The Application of a Non-Radioactive DD-AFLP Method for Profiling of *Aeluropus lagopoides* Differentially Expressed Transcripts under Salinity or Drought Conditions

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Background: *Aeluropus lagopoides* is a salt and drought tolerant grass from Poaceae family, distributed widely in arid regions. There is almost no information about the genetics or genome of this close relative of wheat that stands harsh conditions of deserts.
Objectives: The main aim of this research was to isolation and characterization of salt and drought inducible genes from *A. lagopoides* by Differential Display Amplified fragment length polymorphism (DD-AFLP) method.
Material and Methods: In this research *A. lagopoides* was grown under salt or drought conditions and after modifying the DD-AFLP method several fragments were isolated and after nomination their induction was studied by reverse northern blotting.
Results: DD-AFLP led to the improvement of a non-radioactive method for which many parameters were optimized. Having screened approximately 1600 transcript-derived fragments, 1.4 percent of them showed varied expression levels in response to high salt or drought treatments. The relative abundance of twenty one selected differentially expressed fragments was inspected by reverse northern blotting that affirmed the potential of this applied method. Sequence comparisons revealed that some of the isolated genes are involved in osmotic adjustment, regulation of transcription, cation transportation and stress responses. These data clearly show that the modified DD-AFLP method was a successful and reliable approach for the isolation of differentially expressed genes.

Keywords: *Aeluropus lagopoides*; DD-AFLP; Drought; Inducible genes; Salinity

1. Introduction

A high percentage of agricultural regions are affected by salinity and drought (1). Therefore, these osmotic stresses are considered as the most important environmental factors affecting plant productions. Members of a family, genus or even species use different mechanisms in response to salt stress (2). In Agriculture, different responses to salt stress in close relatives of crops are of interest in order to determine salt tolerant mechanisms. Biological responses and developmental programming of cells are eventually controlled by precise regulations of gene expressions (2). While comparative genetic mapping in plants has provided evidence for a remarkable conservation of marker and gene order (colinearity) between related grass genomes (3), still reliable methods are required to reveal the gene expression profiles in various environmental conditions and/or developmental stages.

Many molecular techniques are now available to identify and clone differentially regulated genes. In 1992, a PCR-based method, collectively referred to as differential display (DD) or RNA fingerprinting was developed in which a set of arbitrary primers were used to amplify cDNA fragments representing

expressed genes (4). This method was widely accepted and many improvements were made to gradually fix technical problems (for a review, see 5). Three years later, amplified fragment length polymorphism (AFLP) method was introduced as a robust marker for the visualization of genomic variations (6). Soon after, these two methods were coupled so as to monitor differentially expressed genes in a more reproducible manner (7). In this method, double-stranded cDNA molecules are restricted and ligated to the defined adaptor sequences. Following amplification of a subset of ligation products with adaptor-specific primers carrying two or more arbitrary nucleotides, gene-representative bands are detected on a polyacrylamide gel (8). This methodology, named differential display-amplified fragment length polymorphism (DD-AFLP), was also subjected to many improvements such that it is now considered as high throughput method in functional genomics. As the technique relies on restriction digestions and the use of arbitrary nucleotides, DD-AFLP patterns of expressed transcripts could be readily simulated for sequenced genomes by computer software such as GenEST (9). DD-AFLP also produces a great deal of information even for undetermined genomes as there is no need for prior sequence information.

Alongside, to reduce the hazards of working with radioisotopes during gel electrophoresis and subsequent band excision, several researchers have explored the use of non-radioactive methodologies such as staining of DNA fragments with silver nitrate on PAGE (10, 11) or ethidium bromide on agarose gel (12) and also digoxigenin labeling of primers (13). In the year 2003, Dubos and Plomion (14) showed the reliability of silver staining simply by comparing with an autoradiogram of the same gel. In addition, DD-AFLP is particularly useful for the study of plant-pathogen interactions because the method facilitates discovery simultaneously in both organisms. These advantages are emphasized by Polesiani and colleagues (2008) (15) in a study about differential gene expression pattern in grapevine infected with *Plasmopara viticola*. Despite all improvements and advantages, both Differential Display (DD) and DD-AFLP methods are essentially employed for amplification of 3´-ends of expressed transcripts. This is advantageous for distinction of expression pattern of gene family members. However, there is a major drawback as the functional annotation is aimed only where the coding region sequence is required.

DD-AFLP is a commonly used, robust, and reproducible tool for genome-wide expression analysis in any species without requirement of prior sequence knowledge. Therefore it is employed for profiling of transcriptional responses of a variety of plants or tissue effects to different treatments and/or conditions to discover responsive genes. Recently, this technique is used for the detection of responsive genes in plants and plant tissues subjected to jasmonate (16). Dae *et al*., (2012) (17) identified 87 salt stress-responsive transcripts using the cDNA-AFLP technique in *Brachypodium distachyon* which 32 TDFs corresponded to *Brachypodium* genes with locus name. In an attempt to identify transcripts induced by heat stress, twenty-day-old rice seedlings of different rice cultivars suffering from heat stress were treated at different times (18). Consequently, more than three thousand different fragments were indentified, and 49 fragments were selected for sequencing and differential expressed genes were classified functionally into different groups (18).

