. This reaction contains three stages: Ongoing stage, cycling stage and melt curve stage. Forty PCR cycles were performed according to the following temperature scheme: 94°C for 10 min, 95°C for 15 s and 60°C for 1 min, 95°C for 15 s, 60°C for 1 h, 95°C for 15 s. The relative value of the expression level of each gene was calculated by the $2^{-\Delta\Delta C}$T method, using the *A. mongolicus Actin* gene as an internal control (Livak and Schmittgen, 2001). The primers utilized in our real time PCR experiments are listed in Table 4 and the gene specificity of real time PCR products was confirmed by sequencing.

Bioinformatics and computational analysis

After performing homology searches and similarity Basic Local Alignment Search Tools (BLASTS) of EST sequence, the results were analyzed on the GenBank database and accessed through the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov/). Functional annotation was performed using the BLAST search algorithm (Altschul et al., 1997). EST

Table 2. Adaptor and primer used for cDNA-AFLP analysis.

***EcoR*I adaptor and primer**	***Mse*I adaptor and primer**
Adaptor: Top strand:5'-CTCGTAGACTGCGTACC-3'	Adaptor: Top strand:5'-GACGATGAGTCCTGAG-3'
Bottom strand:5'-AATTGGTACGCAGTC-3'	Bottom strand:5'-TACTCAGGACTCAT-3'
E_{00} 5'-GACTGCGTACCAATTC-3'	M_{00} 5'-GATGAGTCCTGAGTAA-3'
E-TG 5'-GACTGCGTACCAATTCTG-3'	M-CAA 5'-GATGAGTCCTGAGTAACAA-3'
E-TA 5'-GACTGCGTACCAATTCTA-3'	M-CAG 5'-GATGAGTCCTGAGTAACAG-3'
E-TC 5'-GACTGCGTACCAATTCTC-3'	M-CAC 5'-GATGAGTCCTGAGTAACAC-3'
E-TT 5'-GACTGCGTACCAATTCTT-3'	M-CTT 5'-GATGAGTCCTGAGTAACTT-3'
E-AA 5'-GACTGCGTACCAATTCAA-3'	M-CTC 5'-GATGAGTCCTGAGTAACTC-3'
E-AT 5'-GACTGCGTACCAATTCAT-3'	M-CTA 5'-GATGAGTCCTGAGTAACTA-3'
E-AC 5'-GACTGCGTACCAATTCAC-3'	M-CTG 5'-GATGAGTCCTGAGTAACTG-3'
E-AG 5'-GACTGCGTACCAATTCAG-3'	M-CAT 5'-GATGAGTCCTGAGTAACAT-3'
E-NN 5'-GACTGCGTACCAATTCNN-3'	M-NNN 5'-GATGAGTCCTGAGTAANNN-3'

Table 3. Stress induction of *A. mongolicus.*

Stress	Treatment method	Time (h)	
		AFLP	Real-time PCR
Drought	20%PEG	6	6, 12, 24, 48
Cold	4°C	6	6, 12, 24, 48
Drought and Cold	20%PEG and 4°C	6	6, 12, 24, 48

Table 4. Primers used for real time PCR analysis.

EST	Upstream primer	Downstream primer
EST013	TTCCTTGACTTTGTCCGTGT	CATTGCTATTTGACTCGGACC
EST021	GTAACTCCAACAGACAGGCA	GTGAATCGGTCCCTAAGGAAG
EST029	ACATAAGGAAGGCAATGCTCG	ATTCAGGCTTGTGGTCCAGG
EST083	GTGCCGTATTGATGCCTACC	CTGCGTTCCCAAAGATAAAGTC
Actin	GGTCCACTATGTTTCCGGGT	TTGAAAGGTGCTGAGTGATGC

sequences where blasted against the genomes of *Medicago truncatula* (http://www.medicago.org/genome/) and *Arabidopsis* (http://www.arabidopsis.org/). Sequence alignments were performed in all cases, using DNAMAN software.

