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SALINITY INDUCED CHANGES IN COTTON (*GOSSYPIUM HIRSUTUM* L.)

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Abstract

Cotton (*Gossypium hirsutum* L.) is susceptible to abiotic stresses. High salinity is a common abiotic stress condition that adversely affects plant growth. Altered ion and water homeostasis changes due to NaCI stress, lead to molecular damage, growth arrest and even death. As a consequence of salt stress effects, secondary stresses such as oxidative damage by reactive oxygen species may occur. Reactive oxygen species can alter cellular metabolism through oxidative damage of lipids, proteins and nucleic acids causing lipid peroxidation, protein denaturing and DNA mutation. In recent years, several selective and sensitive assays have been developed to evaluate the effects of environmental stress on vegetal organisms. RAPD is one of them and developed for DNA analysis. In this study, cotton seedlings were used as bioindicator of salinity stress in the range of 50-400 mM. Effects of salinity stress were determined by comparing RAPD profiles of normal and treated cotton seedlings include variations in band intensities as well as gains or losses of band numbers. The DNA polymorphisms detected by RAPD analysis could be used as an investigation tool and useful biomarker assay for observing environmental stresses such as high salinity on vegetal organisms.

Introduction

Salinity is one of the most serious abiotic stresses (Rahman *et al*., 2004). It is a constraint to plant growth and development and causes low productivity in crop species in agriculture worldwide (Hameed *et al*., 2008). Saline soils adversely affect plant growth and lead to plant death due to the effects of water stress, ion toxicity, ion imbalance, or a combination of these factors (Bor *et al*., 2003).

Plants are immobile organisms and constantly exposed to changes in the environment. In saline soils, roots are the primary point of contact with salt. To some extent, plants could overcome osmotic and ionic stress caused by high salinity via developing efficient and specific mechanisms (Mahmood *et al*., 2010). But several cellular stress responses are induced by excess of salt causing damage to different cellular components (Dolek *et al*., 2001).

As a consequence of osmotic and ionic stresses, secondary stresses such as oxidative damage often occur. Reactive oxygen species generated by salt stress are highly reactive and alter normal cellular metabolism causing lipid peroxidation, protein denaturing and DNA mutation (Dat *et al*., 2000; Implay, 2003). Salt stress causes nuclear deformation and subsequent nuclear degradation (Katsuhara & Kawasaki, 1996). Structural changes of nuclei caused by salt stress have been reported (Werker *et al*., 1983).

Chemical and physical agents induce changes in DNA. For DNA analysis, Random Amplified Polymorphic DNA (RAPD) Technique developed by Williams *et al*., (1990) and Welsh and McClelland (1990) is a sensitive method to indicate wide range of DNA damage types. This is done through detecting variations in band intensities, and gain or loss of DNA bands following toxicant exposure that might be an indicator for DNA changes (Uzonur *et al*., 2004; Atienzar & Jha, 2004; Liu *et al*., 2005; Swaileh *et al*., 2008).

Cotton is one of the most important fiber crops, which is widely cultivated throughout the world. In addition to textile manufacturing, it produces seeds with a potential multiproduct base such as hulls, oil, linters and food for animals (Song & Yamaguchi, 2003). Although cotton is classified as a salt tolerant crop (Reinhardt & Rost, 1995), its growth and yield are severely inhibited in higher salinity soil, especially at germination and emergence stages (Ashraf, 2002). Therefore, the aim of this study was to evaluate genetic stability of *G. hirsutum* grown under high salinity stress by using RAPD-PCR method. For detection of possible DNA damages induced by salt, comparisons were performed between the RAPD profiles of control, 50, 100, 200, and 400 mM exposed seedlings.