Aeluropus lagopoides is a halophyte member of Poaceae family growing in the salt marsh regions (19). Also, it is a close relative of bread wheat. Because of the high-salt (20) and drought (21) tolerance potential of *Aeluropus*, we planned to investigate molecular aspects of *Aeluropus* salt stress tolerance. Here, we have reported some modifications and simplification of a procedure for DD-AFLP of plant mRNA species by systematic examination of several parameters. The methodology was extended to represent both 3´-end and internal fragments of expressed genes for the first time. The optimized method was used to isolate salt and drought- responsive transcripts from *Aeluropus lagopoides* shoots exposed to salinity and drought treatments.

2. Material and Methods

2.1. Plant Material
Seeds of *Aeluropus lagopoides* collected from central arid regions of IR Iran were sterilized and cultured on 1cm^2 pieces of steel mesh placed on the solid MS (22) medium. After 21 days of growth at 25-30ºC with a photoperiod of 16 h light/8 h darkness, seedlings were transferred into liquid ½ strength MS medium and grown in the above conditions as described previously (23). Medium was refreshed every 3 days. For high salinity treatment, up to 600 mM NaCl was added to the above medium. After 10 days, shoots were collected and stored at -80ºC. Drought treatments was done by 18 days of water withholding as described by

Mohsenzadeh *et al.*, (2006) (21). Control plants were watered every other day. All treatments were conducted in at least two independent biological replicates.

2.2. DNA Template Preparation

Harvested shoots were ground to fine powder in liquid nitrogen with a mortar and pestle and used for direct mRNA extraction as described by Schaeffer (24). Double-stranded cDNA synthesis was carried out using 0.3-2 µg mRNA according to cDNA Synthesis System Kit instructions (Roche Biochemical, Manheim, Germany). cDNA synthesis products were cleaned up with phenol:chloroform:isoamyl alcohol (50:49:1, v/v) and ethanol precipitation. Double-stranded-cDNA molecules were restricted by a tetramer restriction enzyme such as *Taq* I.

An asymmetrical adaptor was made using 50 µM of synthetic oligo-nucleotides named L-4 and L+4T2 (Table 1) in a solution of annealing buffer composed of 10 mM Tris-HCl pH 7.5, 5 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. The oligonucleotide solution was heated at 95ºC for 5 minute and slowly cooled to 39ºC for proper annealing. About 1 µg of digested cDNA molecules were ligated to 60 pmole adapter in the presence of 1.5 units T4-DNA ligase in a standard reaction (25).

2.3. DD-AFLP Reactions

Several variables were examined to establish the optimized DD-AFLP protocols as described below: For PCR1, diluted adapter-ligated cDNA fragments (see Results) was used in a volume of 20 µl PCR reaction consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.25 mM of each dNTP, 6 pmole primers, 2 mM MgCl$_2$, 1 unit *Taq* DNA polymerase (Ampliscence). The primer pairs for 3´-end or internal gene fragments were L-4 and DTNN or L-4 and MT (Table 1), respectively. The reaction was incubated at 72ºC for 1 min to fill in the adaptor ends, prior to PCR1 reactions. Pre-amplification of internal gene fragments was carried out with a 12 cycles of 94ºC for 30 s, 52ºC for 1 min, 72ºC for 2 min and a final extension step at 72ºC for 5 min. For amplification of 3'-end gene fragments, the PCR1 reaction was performed with a one cycle of 94ºC for 30 s, 52ºC for 1 min, 72ºC for 2 min, and the 12 cycles of 94ºC for 30 s, 58ºC for 1 min, 72ºC for 2 min and followed by final extension at 72ºC for 5 min. For PCR2, diluted PCR1 products (see Results) were added to 20 µl PCR reaction (as above) containing 6 pmole of one of the L-4TNN, the arbitrary primers (Table 1). PCR2 was carried out with the following

Table 1. A list of oligonucleotides that were used for adapter preparation and DD-AFLP reactions

Name	Sequence	T$_m$(ºC)
MT	TTT TGA ATT CTT TTT TTT TTT TTT TTT T(AGC)	52
L-4	GTG TGG ATC CTG GGT GTG GTG T	64
L+4T2	CGA CAC CAC ACC CAG GA	58
DTNN	TTT TTT TGA ATT GTT TTT TTT TTT TTT TCC	58
L-4TNN	GTG TGG ATC CTG GGT GTG GTG TCG ANN	>62

program: 94ºC for 1 min, then 35 cycles of 94ºC for 30 s, 64ºC for 30 s, 72ºC for 2 min, and final extension at 72ºC for 5 min.

2.4. Visualizing DD-AFLP Products and Re-amplification

PCR2 products were loaded onto a 4-5% polyacrylamide gel and run at 90V for about 3 h. Gels were silver-stained according to Bassam and colleagues (1991) (26). Differentially expressed cDNAs were cut from the gels, frozen in liquid nitrogen, and thawed in 20 µl sterile double-distilled water. One µl of the eluted DNA in water was used for re-amplification using the same primers and conditions as PCR2 reaction. The amplified fragments were cloned in pBluescript plasmid (Clontech, Palo Alto, CA) or TA cloning vector (Roche, Ins T/A clon PCR product) for sequencing and cross hybridization among clones. Analysis for sequence similarity and structural features were conducted by the use of online databases and related software that included searches against EST sequences in gramineae plants gene index section of TIGR database or GenBank by BLAST softwares (27).

