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Expression of Chloroplast-Related DNA Sequence in Salt Sensitive Sunflower Line by Salt Stress

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Abstract

Adverse environmental conditions including high salt concentration, temperature fluctuations, drought, and poisonous gas stress can result in a series of physiological metabolic reactions in plants. Response to these stresses comes out in series of adaptations in the prevailing environment by altering the gene expression to synthesize the most appropriate proteins. The study conducted was aimed to analyze the expression of differentially expressed DNA-sequences in salt sensitive sunflower line subjected to stress conditions. Differential display RT-PCR was performed on salt sensitive line of sunflower plants to investigate expression pattern of related transcripts under salt-stress. The plant leaf tissues were subjected to salinity stress for a short duration (24 h time period) and analyzed for the expression pattern of transcripts accumulated during that period of stress. Only the upregulated transcripts were taken under considerations so as to determine the salinity stress indicators. Sequencing revealed the amplified upregulated cDNA fragment (SS-A3H28), residing in chloroplast part of the plant cell, to be the potential indicator for stress related expression in sunflower. It was concluded that the plant promptly responded to the stress conditions by altering the gene expression of various regulatory metabolic processes, especially the photosynthesis.

Keywords: Salt stress, Sunflower, Differential Display-PCR, Chloroplast

Introduction

High salt concentration is among the significant abiotic stresses adversely affecting plant development and yield for a wide range of crops (Jaleel et al., 2007; Athar et al., 2008). The distressing effect of and Flowers, 2005). The outcomes have been ion toxicity and osmotic stress experienced by plants (Mandhania et al., 2006), following damage at molecular level that has the consequences in limited growth (Jaleel et al., 2008). Modifications at molecular level in resistance-concerning metabolic pathways in different plant species, proved successful in enhancing the salt tolerance and then applying these to the experimental species.

Plant species have been studied for their responses (physiological/molecular) when subjected to different stresses (Zhu et al., 2005; Selvam et al., 2009; Kang et al., 2010). The differential screening has been helpful at molecular level in sorting out the novel endurance mechanisms (Amtmann et al., 2005; Bohnert et al., 2006), subsequently to find out the key responses (molecular) for model species (Cushman and Bonhert, 2000; Shinozaki et al., 2003; Valliyodan and Nguyen, 2006).

Sunflower (*Helianthus annuus* L., Asteraceae) having an electric conductivity (EC) value of 8 (McKenzie, 1988) can moderately tolerate the drought and saline environmental conditions (Connor and Hall, 1997). It is an important oil producing crop for both the mono-unsaturated and polyunsaturated fats but with low saturated fat levels.

With its particular importance for human health, sunflower was used in this experiment to explore the salt stress effects on its genome. The effects of salt stress were examined in the slat sensitive line, by observing the upregulated expression of DNA sequences at that particular condition. In this work, differential display reverse transcription-PCR (DDRT-PCR) method was used for identifying the cDNA fragments induced in sunflower seedlings following NaCl treatment for short duration (up to 24 h time period). The expression pattern was analyzed in leaves of the salt-sensitive sunflower line. Out of a pool of cDNA fragments differentially upregulated under salt-stressed conditions, only one PCR fragment showed the possible homologies identified in the chloroplast region among the *Brassicaceae* species.

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Materials and Methods

Plant growth conditions and salt treatment

Seeds of sunflower salt sensitive line S-278, were obtained from National Agriculture Research Center (NARC), Islamabad, Pakistan and germinate in washed moist sand filled in plastic pots (sand culture). The experiment at seedling stage was carried out in a controlled growth room of Department of Botany, University of Agriculture Faisalabad. The growth temperature was maintained at 26°C with a 16/8 h light/dark photoperiod and light intensity of 150 µ Em-2S-1. Five days old seedlings were fertilized with full strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). After 2-weeks, the control plants remained in the nutrient medium but the experimental plants were irrigated with 150 mM NaCl added to the Hoagland's nutrient solution.

