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Research Article

Gene Isolation and Expression Analysis in *Pistacia* L. under Salt Stress Condition

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Abstract

Soil and water salinity are one of the limiting factors for growth and productivity of *Pistacia* in the worldwide. Among trees, *Pistacia* is one of the most tolerant to salinity. The knowledge on gene expression patterns with cellular proteins functionalization can be useful to produce more tolerant plants to stress. Herein we report a new sequence of β-*Actin* transcript in pistachio plant along with expression analysis result of two salinity responsive genes. Two cultivars of *Pistacia* (*UCB-1 Pistacia* (*eBR1*) and *Pistacia atlantica* Desf.(Baneh)) were treated using NaCl (EC: 7 to 12ds/m) To evaluate the physiological plant stress, the differences between the mean of total dry weight among control and salt stressed plants were measured in Baneh and eBR1. The effect of salt stress was studied at a molecular level by expression analysis of the *NAC1* and *SOS1* genes using qRT-PCR method. A partial mRNA of *β-Actin* gene was isolated as a housekeeping gene and submitted in gene bank with the accession number of JZ898211. The results show that dry weight in eBR1and Baneh increased and decreased, respectively in salt stress condition. The expression of *NAC1* gene increased significantly under salt stress in Baneh and UCB1 species. The *SOS1* gene expression was also upregulated during salinity stress in eBR1 but it decreased in Baneh.

Keywords*: Pistacia* L; Gene Expression Analysis*; β-Actin*; NAC; SOS

Introduction

The genus *Pistacia*, in the family of Anacardiaceae (cashew family), contains nine species and five subspecies [22]. *Pistacia*. L. (pistachio) is cultivated widely in the Mediterranean regions of Europe, the Middle East, South Asia, North Africa, china, and more recently, in other regions such as California (USA) and Australia [11]. The tree is a semi tropical plant which it can be habituated to moderate salt stress [21]. Salinity is one of the most serious environmental stress that affects plant production and growth in many arid and semi-arid regions [3,15]. More than 20% of cultivated land worldwide (~45 hectares) is affected by salt stress and the amount is increasing day by day [9]. Most of *Pistacia* cultivation is done in salin soil with EC > 6 ds/m in all over the world. Drought and saline stress have a negative effect on yield *Pistacia* of [14]. Although, *Pistacia* trees are acclimated to the variety of soil and they are probably more tolerant to alkaline and salin soil than other trees. Furthermore, *Pistacia* trees grow vigorously in hot, dry, desert-like condition [23]. To cope with saline stress, plants employ various mechanisms, at both the whole plant and cellular levels, which are controlled by a variety of genes and signaling pathways. These genes are expressed and activated at different times during the life of a plant [17]. Transcription factors play essential roles in stress responses by regulating their target genes through binding to the cognate cis-acting elements [27]. NAC family is one of the largest plant transcription factor families, which are only found in plants. NAC TFs regulate several target genes by binding to the CATGTG motif in the promoter region of them to activate transcription in the response to drought stress [18]. Salt stress is also signaled via the SOS pathway where a calcium-responsive SOS_3-SOS_2 protein kinase complex which controls the expression and activity of ion transporters such as *SOS1*. The *SOS1* encodes a plasma membrane NA^+/H^+ antiporter that export Na^+ from the cell. SOS_2 gene also encodes a Suc non-fermenting-like (SNF) kinase, and SOS_3 encodes a Ca^{2+}-binding protein [7]. Salt stress leads to a Ca^{2+} oscillation that activates the SOS_3-SOS_2 kinase complex. These phenomena activated kinase complex phosphorylates and activates *SOS1* and AtNHX1 [6].

In the present study, to investigate the salt stress effects on the expression of key genes involved in abiotic stress response in *Pistacia,* it was analyzed the expression of *NAC1* and *SOS1* genes in two different species of this plant. We isolated a partial sequence of *β-Actin* gene transcript, as well.