2.5. Cross-hybridization and Reverse Northern Blotting

Cross-hybridization and reverse-northern blotting were performed following the procedure of Mohsenzadeh *et al.*, (2006) (21). Duplicate H$^+$ nylon membranes (Roche Biochemical, Manheim, Germany) were prepared by placing dots of reamplified, alkaline-denatured DNA inserts of the isolated clones. To synthesize labeled cDNA molecules, 10 µg heat-denatured total RNA isolated from stressed and non-stressed plants were added into cDNA synthesis reactions consisting of 80 units of Expand reverse transcriptase, 50 mM Tris-HCl, pH 8.5, 8 mM MgCl$_2$, 150 mM DTT, 1.25 units.µl^{-1} RNase inhibitor, 0.5 mM dNTP mixture, 0.13 mM dTTP, and 0.07 mM DIG-

dUTP (Roche Biochemical, Manheim, Germany) incubated at 42°C for 2 h.

Prehybridization and hybridization reactions and stringent washes and detection steps were carried out according to the DIG Labeling and Detection Kit instructions (Roche Biochemical, Manheim, Germany). The intensity of each dot was quantified by TotalLab software (Phoretix International, Tyne House, Newcastle, UK). All data were normalized by dividing the given volume value for each dot by that of alpha-tubulin or genomic DNA. The relative expression level was calculated as the ratio of dot intensities hybridized with labeled cDNA derived from stressed plants by that of the non- stressed ones.

3. Results

3.1. Salt and Drought Treatments

Hydroponically-cultured seedlings of *A. lagopoides* were treated with different concentrations of NaCl. Based on preliminary physiological studies (data not shown), 10 days of treatment with 600 mM NaCl concentration was chosen as harsh, long-term salt treatment.

Additionally, vegetatively propagated plants were grown for one month in green house. Samples were collected after 18 days of water withholding. At the same time, control plants were well-watered.

3.2. Optimization of DD-AFLP Protocol

As schemed in Figure 1, DD-AFLP method consisted of two major steps: (1) template preparation by the synthesis of cDNA and ligation of adapters to the restricted cDNA fragments; (2) visualization of cDNA fragments representing expressed genes by PCR with primers carrying two or more arbitrary bases. The latter step requires deliberate optimization for production of clear, consistent and reliable banding patterns that represent gene expression profiles in treated cells. Here, we used only one 4-mer cutting enzyme which leads to creation DNA templates for amplification of both internal or 3′-end fragments of genes. For the ease of the work, the optimization steps were examined by small PAGE proceeded with long gels in the terminal parts.

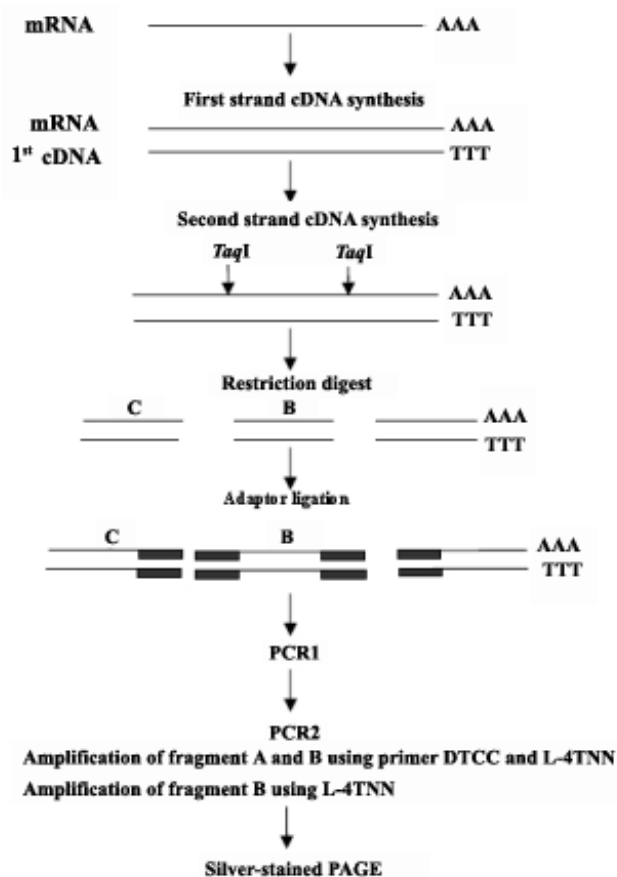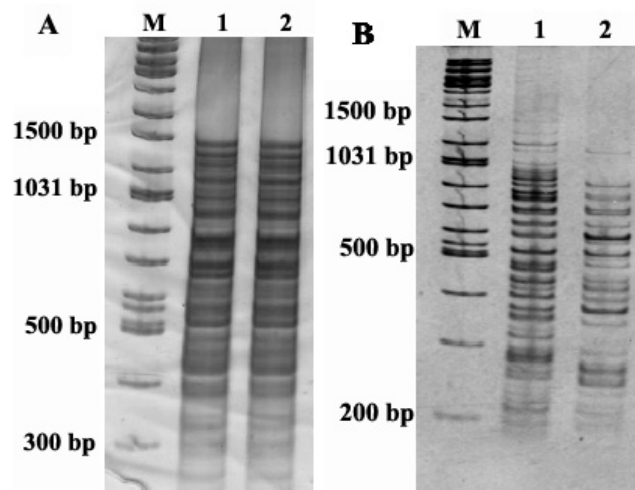Empirically, the best banding pattern for the inter-