The seedlings were harvested after 3 h, 6 h, 12 h and 24 h of salt irrigated water to examine the effect of the salinity shock on gene expression. Plant samples were collected and leaves of each seedling were separated for further work, snap frozen in liquid nitrogen and stored at -80 oC, in plastic bags.

RNA extraction and cDNA synthesis

Total RNA was isolated from leaf tissues of frozen plant samples (approximately 100 mg)using the plant RNeasy system (Qiagen, Canada), following the manufacturer's instructions. The total RNA was quantified on a GeneQuant Pro (Amersham Biosciences) spectrophotometer and the quality of RNA was analyzed electrophorizing the samples on 1% agarose gel (Sambrook and Russel, 2001). The sequences of the primers (anchored and arbitrary) used were as described by the Deng et al. (1999). These were;

Anchored primers:

HT11M: 5/-AAGCTTTTTTTTTTTA/G/C-3/

Arbitrary primers:

HAP25: 5/-AAGCTTTCCTGGA-3/; HAP26: 5/-AAGCTTGCCATGG-3/;

HAP27: 50-AAGCTTCTFCTGG-3/; HAP28: 5/-AAGCTTACGATGC-3/;

HAP29: 5/-AAGCTTAGCAG-CA-3/; HAP30: 5/-AAGCTTCGTACGT-3/;

HAP31: 5/-AAGCTTGGTGAAC-3/; HAP32: 5/-AAGCT -TCCTGCAA-3/

Differential Display for total RNA was performed as described by Song et al. (1995) for every anchored primer, three reverse transcription reactions were set up for each RNA sample. To synthesize the first strand of cDNA, 2 µg of total RNA was taken in a 0.5 mL microcentrifuge tube and made the final volume up to 25µL with DEPC treated water. The tube was heated for 10 min at 65°C water bath. After the specified period, 10µL of 5X M- MuLV RT buffer, 5µL of 2.5 mM mixed dNTPs, 6µL of 20µM anchored oligo-dt primer and 1 µL of RNasin were added to the tube and incubated at room temperature for 15 min. Reverse trnscriptase, M-MuLV RT, 1.5 µL was then gently mixed in the reaction mixture and incubated again at 37°C for 1 hour. Finally the reaction mixture was heated in 95°C water bath for 5 min and the cDNA was stored at -20°C for later use.

PCR amplification

The PCR reaction was set by adding to a 0.5 mL microcentrifuge tube, 4.0 µL cDNA, 2.5µL mixed dNTPs (100µM), 2.5µLTaq DNA polmerase buffer (10X), 2.5µL MgCl2 (25 mM), 2.5µL anchored oligo-dT primer (20µM), 1.0 µL 13-mer arbitrary primer (20µM). Taq DNA polymerase, 0.5 µL was added at the end and the reaction mixture was brought to 25µL final volume with sterile water. All the contents were mixed gently. PCR reaction was run as follows: 94°C for 30 sec, 42°C for 1 min, 72 °C for 30 sec, 40 cycles, followed by a 5 min final extension at 72°C. Before loading to the gel, PCR samples (3.5 mL of each) were incubated immediately with 2 mL of loading dye (Fermentas) at 80°C for 2 min.

Denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (6%) was prepared following the protocol described by Sharma and Yadav (2009) in mini-PROTEAN Electrophoresis Cell (Bio-Rad) apparatus. Denatured the PCR products (5 µL) in 2X loading dye (20 mM EDTA, 0.05% Bromophenol blue, prepared in 95% formamide) and incubated for 3 minutes at 95 ºC and loaded on gel. The gel was run at 80 V in TBE buffer until the dye reached the bottom of the gel. Silver staining of the polyacrylamide gel was performed using Bio-Rad silver staining kit following the instructions as described by the Bio-Rad Silver Stain Handbook with slight modification as fixing the gel in 7.5% acetic acid (v/v) at once after electrophoresis, and ending the developmental reaction using 7.5% acetic acid (v/v).