RESULTS

Differential expression analysis

To determine the gene expression of *A. mongolicus* under drought, cold and a combination of drought and cold stress, samples were collected at 0, 6, 12, 24 and 48 h, after the aforementioned treatments. The cDNA expression profiles were determined by PCR selective amplification, using 64 different primer combinations. In total, 4093 cDNA fragments were screened. A section of a typical AFLP gel used in this analysis is shown in Figure 1. As anticipated, the majority of the transcript-derived fragments (TDFs) showed similar levels of accumulation in samples from all six time-points. Typical primer combinations producing differentially expressed TDFs were selected and the experiments were repeated three times to confirm the reproducibility of the data.

395 specific bands were found among the 4093 bands, with a 9.7% specific efficiency. A total of 220 differentially accumulated TDFs, ranging in length from 50 to 300 bp (Table 5), were recovered from gels, re-amplified with the corresponding cDNA-AFLP primer combinations, sub-cloned and sequenced successfully. Among the 220 successfully sequenced TDFs, 30 (13.6%) differential expression fragments derived from samples that had not undergone treatment, 39 (17.7%) differential expression fragments derived from samples that had undergone drought treatment, 70 (36.9%) differential expression fragments derived from samples that had undergone cold

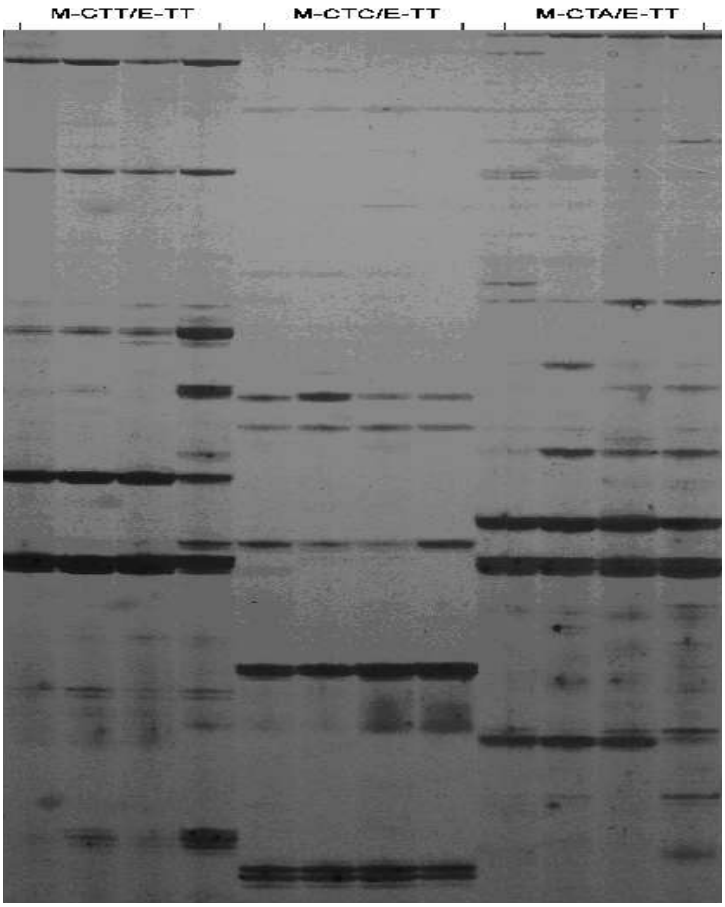

Figure 1. The patterns amplified by M-CTT/E-TT、M-CTC/E-TT、M-CTA/E-TT primer combination (partial). M-CTT, 5'-GATGAGTCCTGAGTAACTT-3'; E-TT: 5'-GACTGCGTACCAATTCTT-3'; M-CTC, 5'-GATGAGTCCTGAGTAACTC-3'; M-CTA: 5'-GATGAGTCCTGAGTAACAT-3'.

stress and 81 (36.9%) differential expression fragments derived from samples that had undergone both drought and cold treatments were found.

According to the stress induced gene classification method (Kasuga et al., 1999), differential expression genes of *A. mongolicus* were classified as regulator (genes known to be involved in signal transduction, transcriptional regulation and the creation of transcription factors) and functional genes (genes known to be involved in protein modification, senescence defense, translocation, cell wall synthesis, photosynthesis, metabolism, growth and development). Excluding the interference sequence (*E. coli* sequence) and the redundant sequence, 99 homologous sequences were found after BLASTing. Among these TDFs, 9 sequences were related to protein modification, 12 sequences were related to transcriptional regulation and translation, 9 sequences were related to metabolism, 11 sequences were related to senescence defense genes, 1 sequence was found to be related with signal transduction genes, 3 sequences were related to cell development, 3 sequences were related to photosynthesis and 2 sequences were related to translocators. Interestingly, 49.5% (49 TDFs) show significant similarity to unknown genes with no assigned function from various genome projects. These fragments represent new candidate proteins that may be involved in cell fate determination, differentiation, cell wall remodeling and cell death.