Materials and Methods

Plant material, growth condition and stress treatments

Six plants of UCB-1 *Pistacia* cultivar (eBR1) obtained from a cross between "atlantica" as female and "integerima" as male and six plants of *Pistacia atlantica* Desf. (Baneh) were used in this study. The Plants were individually grown in plastic pots contain of mixture of 40% soil, 40% compost and 20% sand. Temperature was maintained at 25/18 ± 2°C (day/night) and relative humidity was about 65–85 ± 5%. Supplementary light giving, an approximately 16-h light and 8-h dark period, was maintained during experiment. Plants in the well-watered treatment were irrigated two times in a week at field capacity. To impose salinity stress, the plants were irrigated by NaCl (with ratio of 14 to 1 NaCl and $CaCl_2$) solution. Salt was added to pots as irrigation step by step (each two days) by to Ec of 12 ds/m. The irrigation was imposed to field capacity for 21 days. The samples (leaves) were harvested after 72 hours of the completion of salt stress and stored at -80°C until RNA extraction.

RNA Extraction

The modified CTAB protocol [2] was used to extract total RNA. We used the proteinase K instead of two compounds of extraction buffer, β- mercaptoethanol and spermidin. The mixture was incubated at 65ºC for 20 min for dissolving of buffer. Sodium acetate and ethanol were also used for depositing RNA in this method. The RNA is kept at -80ºC for at least 2h and then the pellet of RNA was washed with NaCl (5M) and 70% ethanol. After a short drying time at room temperature, the pellet was dissolved in DEPC water and total RNA solution kept in -80°C until qPCR. Quality and quantity of extracted RNA were evaluated with spectrophotometer (NanoDrop 2000C spectrophotometer, Thermo scientific, USA).

Primer designing for genes amplification

Since the complete sequences of *β-Actin* gene have not still been reported in pistachio. BLAST and ClustalW software were used for sequence homology and conserved regions of *β-Actin, NAC1* and *SOS1* genes among the green plants. The specific primers were designed with Primer 3 software table 1. These primers were used in the PCR and qPCR methods. For *β-Actin* gene, it was designed a pair of primers (*β-Actin*-Long) for isolation a longer sequence of the gene transcript in first step and then, exact primers were designed based on obtained partial sequence of *β-Actin* mRNA for qPCR analysis.

Gene abbreviation	Forward primer sequence (5´-3´)	Reverse primer sequence (5´-3´)	Tm (°C)
β-Actin	AGCATGGTATTGTGAGCAACTG	TGGGTCATCTTCTCTCTGTTAGC-	60
β-Actin-LONG	ACAATGGAACTGGAATGGTGAAG	CCTTCATGTCACGGACAATTC	61
SOS1	GAGCTGGTCAACATCATTGT	CCACAACAGCCACAGGATC	60
NAC1	GCTGAGAACTAACCGAGCAAC	CAATGTCTTCCTCATCCCAAC	60

Table 1: Primer sequences used in quantitative real-time PCR for *β-Actin*, *SOS1* and *NAC1* and partial sequence isolation of *β-Actin* gene in *Pistacia. vera.*

cDNA synthesis and PCR

cDNA synthesis was done by Thermo scientific cDNA synthesis kit according to manufacturer's instruction. As a brief, 5 µg of total RNA with 200U of Superscript III reverse transcriptase (Thermoscientific, USA) using 0.5 µM of oligo dT and 100 pmol of Random Hexamer primers.

Conventional PCR was done to check the specification of designed primers. 20 µL of PCR reaction was included of 3 µl cDNA, 10µl 10X buffer (MgCl2 2M), with 0.5µl of Forward and Reverse primers (10µM) and distilled water up to 20 µl. PCR was performed according to following thermal condition: denaturing at 94°C for 3 min followed by 35 cycles of 94°C for 30s, 72°C for 70s, 72°C for 1.5

min. PCR products were electrophoresed on 2% Agarose gel. To sequence of *β-Actin* transcript in partial, the single band obtained using *β-Actin*-long primers was isolated and purified from gel and sequenced by Macrogen Inc. (Seoul, South Korea).

Quantitative PCR

Quantification of *SOS1* and *NAC1* genes expression levels in the samples were measured by qPCR using Hot Firepol EvaGreen qPCR master mix according to manufacturer's instruction (solis BioDyne, Estonia). qPCR was performed using primers table 1 by a Qiagen Real-time PCR System (Rotor-Gene Q, Germany). The specificity of real-time PCR amplification was confirmed by the following criteria: (1) a single peak in melting temperature curve analysis of real- time PCR-amplified products; (2) a single band on agarose gel electrophoretic analysis. The relative expression levels were calculated according to [19].