Figure 1. A schematic representation of major DD-AFLP steps in which the amplified fragments corresponding to 3'-ends (A) and internal regions (B) of expressed genes are shown

Figure 2. Illustration of the effect of annealing temperature during PCR1. A: DD-AFLP of the internal gene fragments; L-4 and MT primers were used in PCR1 reactions with annealing temperature 52°C in all 12 cycles. B: DD-AFLP reactions for the 3'-end; L-4 and DTCA primers were used in PCR1 reactions run at 52°C as annealing temperature for the first cycle followed by 12 cycles at 58°C. Lane 1 and 2 are PCR replicates of the same samples. All PCR2 reactions were performed with annealing temperature of 64°C. Lanes M are 100-bp molecular weight ladder
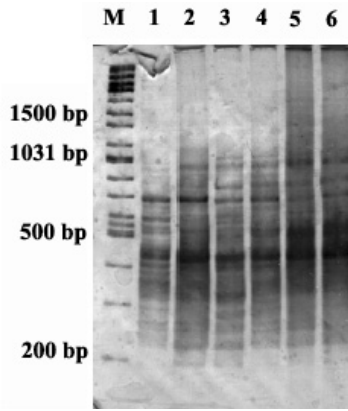
Figure 3. Illustration of the effect of MgCl$_2$ concentrations in PCR2 reaction. In this selected image, the final MgCl$_2$ concentrations in PCR1 was 2 mM. The final MgCl$_2$ concentrations in PCR2 were 1, 1.5, 2, 2.5, 3, 3.5 and 4 mM in lane 1 to 6, respectively. Lane M is 100-bp molecular weight ladder
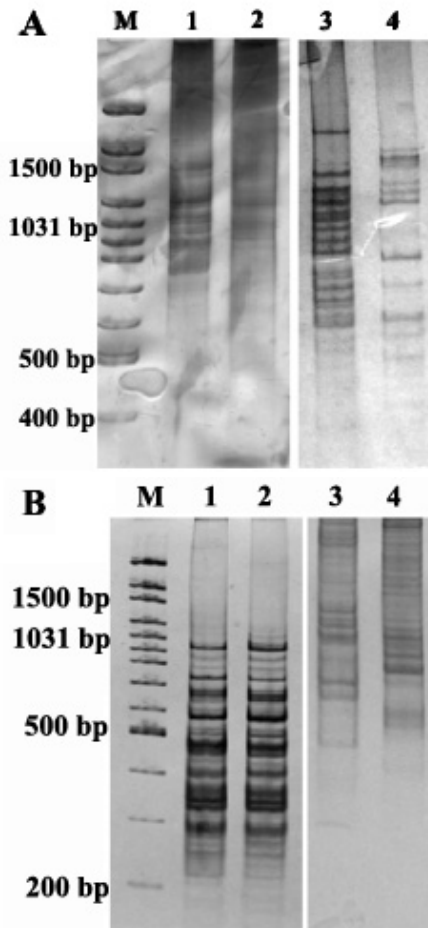

Figure 4. A: Illustration of the effect of extension time for 1 min (lanes 1 and 2) and 2 min (lanes 3 and 4). In this panel, mRNA samples deriving the DD-AFLP reactions in lane 1 and 3 or lane 2 and 4 were the same extracts. B: Illustration of the effect of PCR1 cycle numbers for 12 (lanes 1 and 2) and 20 cycles (lanes 3 and 4). In this panel, each pair of lanes represents the same mRNA samples. Lanes M are 100-bp molecular weight ladder

nal gene fragments was observed when annealing temperature was adjusted at 52°C for all 12 cycles in PCR1 (Figure 2A). However, for the 3'-end gene fragments it was necessary to set annealing temperature of PCR1 at 52°C for the first cycle and increased to 58°C for the other 12 cycles (Figure 2B) so that templates with high-Tm priming sites were enriched.

To find out the best MgCl$_2$ concentration, various levels of MgCl$_2$ were used in both PCR1 and PCR2 reactions. We generally obtained clearer banding patterns when they were obtained between 1.5 to 2.5 mM MgCl$_2$ in either PCR reactions. Since a wide range of sizes, high resolution and uniformity of amplified bands are desired in DD-AFLP, 2 mM MgCl$_2$ was found to be the optimal concentration (Figure 3).

The extension time and the cycle numbers were both effective in obtaining consistent results. When the duration of extension time was increased from 1 to 2 min, the amplification of ESTs with the size of 200 to 1500 bp was allowed leading to clear banding patterns (Figure 4A). The most pronounced effect was observed for PCR1 cycle numbers as 12 cycles provided better banding pattern than 20 cycles (Figure 4B), probably due to lesser over-representation of amplified molecules.