Expression patterns analysis by real time PCR

To validate the cDNA-AFLP expression profiles, a real time PCR study was designed to investigate differentially accumulated TDFs, using total RNA populations harvested from tissues at the same time points that were used for the cDNA-AFLP analysis. EST013 (translation-related), EST021 (related to translation and transcriptional regulation), EST029 (ATP-dependent RNA helicase- related) and EST083 (cell wall modification-related) were selected for further analysis.

Real Time PCR analysis showed that the expression

Table 5. Sequence analyses of differential cDNA-AFLP fragments.

TDF number	Length (bp)	Annotation
EST001	71	Metabolic process
EST002	151	Inflorescence development, phyllome development
EST003	65	Metabolic process
EST004	183	Unknown
EST005	134	Protein amino acid phosphorylation
EST006	104	Unknown
EST007	163	Gravitropism, ethylene mediated signaling pathway, nuclear-transcribed
EST008	134	Response to cold, heat, response to virus
EST009	163	Member of cyclic nucleotide gated channel family
EST010	186	Translation, ribosome biogenesis and assembly
EST011	64	Proteolysis
EST012	154	Metabolic process
EST013	95	Transcription (chloroplast)
EST014	98	Protein amino acid phosphorylation
EST015	111	Unknown
EST016	116	Metabolic process
EST017	155	Unknown function (*M. truncatula*)
EST018	186	Unknown
EST019	199	Induced by salicylic acid (Soybean)
EST020	137	Regulation of transcription
EST021	189	Translation
EST022	116	Negative regulation of transcription, regulation of transcription, DNA-dependent, response to auxin stimulus, leaf senescence
EST023	109	Unknown (*M. truncatula)*
EST024	140	Unknown
EST025	186	Metabolic process
EST026	120	Translation
EST027	212	Protein folding(Calcium ion binding)
EST028	112	Photosynthesis
EST029	224	ATP-dependent RNA helicase
EST030	103	Pyruvate decarboxylase
EST031	184	Photosynthesis
EST032	155	Photosynthesis
EST033	121	Autophagy
EST034	232	Pyruvate kinase (*M. truncatula*)
EST035	95	Response to cold (*P. angustissimus*)
EST036	242	Treated with oligogalacturonides(root, *M. truncatula*)
EST037	183	Unknown (*G. max*)
EST038	95	Unknown (*P. angustissimus*)
EST039	89	unknown (Glycine)

levels of these genes were up-regulated under the drought, cold and drought/cold stresses treatments. EST 083, however, was an exception showing unexpected expression levels under cold induction and differences in the time-points at which maximum transcript levels were reached.

As shown in Figure 2, all four EST fragments derived from samples that had undergone a 20% PEG treatment continued to increase from 0 to 48 h. EST013 reached its maximum expression level at 48 h, while EST021, EST029 and EST083 reached their maximums at 24 h and then, decreased. Significance analysis shows that

Table 5. Contd.

TDF number	Length (bp)	Annotation
EST040	112	N-terminal protein myristoylation，glutamine metabolic process
EST041	87	Response to drought
EST042	162	Translational initiation
EST043	147	Response to infection and propagation
EST044	123	Unknown
EST045	179	Similar to auxin and ethylene responsive GH3-like protein (*P. trifoliata*)
EST046	159	Induced by salicylic acid (*G. max*)
EST047	133	Galactose metabolic process
EST048	131	Unknown
EST049	88	Unknown (*M. lambda* Zap)
EST050	148	Carbohydrate metabolic process
EST051	123	Unknown (*G. max*)
EST052	176	Unknown (*Vigna*)
EST053	129	Translational initiation
EST054	168	Unknown
EST055	145	Unknown
EST056	97	Unknown
EST057	136	Unknown
EST058	81	Proteolysis
EST059	103	Unknown
EST060	173	Metabolic process
EST061	93	Translation
EST062	76	Protein ubiquitination
EST063	73	Translation
EST064	182	Unknown
EST065	167	Unknown
EST066	165	Unknown
EST067	147	Unknown
EST068	91	Unknown
EST069	182	Unknown
EST070	184	Unknown
EST071	148	Transport
EST072	71	Unknown
EST073	140	Unknown
EST074	93	Unknown
EST075	61	Unknown
EST076	126	Unknown
EST077	71	Unknown
EST078	61	Unknown