Recovery and reamplification of cDNA fragments, sequencing and bioinformatics analysis

The differentially upregulated displayed bands were excised from the gel, cut into small pieces and transferred to a microcentrifuge tube. The tubes were incubated for 15 min at room temperature in 200 µL sterile water, boiled for 10 min and centrifuged for 1 min at 8,000xg. The 10 uL of DNA from the supernatant was reamplified in a 25 µL reaction mixture with the same set of primers and PCR conditions as described earlier. The reamplified PCR products were run on 1% agarose gel in 1X TAE buffer and visualized after staining in ethidium bromide.

The Reamplified DD-PCR products were extracted from agarose gel and sequenced from Centre of

Excellence in Molecular biology (CEMB), Lahore, Pakistan. The homology of the sequences was analyzed using BLAST (Basic Local Alignment Search Tool).

Results and Discussion

Differential Display of sunflower DNA sequences

Plants have the potential to survive under stress conditions through alteration in expression of specific genes (stress-responsive genes). The objective of the current study was to appraise the expression of gene/DNA sequences controlling the key metabolic processes of salt tolerance in sunflower *(Helianthus annuus)*. The identification of differentially expressed transcripts is a preliminary step for identification of specific genes and their regulatory elements leading to characterization of metabolic pathways predicted to be the responses of plants under salt stress.

The expression pattern of DNA sequences in salt sensitive cultivar of sunflower seedling both from control plant and from the treated ones after the NaCl treatment for various durations was comparatively analyzed. The Differential display was used to detect the fragments of gene/DNA sequences, expressed differentially under stress conditions. Total RNA was obtained from the leaves of NaCl treated (24 h) and untreated (0 h) seedlings of sensitive sunflower line. The amplified transcripts of RNA were separated by urea-PAGE (Fig.1) and visualized by silver staining (Deng et al 1999). Using the differential display method both the down- and up-regulated DNA sequences were isolated and characterized. Many stress downregulated genes have also been reported (Shinozaki and Yamaguchi-Shinozaki, 2000; Ramanulu and Bartels, 2002).

We isolated the cDNA fragments that were upregulated in the sensitive line. The objective of the study was to characterize the main affected site from salinity. The upregulated sequences may either be the genetic indicator of salt-toxicity or be the expression for the protective measures from salinity threat. Liu and Baird (2004) identified from *Helianthus annuus* (asteraceae) a gene, HaABRC5, reported to be upregulated in response to drought, salinity, and abscisic acid, using the same technique (differential display of mRNA transcripts). The full-length cDNA was found encoding 141 amino acids containing a "bipartite nuclear targeting sequence" but showed no homology to any known genes from the database. Here, we investigated the upregulated sequences expressed under salt stress, so that to characterize the stress induced genes/ DNA sequences to understand the molecular mechanism of stress tolerance.

The upregulated differentially expressed bands were extracted from urea-PAGE gel and reamplified on 6% agarose gel as shown in the Fig. 2 (200 and 150bp sized reamplified DNA bands).

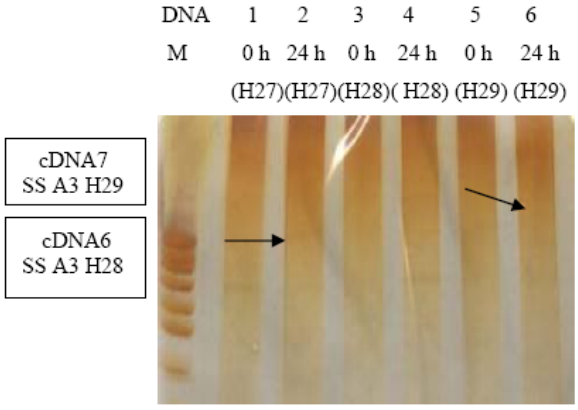

Fig 1 A representative presentation of differential display (DDRT-PCR) from leaves of sensitive sunflower line, S-28. Lane1: 1 kb DNA ladder, Lanes 1, 3 and 5 represent the control samples (0 h). Lanes 2, 4, and 6 represent salinity stressed samples (24 h post-stressed), RT-PCR reaction were performed using anchored primers; A:3 (HT11C) and arbitrary primers; Hap:27, Hap:28 and Hap:29. cDNA fragments which appeared to be differentially expressed in treated samples are indicated by arrows.