We consistently observed that DNA banding became randomized when initial amounts of DNA templates were too low in PCR1 or 2. Similarly, Vos
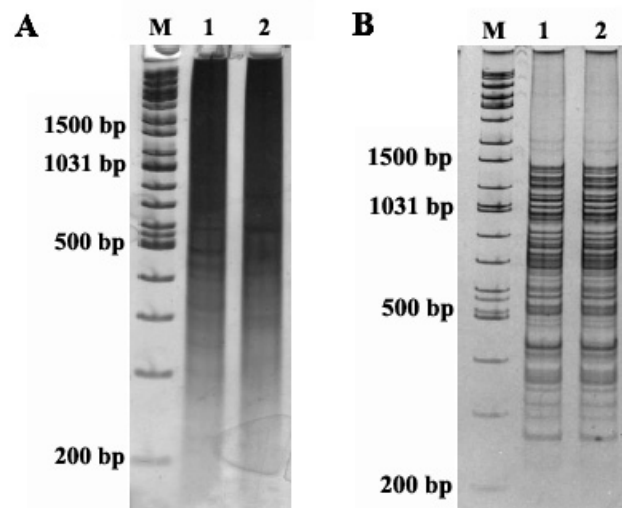

Figure 5. A: Illustration of the effect of using primers with two arbitrary nucleotides for 3'-end or internal gene fragments in PCR2. B: Illustration of the effect of using primers with four arbitrary nucleotides for 3'-end or internal gene fragments in PCR2. In PCR1, we have used L-4 and MT primers. Primers L-4TGT and DTCC (A) or L-4TGT alone (B) were used in PCR2 reactions for 3'-end or internal gene fragments. Lanes M are 100-bp molecular weight ladders
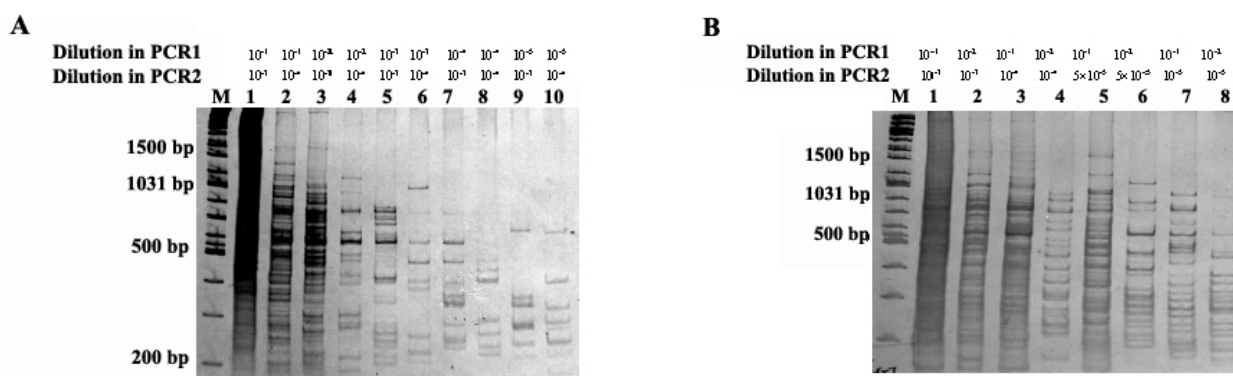
Figure 6. A: The effect of reducing initial amounts of template DNA in PCR1 B: The effect of reducing initial amounts of template DNA and PCR2. Panel A, ligation products were diluted for 10^{-1} to 10^{-5} times as shown, before addition 1 µl of each into the PCR reactions. Two dilutions of each PCR1 products (10^{-3} and 10^{-4}) were used in PCR2 reactions. Panel B, after first amplification of two dilutions of ligation products (10^{-1} and 10^{-2}), PCR1 products were diluted for 10^{-3} to 10^{-5}. Five µl of each dilution was added to PCR2 reactions. Lanes M are 100-bp molecular weight ladders

and colleagues (1995) (6) reported that if the initial DNA template were reduced to below one picogram, the banding patterns were not reproducible any more. On the other hand, when we used high amounts of DNA template with primers carrying only two arbitrary nucleotides, numerous amplified bands produced smeary patterns. We concluded that this is due to the presence of too many DNA templates resulting from the digestion by a 4-mer restriction enzyme. When the number of arbitrary nucleotide was chosen to be four, two on each side, the variation in banding patterns was greatly reduced (Figure 5 and also see the next section).

The effect of several dilutions in initial DNA template was also examined in PCR1 and PCR2 reactions. It appeared that by reducing the amounts of initial DNA template, the banding pattern became clearer. It was essential to keep the overall dilution coefficient around 10^{-5} as shown by using several dilution levels of template DNA in both PCR1 and PCR2 (Figure 6A and B).

As explained above, the use of single adapter-specific primer for PCR2 of DNA fragments allows visualization of sequences with digestion sites at both ends. These correspond to the sequences located in the middle of expressed genes. Again, the banding pattern became randomized if template DNA was too diluted, e.g. at overall dilution factor of 10^{-6} or below. Unlike PCR1, little effect was observed after increasing the amounts of DNA templates to initiate PCR2 reactions (Figure 6B).