the difference observed between EST021 and EST083 is significant, while the difference observed in EST029 is not.

As shown with the cold treatment (Figure 3), EST013 showed sharply decreasing expression levels during the first 12 h, after which it then increased drastically, showing a significant statistical difference. The expression level of EST021 ascended, reaching its peak at 24 h, after which its expression decreased with the lengthening cold treatment. EST029 and EST021 showed similar expression patterns. However, EST029 reached its peak at 12 h. The expression of EST083 decreased significantly during the time of treatment, reaching its nadir at 12 h.

With respect to the combination of drought and low temperature treatment (Figure 4), EST013 was up-regulated and reached its highest level at 24 h of stress. EST021 and EST029 had a similar expression pattern. In both cases, expression increased with the stress treatment and reached its peak at 24 h of treatment,

Table 5. Contd.

TDF number	Length (bp)	Annotation
EST079	127	Unknown
EST080	44	Unknown
EST081	89	Proteolysis
EST082	67	Unknown
EST083	60	Cell wall modification
EST084	91	Metabolic process
EST085	71	Unknown
EST086	75	Unknown
EST087	62	Unknown
EST088	88	Plant-type cell wall organization and biogenesis
EST089	89	Translation
EST090	146	Unknown
EST091	103	unknown
EST092	77	Unknown
EST093	104	Unknown
EST094	243	Unknown
EST095	76	RING-H2 finger protein (biological process unknown)
EST096	102	Unknown
EST097	176	Unknown
EST098	105	Unknown
EST099	106	Unknown

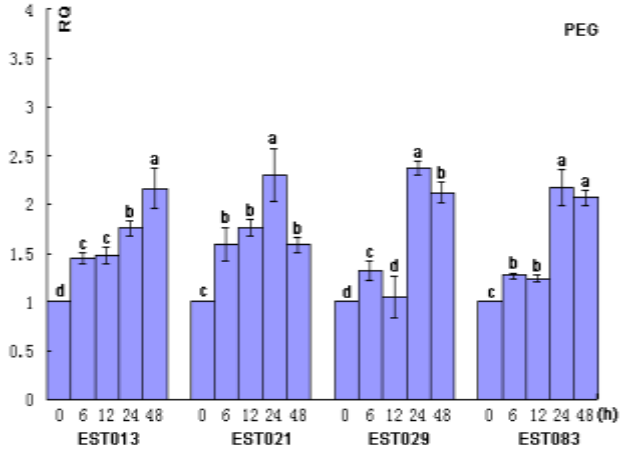

Figure 2. Expression trends of 4 EST sequences under PEG treatments in *A. mongolicus*. *A. mongolicus* were treated by PEG for 0, 6,12,24,48 h, respectively.

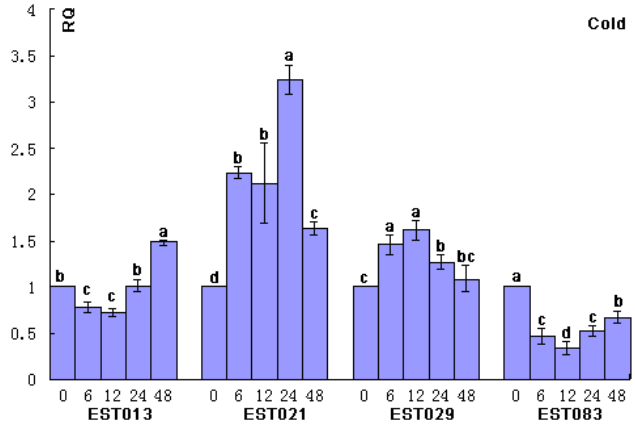

Figure 3. Expression trends of 4 EST sequences under cold treatments in *A. mongolicus*. *A. mongolicus* were treated by cold for 0, 6,12,24,48 h, respectively.

decreasing fiercely and showing a significant statistical difference. While EST083 decreased significantly at 6 h of treatment, compared with the control, it continued to increase and reached its vertex at 24 h. After this point, it decreased slightly, showing an un-significant statistical difference.