Materials and Methods

Plant sampling and germination: Seeds of cotton var. Nazilli 84S, which is one of the commonly planting varieties in Western Turkey, were obtained from Nazilli Cotton Research Institute, Aydin-Turkey. Before surface sterilization, cotton seeds were kept under flowing tap water for 1 h and they were surface sterilized by immersion in 70% ethanol for 3 min., followed by stirring in 20% commercial bleach for 20 min. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 min. and they were dried onto filter papers. Seed coats were removed with sterile scalpel and tweezers prior to germination. The seeds were germinated on hormone free MS (Murashige and Skoog) medium which contained 4.3 g basal salt mixture, 30 g sucrose and 2.2 g phytagel. The pH of the media was adjusted to 5.7 with 1 M NaOH before autoclaving. After autoclaving, 1 mL MS vitamin solution, sterilized by micro filter was added into MS media. 20 mL MS media was poured into Magenta vessels and 1 seed was germinated in each Magenta vessel. Seeds were kept at growth chamber with photoperiod of 16 h light (7500 lx) and 8 h dark, at 25^{o}C and 70% humidity. After 15 days of germination period,

young plants were transferred into sterile jars with MS (250 mL) containing different levels of NaCI (0, 50, 100, 200 and 400 mM) for one month. After one month growing period, seedlings were harvested. Some growth parameters such as stem length, fresh and dry weight of leaves and stems and leaf area were measured.

The standard error values of the means were calculated to compare the site categories. Statistical analysis was performed using a one way ANOVA (for $P < 0.05$). Based on the ANOVA results, a Tukey test for mean comparison was performed, for a 95% confidence level, to test for significant differences among treatments.

DNA isolation and RAPD-PCR applications: DNA isolation was carried out using the DNeasy Plant DNA Extraction Mini Kit (Qiagen), according to the supplier's instructions. DNA concentrations and sizes were estimated by comparing them with a standard sample (GeneRuler^{TM} 100bp DNA Ladder, ready-to-use, MBI Fermentas) in an agarose gel (fragment (bp) and DNA quantity in band, (ng) were given, respectively, for 10 bands in descending order of the fragment sizes 1:1031-169, 2:900-147, 3:800-131, 4:700-115, 5:600-98, 6:500–164, 7:400–65, 8:300–49, 9:200–33, and 10:100-16). RAPD amplification was done in a 25µl PCR mix, containing 1xPCR buffer $(NH_4)_2SO_4$, 0.2 µM from each dNTP (2 µM dNTP mix), 25 picomoles of primer OPA-O8 5'CCACAGCAGT3' from QIAGEN Operon RAPD® 10 mer Kits, 20-200 ng of genomic DNA, and 0.5 units of Taq DNA polymerase, and filled up with sterile deionized water to the final volume. PCR chemicals were obtained from MBI Fermentas, except when otherwise stated. Tubes containing all reaction components, except template DNA, were included as controls for each reaction. Amplification was performed in a Techne Endurance TC-512 Gradient Thermal Cycler programmed for 3 min. at 95°C (initial denaturation of template DNA) followed by 45 cycles of 1 min. at 94°C (denaturation), 1 min. at 37°C (annealing temperature), 2 min. at 72°C (elongation), and 5 min. at 72°C (final extension step).

Agarose gel electrophoresis and analysis of DNA profiles: Amplification products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV-light. Results were documented with both GelDoc 2000 (BioRAD) and SONY digital camera with UV filters. The amplified products were run together in groups of either three or ten according to the need for precision in assessment of the extend variation and instability. Clearly observed bands were scored and used to create the genetic profiles of each plant sample.

Results

Effects of NaCI stress on growth parameters: The stem length and the inhibition of stem and leaf growth rates were analyzed in cotton seedlings in response to different NaCI concentrations. The results obtained from the stem length experiment were shown in Table 1. Following exposure to high NaCI concentrations, stem length of cotton seedlings decreased from ~7.37cm (control) to ~3.20 cm (400 mM). Data obtained from Table 1 showed that stem lengths were substantially decreased with the increase of the NaCI concentrations compared with the control seedlings. After 30 days of NaCI exposure, the stem growth rates decreased from 1.379 g to 0.225 g (83.69%) for stem fresh weight and from 0.307 g to 0.142 g (53.75%) for stem dry weight. Also, leaf growth rates decreased from 0.433 g to 0.050 g (88.45%) for leaf fresh weight and from 0.0725 g to 0.018 g (75.17%) for leaf dry weight (Table 1). These results verify that NaCI is indeed a toxic chemical for cotton plants.

Table 1. Some growth parameters of cotton in different salinity levels (0, 50, 100, 200 and 400 mM) in one month of growing period. According to the results of variance analysis and Tukey test, the mean difference is significant at p<0.01 (*) and p<0.05 (**) levels.