One may also be interested in monitoring differential expression at the 3'-end of mRNA where the high-est sequence diversities among gene family members are expected. However, since oligo-dT primer needs low annealing temperature, mis-priming leads to smeary patterns such that further modification in the reaction conditions is required. Using a longer oligo-dT primer with two arbitrary nucleotides along with adapter-specific primer, we achieved a reliable banding pattern (Figure 2B).

Putting together, we concluded optimized protocols for DD-AFLP as detailed in Material and Methods. A typical example for the application of this methodology is shown in Figure 7. Using a long polyacrylamide gel electrophoresis system, 80-100 bands can be resolved in each lane. On average, 1.4 percent of bands were found to be differential when samples of drought or high-salt treated and control *Aeluropus* plants were compared.

3.3. Characterization of Differentially Expressed Fragments

Differentially represented DNA fragments were cloned and subjected to cross hybridization to find if there is more than one type of DNA fragment in each DD-AFLP band and if distinct bands correspond to the same gene fragments amplified by different sets of primers (Tables 2 and 3). In this way, the cross-hybridized fragments were sorted into distinct groups as they must carry highly similar sequences. One representative of each group was sequenced and stored as an expressed sequence tag (EST) related to salinity or drought in GenBank under accession numbers CX79913 to CX79924 and CV225297 to CV225306, respectively. The corresponding genes were named as
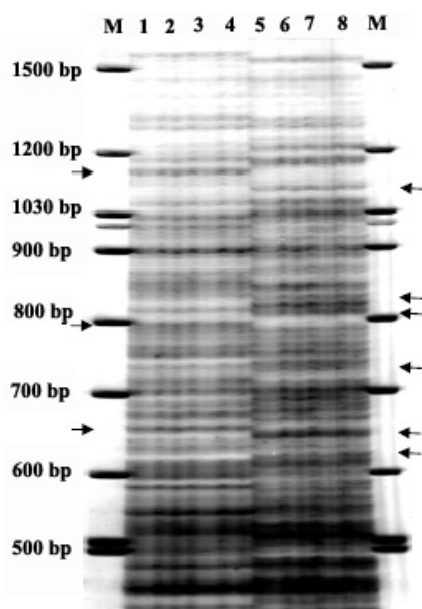
Figure 7. A typical example of cDNA-AFLP products corresponding to the internal fragments of genes separated on a 40-cm long PAGE. mRNA samples were extracted from either drought-treated (lane 1 to 4) or control (lane 5 to 8) shoots. For each one, two PCR reactions of two independent biological samples were used. Some differentially represented bands are well distinguishable (arrows). Lanes M are 100-bp molecular weight ladders

Aeluropus salt response, *asr*, (Table 2) or *Aeluropus* drought tolerance, *adt*, genes (Table 3). BLAST searches (28) succeeded to predict full length gene sequences in some cases, such as *asr4*, *asr5* based on EST and TC data produced in genome projects of cereals such as wheat, rice and barley. Translated sequences were used for both homology and motif searches to bring about possible annotations based on available data in primary and secondary databanks. These analyses revealed the involvements of some known salt stress responsive proteins in osmotic adjustment such as betaine aldehyde dehydrogenase. Some of the encoded proteins were also related to signal transduction pathways.

3.4. Expression Analysis of the Isolated Fragments

In order to confirm that the identified genes are inducible, the expression of the cloned fragments in *Aeluropus lagopoides* shoots was analyzed by reverse northern blot. Total cDNA probes were made using total RNA extracted from the shoots of plants (29) cultured hydroponically in the absence or presence of 600 mM NaCl. As shown in Figure 8, all of the fragments were induced by salinity except for *asr1,* confirming the robustness of the improved DD-AFLP method for

Table 2. Sequence similarities of *A. lagopoides* ESTs, isolated under salinity, classified as functional and regulatory protein groups

A. lagopoides EST	Length (bp)	GenBank Accession Number	Relative expression level[1]	Known motives	spices	E-value[2]	Tentative annotation
asr1	296	CX779716	+1	-	*Cytophaga hutchinsonii*	7.2	histidine kinase
asr2	666	CX779719	+5	ZF-CCHC	*A.thaliana*	0.037	Putative DNA-bindin
asr3	538	CX779720	+5	-	*O.sativa*	4e-09	Protein
asr4	661	CX779721	+5	-	*T.aestivum*	7.7e-08	Retrotransposon protein
asr5	170	CX779722	+2	Cys-Thre-Gly-rich regions	-	-	BADH Unknown
asr6	140	CX779723	+7	-	*T. aestivum*	7.3e-12	Low temperature-responsive RNA-binding protein
asr7	301	CX779718	+3	-	*A. thaliana*	5e-04	RNA-binding protein
asr8	387	CX779724	+6	Alpha-2B adrenergic receptor signature	*H. vulgare*	2.1e-05	Transmembrane protein
asr9	349	CX779717	+5	AT-hook	*O. sativa*	1e-09	Retrotransposon protein
asr10	412	CX779715	+3	-	-	-	Unknown
asr11	498	CX779714	+1.1	2FE2S-FER1	*O. sativa*	5e-10	Copia-type Polyprotein
asr12	482	CX779713	+6	Proline-rich protein signature	*Z. mays*	1.2e-36	NBS-LRR-like resistance protein