In addition, we compared the expression levels of these four TDFs under all three types of treatment. The result shows that expressions induced by a combination of drought and cold was higher than expression induced by either drought or cold alone.

DISCUSSION

In this work, cDNA-AFLP approach was used to enrich and identify drought and cold tolerance-related genes from the drought-cold-resistant plant species *A. mongolicus*. We focused on the early stages of severe

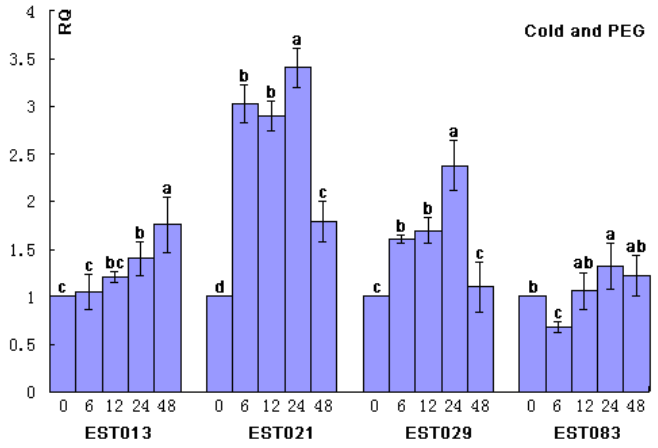

Figure 4. Expression trends of 4 EST sequences under PEG together with cold treatments in *A. mongolicus* were treated by PEG and cold for 0, 6,12,24,48 h, respectively.

drought and cold stress treatment in leaf tissues, because of the important roles that are played by early response genes in mediating the effects of drought and cold stress. A total of 220 differentially expressed fragments responsive to drought or cold were obtained and analyzed. After BLASTing against the genome of *Arabidopsis* and *M. truncatula*, the homologous sequences of 99 TDFs were found. Among these TDFs, 50 TDFs posses functional annotation and 49 TDFs are of unknown significance. However, most of these function-unknown TDFs were up-regulated by the applied stress; it is therefore postulated that, these unknown TDFs play important roles in stress resistance mechanisms. In addition, the differential expression patterns of a subset of four genes were further confirmed by real time PCR analysis.

To date, molecular marker technologies have been used exclusively in the research of plant genes important to stress resistance. In this study, we chose cDNA-AFLP analysis to look into stress resistance mechanisms in *A. mongolicus* because of advantages such as low false positive readings, high reproducibility, precise reporting of gene expression and an unparalleled access to full-scale expression information of transcriptomes, etc (Bachem et al., 1996). Although, cDNA-AFLP is a comparatively ideal technology for genomic analysis, it does harbor certain disadvantages, being time-intensive, expensive and quite sensitive to the quality and purity of the required RNA. We found these disadvantages to be acceptable in light of the many advantages already described.

To isolate the high-quality RNA required during cDNA-AFLP analysis, we employed a 2×CTAB extraction method. DTT, containing certain RNase inhibitors, replaced 2-mercaptoethanol in our RNA extraction buffer. Another factor of very real importance to cDNA-AFLP analysis is the selection of restriction enzymes. Our experiments utilized the *EcoR* I and *Mse* I restriction enzyme and we screened 64 pairs of primers for amplification. The selection of this enzyme allowed us to use less primer in our reactions and we were, as a result, able to analyze a greater number of differentially expressed mRNAs. In addition to this, we determined that our gels should be run for 30 min and that the temperature should not rise above 50°C, to prevent the bands from blurring or diffusing. Furthermore, the optimum time for photography is 3 to 5 min.

As shown in Figure 5, we found 49 function-unknown TDFs, 9 TDFs related to protein modification, 12 TDFs related to transcription and translation, 9 TDFs related to metabolism, 11 TDFs related to senescence defense genes, 1 TDF related to signal transduction, 3 TDFs related to cell development, 3 TDFs related to photosynthesis and 2 TDFs related to translocation after sequencing and BLASTing. According to the expression, trends observed in samples under drought-stress, cold-stress and a combination of drought and cold-stress were placed these into 7 classes. The details of this classification may be seen in Figure 6.