	Control	50 mM NaCl	100 mM NaCl	200 mM NaCl	400 mM NaCl
Stem length (cm)	$7.369 \pm 0.156^{**}$	$6.053 \pm 0.089^{*}$	$4.596 \pm 0.266^{**}$	$3.679 \pm 0.195^{**}$	3.195 ± 0.401
Stem fresh weight (gr)	$1.379 \pm 0.027^{*}$	$1.299 \pm 0.060^{*}$	$0.963 \pm 0.074^{**}$	$0.547 \pm 0.052^{**}$	$0.225 \pm 0.047^{**}$
Stem dry weight (gr)	$0.307 \pm 0.012^{*}$	$0.384 \pm 0.014^{*}$	$0.227 \pm 0.027^{**}$	$0.150 \pm 0.021^{*}$	0.142 ± 0.001
Leaf fresh weight (gr)	$0.433 \pm 0.013^{**}$	$0.443 \pm 0.014^{**}$	$0.356 \pm 0.020^{**}$	$0.278 \pm 0.038^{**}$	$0.050 \pm 0.006^{**}$
Leaf dry weight (gr)	$0.0725 \pm 0.003^{**}$	$0.074 \pm 0.002^{**}$	$0.0527 \pm 0.005^{**}$	$0.043 \pm 0.007^{**}$	0.018 ± 0.002

Effect of NaCI stress on RAPD-PCR profiles: The genetic effects of varying NaCI concentrations were analyzed with comparison of the RAPD-PCR profiles. The RAPD-PCR analysis was performed with DNA extracted from leaf of the A and B plants, each group treated with different NaCI concentrations (0, 50, 100, 200, 400 mM).

OPA08 primer (GTGACGTAGG, QIAGEN Operon RAPD® 10 mer Kits) that generated specific and stable band profiles. OPAO8 was selected because it is a reproducible, efficient and widely applicable primer for different species (Uzonur *et al*., 2004). The molecular sizes and DNA concentrations of bands were automatically estimated according to the marker (GeneRulerTM 100 bp DNA Ladder, MBI Fermentas) using the Gel-Doc 2000 analyzer system and Quantity One Program 4.4.1 version.

The comparisons were done on the basis of alterations in RAPD profiles of NaCI treatment groups and control for cotton. The alterations in RAPD profiles included variations as increase in band intensities, loss and gain of bands compared with the RAPD-PCR profiles of the control plants (Figs. 1 & 2 and Tables 2 & 3). The improved RAPD-PCR methods enabled the determination of variation in band profiles both for exposure groups and control plants.

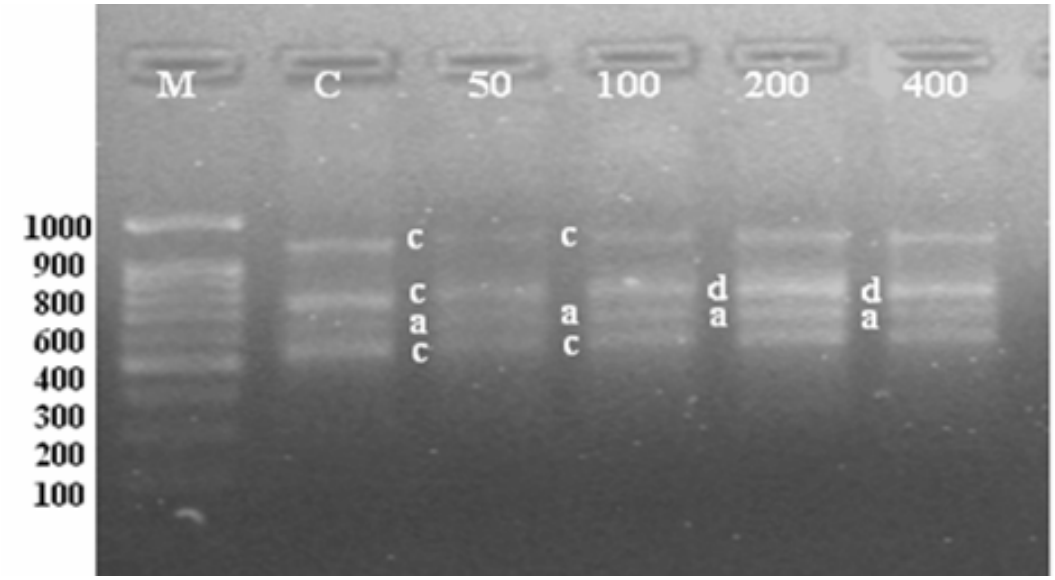

Fig. 1. RAPD profiles of genomic DNA from leaves of cotton seedlings (A) exposed to different NaCI concentration. a: indicates appearance of new bands, b: disappearance of normal bands, c: decrease in band intensities, d: increase in band intensities.