[1]Before calculating the relative intensities, darkness of corresponding dots were normalized with that of genomic DNA. Plus and minus signs show up or down regulation as a result of drought treatment, respectively
[2]*E*-value is the chance of relatedness calculated for the best matching conserved motif sequence search in secondary protein data bases

Table 3. Sequence similarities of *A. lagopoides* ESTs, isolated under drought, classified as functional and regulatory protein groups

A. lagopoides EST	Length (bp)	GenBank Accession Number	Relative expression level[1]	Known motives	spices	E-value[2]	Tentative annotation
adt1	470	CV225297	+2	ZF-RING finger	*Triticum monococcum*	3e^{-20}	Nuclear protein
adt2	347	CV225298	+1.5	Aspartic acid and glutamic-rich regions	*Triticum monococcum*	1.3e^{-2}	Putative transcription factor
adt3	515	CV225299	+1.5	Integrase core domain	*Orysa sativa*	1.1e^{-34}	Integrase
adt4	243	CV225300	+2.5	WD-40 domain	*Orysa sativa*	3.7e^{-12}	Signal transduction component
adt5	737	CV225301	+1.6	Zinc knuckle	*Triticum monococcum*	1.8e^{-5}	Putative polyprotein
adt6	290	CV225302	-12	-	*Triticum monococcum*	-	Unknown
adt7	321	CV225303	-2	Glycine and pro-line-rich regions	*Triticum monococcum*	8e^{-9}	ATP-binding transferase
adt8	482	CV225304	+2.4	Catalytic domain of protein kinase	*Sorghom bicolar*	5e^{-70}	Protein kinase
adt9	607	CV225305	-2.7	Cation transport ATPase	*Triticum monococcum*	2.1e^{-11}	Cation transporter
adt10	634	CV225306	+1.7	Tyrosine kinase	*Triticum monococcum*	2e^{-41}	Tyrosine protein kinase

[1]Before calculating the relative intensities, darkness of corresponding dots were normalized with that of genomic DNA. Plus and minus signs show up or down regulation as a result of drought treatment, respectively

[2]E-value is the chance of relatedness calculated for the best matching conserved motif sequence search in secondary protein data bases

the screening of differentially expressed genes.

In addition, we isolated a total number of 20 fragments using DD-AFLP in plants exposed to drought of which 11 bands were up-regulated and 9 bands were down-regulated. After cross hybridization, the isolated gene fragments were classified in 7 up-regulated and 3 down-regulated ones that were sequenced and analyzed for preliminary annotations (Table 3). Interestingly, it appears that all the identified genes are involved in regulatory and transportation events. The expression levels of these genes were reconfirmed and quantified by reverse northern blot as shown in Figure 8 and Table 3 (21).

4. Discussion

DD-AFLP method offers a simple, rapid means of monitoring differential gene expressions during physiological events, response to environmental conditions or developmental stages. Here, we have investigated the effect of several influencing parameters such that a couple of detailed protocols for the internal and 3´-end gene fragments were introduced. Also, using one restriction enzyme and an adapter in template preparation and one primer in each PCR reaction were other advantages of modified DD-AFLP method. Besides, silver staining method helps with direct elucidation of bands and also reduces potential hazards associated with the use of radioisotopes.

Several successful applications of DD-AFLP have been reported in a wide range of biological systems (7, 10, 30, and 11). However, because of sophistications due to multiple steps, almost all authors were obliged to introduce some modifications in the procedure. The selection of enzyme is a crucial point for optimizing results since it affects the number of different sequences represented in the fingerprint. Habu and colleagues (1997) (31) showed that a meaningful banding pattern could be produced using *Taq*I alone. This enzyme cuts potato full-length cDNA molecules at a frequency of 92% (8). We also digested ds-cDNA
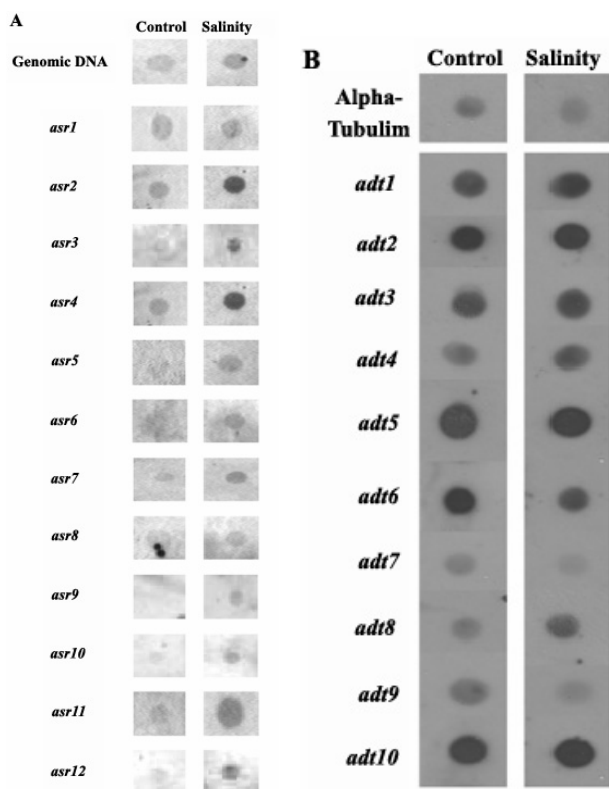
Figure 8. A: Reverse northern blotting results of cDNA-AFLP derived fragments from *A. lagopoides* under salinity B: Reverse northern blotting results of cDNA-AFLP derived fragments from *A. lagopoides* underdrought conditions

molecules by one tetramer restriction endonuclease such as *Taq*I or *Sau*3AI, both of which provide sticky ends for the efficient ligation of adaptor to the ends of restricted fragments. An asymmetrical double-stranded adaptor was then ligated to the digested fragment ends to produce priming sites for subsequent PCR reactions.