Plant tolerance or susceptibility to abiotic stress is a very complex phenomenon. It might involve the co-ordinated expression of multiple stress related genes. When undergoing stress response, signals are transmitted downstream by transduction genes, after which transcription factors combine with the specific loci of stress related genes. This process may induce the expression of functionally related genes, prompting physiological and biochemical adaptation to the abiotic stress. Perception of abiotic stresses and signal transduction is therefore, a critical step in the process of switching on the adaptive response mechanisms meant to ensure the survival and reproduction of plants exposed to adverse environments (Chinusamy et al., 2004). In most plants, cytosolic free Ca^{2+} levels rise after stress treatment. Although, EST009 was found to be related to CaM (Reddy et al., 2002), the detailed function of EST009 has never been reported.

Ethylene, one of the most important endogenous plant hormones, regulates the expression of related genes through signal transduction pathways, manipulating signal molecules and controlling many aspects of higher plant development such as seed germination, root hair formation, development and ageing, participating stress response caused by wound or pathogen, etc. (Fu and Li, 2002; Guilaume et al., 2001). Often, the physiological processes of plants are regulated by the signal response of a specific signal pathway, comprised of plant body components and signal transduction caused by signal activation or passivation (Erwin and Axel, 1998). In this study, EST007 is homological with the sequence of a gene that coded ATXRN4 in *Arabidopsis*. ATXRN4 is related to signal transduction under ethylene and up-regulated under drought. ATXRN4 also participates in the regulation of mRNA degradation (Frederic FS et al., 2004).

To a certain extent, plants seem to respond similarly to

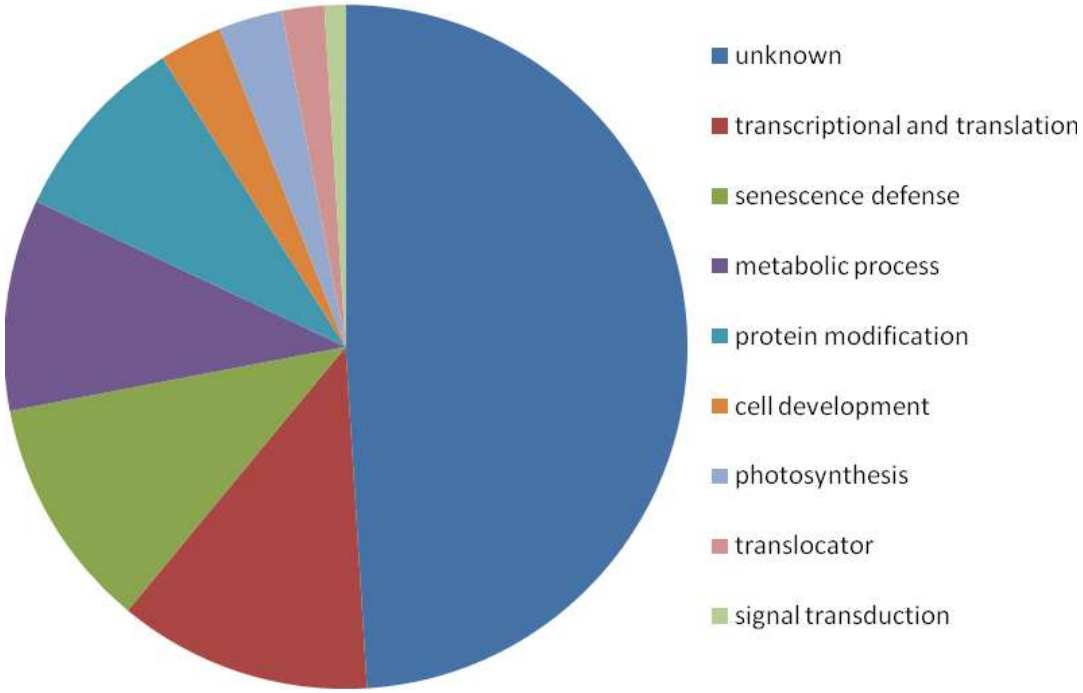

Figure 5. Functional classification of adversity-stressed differentially expressed genes in *A. mongolicus.*