Statistics analysis

Expression levels were calculated from the Ct values obtained from triplicate biological samples. Statistical significance analysis of relative gene expression level compared with reference gene (*β-Actin*) was performed with completely randomized design as factorial experiment. Mean values of relative expression levels were compared by LSD and Duncan tests (P = 0.05, P = 0.01) using SPSS ver. 21.0.

Results and Discussion

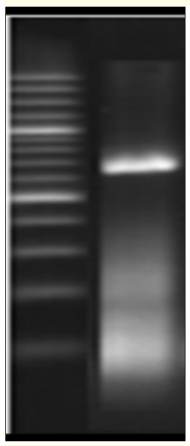
β-Actin gene identification and isolation

In this study, a PCR specific band was obtained in 650bp by using gel electrophoresis and the designed primers Table 1 for *β-Actin* gene from *Pistacia's* leave RNA. This band was separated from the agarose gel and sequenced by Macrogen Inc. This sequence was registered in gene bank, NCBI, with the accession number of JZ898211 figure 1.

Relative tolerance to salt stress between eBR1 and Baneh

Two *Pistacia* L. species, eBR1 and Baneh were selected for salt stress tolerance in this study. eBR1 is a commercial species which is cultivated in most regions and Baneh is a wild type variety of *Pistacia* in Iran.

We performed a long-term salt stress experiment. The differences between the total dry weight mean among well-watered and salt-stressed plants were measured in Baneh and eBR1. Our results showed that salt-stress affected significantly the dry weight of both species. The total dry weight of plants under salt-stress in eBR1 is more than Baneh Figure 2. As shown in Figure 2, eBR1 grew better under salt stress and produced a higher biomass than Baneh after 3 weeks of salt stress Figure 2. Biomass reduction by salt stress was significantly lower in eBR1 than in Baneh figure 2. It means the eBR1 is more tolerant to salt-stress than Baneh. Also results showed that salt-stress reduces the total dry weight in Baneh.



AGGAAGTGGTATAGAAATTATAAGTTTTATTGCTTGTTAAGTAGTTTA
GGCTTGTACTAATCCATTGTGATATGATATGCGTGAATTGCAGGCTGGGT
TTGCCGGTGATGATGCACCCAGGGCAGTGTTTCCTAGTATTGTGGGTAG
GCCAAGACACACCGGTGTCATGGTTGGGATGGGACAAAAGGATGCCTA
TGTAGGTGATGAGGCCCAATCCAAAAGAGGTATTCTTACTTTGAAATAC
CCCATTGAGCATGGTATTGTGAGCAACTGGGATGATATGGAAAAGATCT
GGCATCACACTTTCTACAATGAGCTTCGTGTTGCCCCTGAAGAGCATCC
AGTGCTTCTCACTGAGGCACCCTTAAACCCCAAGGCTAACAGAGAGAA
GATGACCCAGATTATGTTTGAAACTTTCAATGTCCCTGCCATGTATGTTG
CCATCCAGGCTGTGTTGTCCCTCTACGCCAGTGGTCGTACAACTGGTTC
GTATAAATCATGAGTTGAATCTGAACTTTTTAGTCAATTTGTTTTGTCTGA
TGTTTAAATTGCCATCTTCTTGAACAGGTATTGTACTGGATTCTGGTGAT
GGTGTGTCTCATACTGTGCCAATTTACGAGGGTTATGCTCTCCCACACGC
AATTCTTCGTTTGGATCTCGCTGGTCGTGATCTCACAGATGCATTGATGA
AGATTCTTACTGAGAGAGGTTACATGTTCACCACCACTGCCGAGCGGGA
AATTGTCGGAAACATATATGAAAAA

Figure 1: PCR specific band and 650bp of β-Actin mRNA sequence from Pistacia's leave.