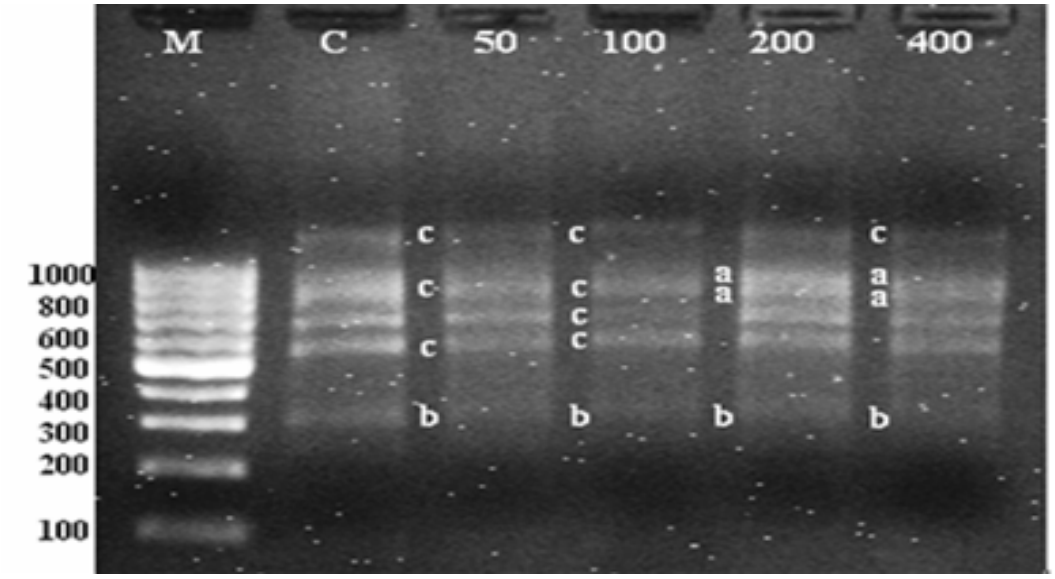

Fig. 2. RAPD profiles of genomic DNA from leaves of cotton seedlings (B) exposed to different NaCI concentration. a: indicates appearance of new bands, b: disappearance of normal bands, c: decrease in band intensities, d: increase in band intensities.

Table 2. Changes of total bands in control, and of polymorphic bands and varied bands in NaCI exposed cotton seedlings.

Primer: OPA08					
NaCI Concentrations (mM)		**Cotton (A)**			
	Total bands	**a**	**b**	**c**	**d**
Control	3				
50 mM	4	1	-	3	-
100 mM	4	1	-	2	-
200 mM	4	1	-	-	1
400 mM	4	1	-	-	1

a: indicates appearance of new bands, **b:** disappearance of normal bands, **c:** decrease in band intensities and **d:** increase in band intensities

Table 3. Changes of total bands in control, and of polymorphic bands and varied bands in NaCI exposed cotton seedlings.

Primer: OPA08					
NaCI Concentrations (mM)		**Cotton (B)**			
	Total bands	**a**	**b**	**c**	**d**
Control	3				
50 mM	4	-	1	3	-
100 mM	4	-	1	4	-
200 mM	4	2	1	-	-
400 mM	4	2	1	1	-

a: indicates appearance of new bands, **b:** disappearance of normal bands, **c:** decrease in band intensities and **d:** increase in band intensities

Table 4. Molecular sizes (base pair, bp) of appearance and disappearance of bands, changes in band intensities.

Primer: OPA08				
NaCI Concentrations (mM)			Cotton (A)	
	a	b	c	d
50 mM	526	-	413, 749, 962	-
100 mM	526	-	413, 962	-
200 mM	526	-	-	749
400 mM	526	-	-	749

Table 5. Molecular sizes (base pair, bp) of appearance and disappearance of bands, changes in band intensities.