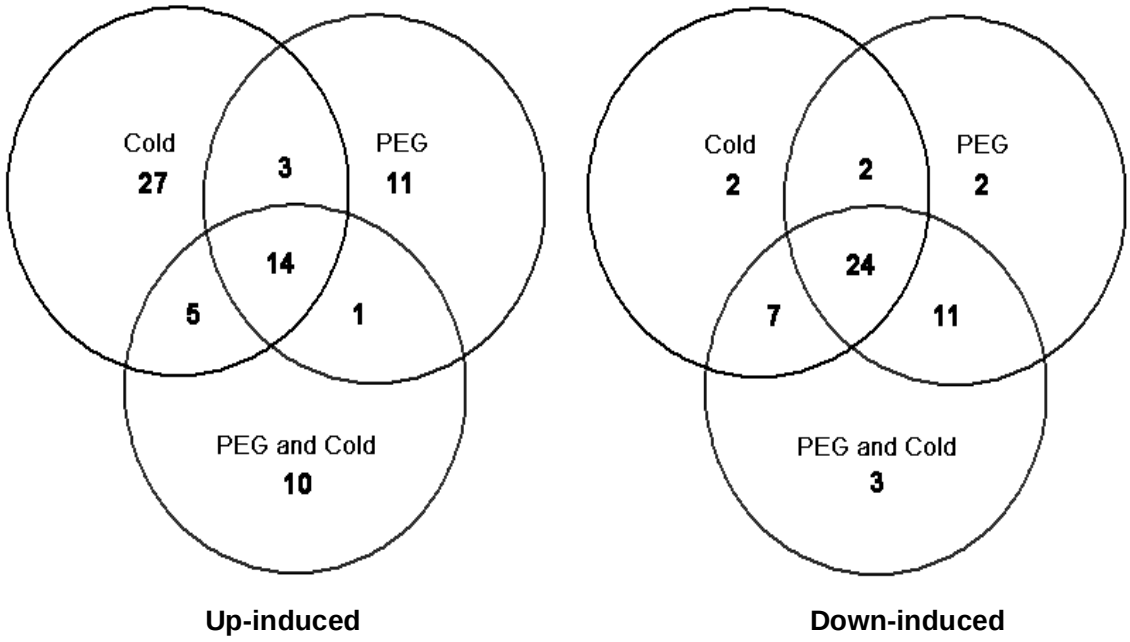

Figure 6. Classification of differential gene expression tendency under different adversity stress.

abiotic stress and senescence under natural conditions, affecting the accumulation of biomass and leaf senescence. Among the genes that are up-regulated during leaf senescence are several whose transcript levels correspondingly accumulate under abiotic and biotic stress (Shimon G et al., 2003).

Leaf senescence is regulated by the coordinated expression of specific genes. A portion of these genes control the degradation of macromolecules and the subsequent mobilization of components. The other portion resists the process of senescence and keeps the cell viable (Buchanan-Wollaston, 1997).

In this study, we found that EST022 is related to senescence. It is up-regulated under both drought and cold-stress.

As we know, photosynthesis is affected by stress and a change in a plant's ability to photosynthesize, is one obvious indicator implemented in the onset of senescence. We obtained 3 photosynthesis-related ESTs that were down-regulated under stress treatment. Many stress resistance mechanisms have formed over time as plants adapted to their stressful environments (Smart and Flemlng, 1996). When a plant is suffering drought, cold or stress of a different origin, harmful substances accumulate. It is also important to note however that, metabolism and transportation are also affected in this scenario, increasing the content of soluble small organic molecules and decreasing the penetration of harmful aggregates. For this reason, deleterious substances that accumulate as a result of stress, such as K^+, Na^+, amino acids, amino acid derivatives, saccharides, etc., are reduced. Among the EST sequences obtained during this study, EST071 is notable and it is related to transportation and down-regulated under cold induction. Most of the ESTs related to metabolism are down-regulated under stress, with exception to EST025, whose detailed function has not yet been reported. Protein modification related ESTs for example; EST011, EST058 and EST081 are up-regulated under stress. It is possible that *A. mongolicus* resists harm by bringing about a change in its metabolism and accumulating osmotic substances known to be co-regulated by these stress related genes.