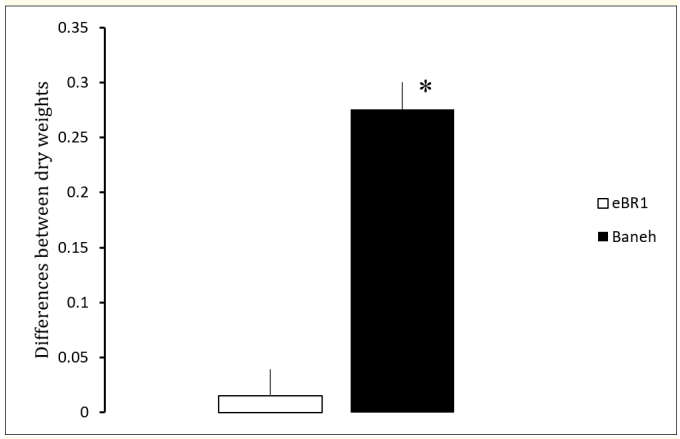


Figure 2: The reduction ratio of biomass (dry weight) in eBR1 and Baneh after salt treatment for 3 weeks. The Dry weight mean of three plants in salt stress condition has been subtracted of 3 plants in non-stressed plant.

Expression changes of the NAC1 and SOS1 genes in response to salt stress

As shown in figure 3 both of *NAC1* and *SOS1* genes are significantly up-regulated in Baneh and eBR1 under salt stress compared with control. The expression ratio of *NAC1* in Baneh is more than eBR1. In opposite, the expression ratio of *SOS1* in eBR1 is more than Baneh. Figure 4 shows relative expression level of two genes in two species. As shown in this figure 4, the relative expression of *NAC1* in Baneh is the most and then it seems, the salt stress affects this responsive gene in Baneh in comparison to *SOS1.* Oppositely, in eBR1, *SOS1* is major responsive gene compared with *NAC1*, so that under salinity treatment, its expression is increased with more ratio than *NAC1.*

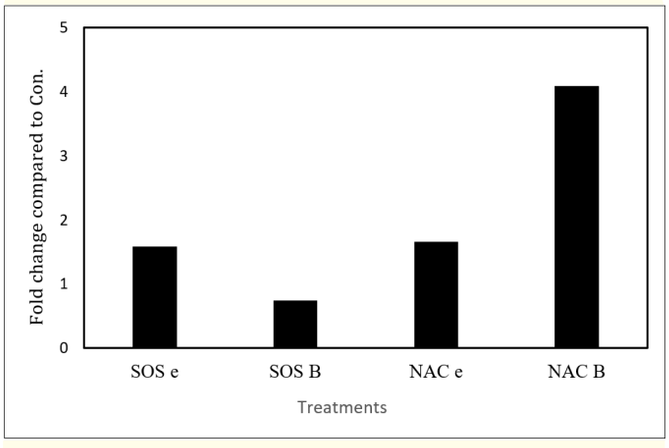


Figure 3: The level of expression of *NAC1* and *SOS1* genes under salt-stress in comparison to well-watered condition. SOS e: *SOS1* in eBR1, SOS B: *SOS1* in Baneh, NAC e: *NAC1* in eBR1, NAC B: *NAC1* in Baneh.

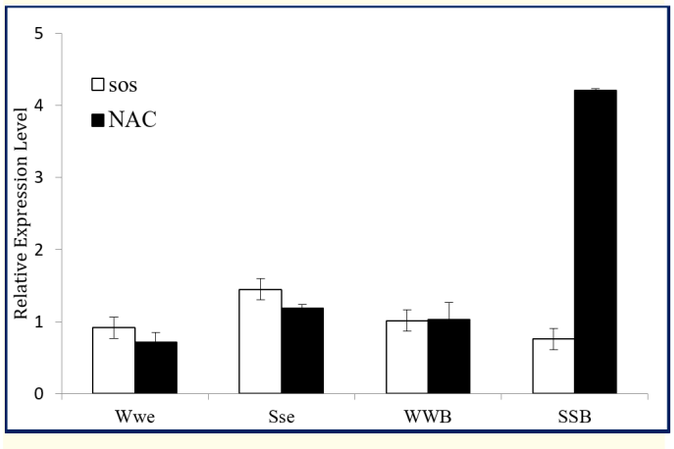


Figure 5: The relative expression of SOS1 and NAC1 genes under well-watered and salt-stress conditions in eBR1and Baneh. WWe: Well- Water Condition eBR1, SSe: Salt Stress Condition eBR1, WWB: Well- Water Condition Baneh, SSB: Salt Stress Condition Baneh.

The *NAC1* gene expression increased significantly under salt-stress condition, while the up-regulation of the *SOS1* gene expression did not show statistical significance.