Primer: OPA08				
NaCI Concentrations (mM)			Cotton (B)	
	a	b	c	d
50 mM	-	312	1081, 822, 526	-
100 mM	-	312	1081, 822, 749, 526	-
200 mM	848, 822	312	-	-
400 mM	848, 822	312	1081	-

In A, extra bands of molecular size approximately 526 bp shown at all NaCI concentrations (50, 100, 200, 400 mM) and in B, extra bands of molecular size 848 and 822 bp shown at 200, 400 mM (Tables 4 & 5). There was no missing band in A whereas in B there was a missing band at all concentrations. Decreasing of band intensities were especially at 200 and 400 mM NaCI exposure in A and at 50, 100, 400 mM NaCI exposure in B. Increasing of band intensities were shown at 200, 400 mM NaCI in A whereas no increments in band intensities were observed in B (Figs. 1 & 2 and Tables 4 & 5).

Discussion

Saline stress may cause alterations in DNA (e.g. rearrangements, structural distortion) because of secondary stresses such as oxidative damage linked to the production of toxic reactive oxygen intermediates like superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals. More importantly, salinity stress causes nuclear deformation and subsequent nuclear degradation (Werker *et al*., 1983; Katsuhara & Kawasaki, 1996). RAPD technique has been successfully used to detect genetic instability in bacteria, plants, invertebrate and vertebrate animals (Savva, 1996, 1998; Atienzar *et al*., 2000). Detection of DNA alterations using RAPD technique is based on the changes in amplified band intensities, disappearance of bands and appearance of new bands in the RAPD profiles generated from stress-exposed organisms in comparison to the control.

Similarly, in this work, genetic instability induced by NaCI-treated seedlings of cotton was reflected by changes in RAPD profiles: decrease or increase in band intensity, disappearance of bands, and appearance of new bands occurred in the profiles in comparison to the controls (Figs. 1 & 2, Tables 2 & 3). Modifications of the RAPD patterns are likely to one or a combination of the following events: (1) alterations in oligonucleotide priming sites due to genomic rearrangements and DNA damage, and (2) interactions of DNA polymerase in cotton seedlings with damaged DNA. These events could be preventative or reducible for polymerization of DNA in the PCR reaction (Nelson *et al*., 1996; Atienzar *et al*., 2002a). In this study, existing RAPD profiles of cotton shows that appearing increase in band intensity occurred for higher NaCI concentrations (200 and 400 mM) (Fig. 1) whereas appearing decrease in band intensity were observed for cotton exposed to 50 and 100 mM NaCI (Figs. 1 & 2). The missing band was obvious for cotton seedlings exposed to NaCI (Fig. 2 and Table 3). Also, new PCR products were found on RAPD profiles. The reason for appearance of new PCR products is that some oligonucleotide priming sites could become accessible to oligonucleotide primers due to structural changes resulting in new annealing events, large deletions, and/or homologous recombination in DNA sequences (Atienzar *et al*., 1999).

In the present study, NaCI had an inhibitory effect on stem and leaf growth and also stem length of cotton. The changes in stem length of cotton seedlings exhibited an inverse relationship with NaCI concentration. In addition to this, a significant reduction in stem and leaf growths of cotton seedlings correlated well with changes in RAPD profiles (Figs. 1 & 2). The results from this study indicate that there is positive correlation between genomic template stability and other parameters (stem and leaf growths and stem length) at above NaCI concentration.

In conclusion, RAPD technique has been used successfully in assessing DNA alterations caused by a variety of compounds such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals produced by salinity stress. In this work, using RAPD technique DNA alterations created by high amount of NaCI in cotton seedlings were detected and it was proved that RAPD could be used as an investigation tool for environmental toxicology. OPA08 primer used in this study was informative that may have great potential for detecting NaCI-induced specific DNA alterations but there are several constrains in applying RAPD technique involving amplification and/or electroporetic separations like DNA contamination, amplification competition and band homology (Backeljau *et al*., 1995; Harris, 1999). Therefore, amplicons found on the RAPD profiles must be analyzed by specific methods such as sequencing, probing etc. (Atienzar *et al*., 1999, 2002b).

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