Plant cell wall, capable of enhancing the cell's mechanical strength, can affect cell growth rate, transportation and participate in the water potential regulation and intercellular signal transduction. Cell wall is composed of cellulose, hemicellulose, pectin, structural protein and activate protein. EST083 and EST088 are related to cell wall modification and tissue synthesis, respectively and their expression levels increased after stress induction. EST083 is a protein phosphatase methylesterase (PME) that functions during fruit development and cell growth (Schwacke et al., 1999). We predicted that *A. mongolicus* would resist the adverse environmental conditions simulated during stress induction via cell wall modification.

In addition, defense related genes EST008, EST035, EST041 and EST043 were detected. These genes were up-regulated under stress. Some ESTs were found to relate to protein modification, for example, EST 005, EST 014 were related to protein phosphorylation. EST014 shows high sequence homology with *Arabidopsis* ARK3, whose function has yet to be reported. Protein phosphorylation is known as one of the most important posttranslational modifications and was thought to be a dynamic biological regulating process. 1/3 of the proteins in a cell are thought to be modified by phosphorylation (Zolnierowicz and Bollen, 2000). Protein phosphorylation and dephosphorylation are two key processes of expression regulation in eukaryotic and prokaryotic cells.

These processes regulate cell signal transduction, cell differentiation, cell growth and almost every life activity. Thus, protein phosphorylation and dephosphorylation have been designated as the molecular switch of cell physiological activity. It is possible that a change in plant growth environment could induce protein phosphorylation, leading to the change of protein quantity and type. EST029 is an ATP-dependent RNA helicase. Gong et al., (2005) detected a DEAD-box RNA helicase mutant in *Arabidopsis*. This mutant is related to mRNA transporttation, plant growth and stress response.

A fairly large quantity of function-unknown EST sequences was detected in this study. Some of these sequences were up-regulated under stress, others were down-regulated. For this reason, we conclude that there are still many stress related genes that have not been detected in *A. mongolicus*.

A real time PCR analysis of 4 ESTs was also included in this study. This analysis allowed us to test and verify the results of polyacrylamide gel electrophoresis and also provided us with the expression levels of stress responsive genes. Among the 4 EST sequences analyzed, we found that: EST013 is related to the transcription of chloroplast genes, EST021 is related to translation, EST029 is a RNA helicase and EST083 is related to cell wall modification. The expression regulation of plant genes is a complicated and precise process, one that typically consists of transcription and translation. Transcription always begins in the preliminary stage of gene expression. During this period, transcriptiontion factors combine with the target gene's cis-acting element and then, control the expression of a series of related genes. This process plays an important role in plant growth and development, in the origin of new species and in stress response (Li et al., 2009). RNA helicase participates in all of the RNA related biochemical processes, including RNA transcription, mRNA splicing, mRNA maturation and transportation, protein translation and RNA degradation, ribosomal assembly, gamete and embryo production, cell growth and differentiation and other life activates (Luking and Stahl, 1998; Abdelhaleem, 2005).

EST013, EST021, EST029 and EST083 were related to transcriptional modification, translation, RNA helicase and cell wall modification genes, respectively. These ESTs were selected for real time PCR analysis to verify our polyacrylamide gel electrophoresis results and further investigate expression trends. The analysis showed that, expression trends at 6 h of induction are similar to what was observed using polyacrylamide gel electrophoresis. Expression of EST013 increased consistently under drought treatment and also under a combination of drought and cold treatment. However, expression levels decreased before increasing. Both EST021 and EST029 increased before decreasing under all the three stress inductions. EST083 increased steadily under drought treatment, while it decreased first and then increased,

under cold treatment and a combination of drought and cold treatment.

In summary, the super-xerophytic desert plant *A. mongolicus* possesses multiple stress resistance mechanisms, as well as a complicated signal network capable of regulating abiotic stress response actively. The coordinated expression of multiple functional genes contributes to the ability of *A. mongolicus* to resist abiotic stress. In addition, many function-unknown genes were found in this experiment and we believe that, *A. mongolicus* may have yet more stress resistance pathways. 5' RACE will be used to clone the full length of these EST fragments.

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