β-Actin Gene identification and confirmation

Species specific sequences of several housekeeping plant genes are still not known and for gene expression studies, these sequences are essential for the given plant. qRT-PCR is general and specific technique which used for gene isolation. It is important that the reference gene is not subjected to the same mechanisms of regulation which is induced by environmental stress in the case of resistance genes. Focusing on salinity stress and difficulties in water extraction from the soil, *β-Actin* was recommended as the best reference gene [20]. According to some previous researches, the numerous *β-Actin* genes in various plant species indicate which this gene family is quiet variable.

Generally, the coding sequence of plant Actin multigene families consists of four exons and three introns, preceded by one non-coding leader exon and an intron located in the 5′ UTR in *Arabidopsis thaliana* [16]. This scheme is largely universal, although there are few exceptions. The sequence heterogeneity also exists among members of the plant Actin multigene families. Dilip Shah [4] discovered complete nucleotide sequence of soybean Actin gene, which was translated to 376 amino acids. In addition, Actin genes from genomic libraries of maize and soybean were isolated [24]. And, they suggested that the families of soybean and maize Actin genes may have diverged from a single common ancestral Actin gene long before the divergence of monocots and dicots. The complete nucleotide sequences of maize (MAc1) and soybean Actin gene (SAc1) were also determined [5]. However, the most complete information about a plant Actin gene family is available for *Arabidopsis thaliana*. Here, the family contains 10 Actin genes (ACT), eight of which encode functional proteins and two likely pseudogenes (ACT5 and ACT9). The Actin4 and Actin12 genes structures conserved in plants, as their sequence were detected in *Arabidopsis thaliana* [12]. Zhang [26] used the *Actin alpha* (Act-α) and *Actin beta* (Act-β) as reference gene for gene expression analysis in *Arabidopsis thaliana*. Similarly, Actin alpha (Act-α) also has been reported and validated as suitable internal control gene in cold and drought stress in Ficus carica and C. album [1]. Our sequences generated in current study showing sufficient identical homology with a list of β-Actin genes in databases, hence the β-Actin gene could serve as a reference gene for gene expression analysis in *Pistacia* L.

Gene expression analysis under salt stress

Plant salt tolerance is a complex behavior, which is not well understood yet. A few genes have been shown to be required for

plant salt tolerance, mostly. The *SOS1* is a genetic locus that was previously identified as essential in plant salt tolerance [25]. In present work, the *SOS1* expression increased significantly in eBR1 in salt stress condition. This up-regulation is consistent with the role of *SOS1* in Na+ tolerance. Plants cells do not appear to contain Na+ ATPase but Na^+-H^+ antiporter activities have been detected in plasma membrane. The SOS genes has shown to encode a putative plasma membrane Na^+-H^+ antiporter. The SOS genes in *Arabidopsis thaliana* plants extremely sensitive to Na^+ stress. Overexpression of the *Arabidopsis thaliana SOS1* gene, improves plant salt tolerance in the plan [13]. In their experiment, Transgenic plants showed that under salt stress, the transgenic plants accumulated less Na^+ in the shoot xylem. The *NAC1* gene expression also increased under salinity treatment compared to control condition in cultivars, eBR1 and Baneh, but the rate of its increasing in Baneh was much higher than eBR1.

It has proved that NAC proteins as major plant transcription factors are involved in abiotic and biotic stresses responses. The *NAC1* (SNAC1) is a Stress-responsive transcription factor which has been found involved in drought tolerance in barley and rice [8]. Honghong [10] also elucidated that SNAC1 in rice encodes a NAC transcription factor and is induced predominantly in guard cells under dehydration. The significantly enhanced drought resistance and salinity tolerance of the SNAC1-overexpressing rice plants suggest that this gene may show great promise for genetic improvement of stress tolerance in Plant.

Data Archiving Statement

A partial mRNA of *β-Actin* gene from *Pistacia* L. was isolated as a housekeeping gene and Sequences records are available at GenBank (National Center for Biotechnology Information) under following accessing numbers JZ898211.

Conclusion

In conclusion, it seems the tolerant species (eBR1) by increasing the expression of salt tolerant responsive genes (*NAC1* and *SOS1*) could be overcome against distractive effect of salt stress and more dry weight can prove these molecular results.

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