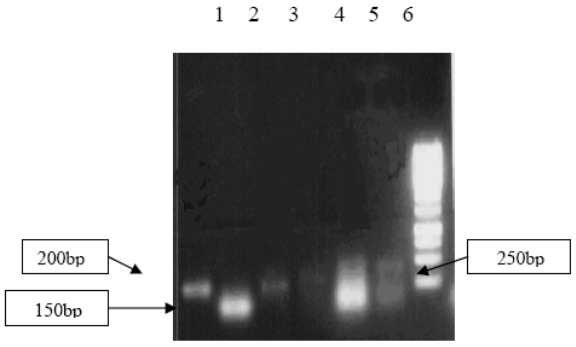

Fig 2 A typical agarose gel representing the differentially expressed reamplified PCR products from sunflower leaves: Reamplification was done using the same primers as used for RT-amplification. Marker: 1 k b (10,000bp) DNA ladder (Fermentas) for which last band is of 250 bp.

Expression of chloroplast-related DNA sequences differentially upregulated from salt sensitive sunflower line under saline conditions.BLAST search found only one cDNA fragment, cDNA6 SS-A3H28 ("SS" represents the sensitive sunflower line),

Table 1 The cDNA6 (SS-A3H28) sequence producing significant alignment with Brassicaceae family members

Accession	*Description*	Max score	Total score	Query coverage	E value	Max identity
AP000423.1	*Arabidopsis thaliana chloroplast DNA, complete genome, ecotype: Columbia*	200	200	65%	3e-48	85%
AP009376.1	*Nasturtium officinale chloroplast DNA, complete sequence*	189	189	55%	7e-45	86%

identified out of many differentially upregulated cDNA fragments for sensitive sunflower line, while rest of the fragments did not show any similarity to the known genes from the GenBank database. Only cDNA bands that were reproducible in three different replications were considered differential. We determined the sequence homology of the upregulated cDNA fragments with other plant sequences using the program BLASTN. The salt stress upregulated cDNA fragment showed sequence similarity to two of the members of Brassicaceae family; 85% homology to the *Arabidopsis thaliana* chloroplast DNA and 86% homology to *Nasturtium officinale chloroplast DNA* (Table1).

The sequence homology has indicated the serious effects of salinity on the chloroplast region of plant cell affecting the most important process of cell's function, the photosynthesis. Along with the plant cell growth retardation, photosynthesis is the prime process affected by drought (Chaves, 1991) and salinity (Munns et al., 2006). The distorting effects have been either the result of decreased CO2 availability to stomata and mesophyll (due to diffusion imitation) (Flexas et al., 2004, 2007), and the alteration of photosynthetic metabolism (Lawlor and Cornic, 2002), or due to the oxidative stress. Oxidative stress encompasses multiple stress conditions (Chaves and Oliveira, 2004) that can affect the leaf photosynthetic machinery very critically (Ort, 2001). Using the differential display, Ansari and Lin (2011) cloned several genes for dehydration in *S. tarmariscina*. They isolated one of the genes, GenBank Accession No. DQ471954, upregulated during dehydration stress with 68% homology with a 18.3 kDa protein gene (TLP18.3 gene, At1g54780) from *Arabidopsis thaliana* thylakoid lumen. The TLP18.3 gene was found with a domain of unknown function (DUF477), a family of uncharacterized proteins. To characterize the TLP18.3 gene, they found it to be up-regulated in *Arabidopsis thaliana* during dehydration and found localized into chloroplast organelle. It has been inferred that plant promptly respond to stress conditions by altering the gene expression of various regulatory metabolic processes, especially the photosynthesis.

Conclusively, the salt stress responsive transcript expression pattern study of sunflower leaf tissues showed the earliest (24 h post salt treatment) effects of salt stress in the chloroplast part of the cell thus may affect the phenomenon of photosynthesis. The upregulated DNA sequences may have the ability to be used as genetic indicators for that particular stress response (salinity stress). This is a baseline study in this important area, which will serve to provide guidance for future research on the expression of salt tolerance genes in plants.

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