In order to make sure that digestion and ligation reactions are working well, our effort was mainly devoted to the optimization of crucial parameters for two consecutive PCR reactions. Ideally, we desired high-resolving banding patterns with an acceptable reproducibility for technical and biological replicates. Beginning with a previously established protocol for genomic AFLP reactions (5), we lowered annealing temperature to 52°C for all 12 cycles of PCR1 to produce initial DNA templates for the internal gene fragments. Then, the stringency was kept high enough during PCR2 by increasing annealing temperature to 62°C or higher. This also reversed possible suppression

effect caused by formation of panhandle-like structure between complementary adaptor sequences ligated to both the ends of denatured DNA molecules.

We also introduced some modifications for DD-AFLP of the 3'-ends of genes in order to get rid of smeary patterns. As short oligo-(dT_{15}) primers may interfere with proper DNA template synthesis by annealing to false sites, we designed DTNN primers with a longer length and two arbitrary nucleotides at 3'-end. However, because of using the above oligo-(dT_{15}) primers during cDNA synthesis step, it was necessary to run the first cycle at low annealing temperature (52°C). Having made new DNA templates, the other 12 cycles were run at annealing temperature of 58°C (Figure 2A and B).

We obtained a better banding pattern when the extension time was increased to 2 min (Figure 4A). This was not surprising as the size of amplified fragments ranged between 200 to 1500 bp. Apparently, the lengthy extension time ensures full DNA synthesis in every cycle. Additionally, further increase in cycle number of PCR1 demolished the banding pattern probably because it over-represented some DNA fragments that are being used as template in PCR2 (Figure 4B).

When working on appropriate dilutions of DNA templates for PCR reactions, we faced a challenge of getting either smeary or randomized banding patterns with the use of high or low amounts of initial DNA concentrations. Vos and colleagues (1995) (6) encountered similar problem with genomic DNA. We reasoned that if too many template molecules are to be amplified, the chance of getting smeary pattern is high. On the other hand, too low amounts causes funder effect on initial template DNA population used for subsequent DNA amplification cycles. Therefore, we decided to reduce the number of DNA template types by having four selective nucleotides (two on each primer) at the cost of increasing initial DNA concentrations (Figure 5). As shown in Figures 6A and B, DNA template at an overall dilution coefficient of 10^{-4} produced consistent band representations of internal gene fragments while that was around 10^{-5} for DD-AFLP of 3'-end fragments. This was expected as there are higher numbers of DNA template molecules to be primed by DTNN oligonucleotide molecules.

Using *Taq*I restriction enzyme and 16 different primers, each of which produced approximately 80-100 bands, we screened around 1600 expressed genes in *Aeluropus lagopoides* plants subjected to osmotic stresses. Approximately, 1.4 percent of the bands were differentially expressed in the shoots of drought or

salt-treated plants. This is very close to estimations obtained by cDNA microarray experiments. For instance, four and three percent of genes were expressed differentially in *Arabidopsis* in response to drought and high salt concentrations, respectively (32).

In this study, we isolated a series of salt and drought inducible *A. lagopoides* ESTs that were expressed differentially at 10 days after 600 mM NaCl treatment or 18-day drought-treated plants using the DD-AFLP method (Tables 2 and 3 and Figure 7). On the basis of the DD-AFLP and reverse northern blot analyses, 22 fragments were identified as salt or drought inducible genes. Some of these genes were previously known to be involved in stress signaling, gene expression regulation, and osmotic adjustment processes. Further characterization of the identified genes, particularly with respect to their functions and roles during salinity and drought is underway.

As cDNA-AFLP is a robust technique in gene identification from organisms suffering different conditions, it is recently used for the differentially genes isolation from *Brachypodium distachyon* (17) and rice (*Oryza sativa* L.) (18) under salt and heat stresses, respectively. Better understanding of the mechanisms of salt or heat stresses tolerance in *Brachypodium* and rice would be very useful for the breeding and genetic engineering of salt or heat tolerance varieties in other Poaceae family members, including wheat, barley, and rice.

Additionally, in an independent study on *Rosa hybridae* by the modified cDNA-AFLP under stringent PCR conditions, we identified transcripts that were strongly expressed during petal senescence of two cut rose cultivars, 'Black magic' and 'Maroussia', with different longevities. About 1,600 cDNA fragments, ranging in size from 250 to 1,200 bp, were reproducibly detected. This allowed the identification of 22 differentially expressed cDNAs corresponding to genes belonging to different functional categories related to senescence, signal transduction pathway, carbohydrate metabolism, energy and other catabolic events such as protein, lipid and cell wall degradation (33 and 34).

Acknowledgments

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