Salt-Induced Differences During the Gene Expression of Telomerase Enzyme in Sunflower

Mahdi Teymourí1, Maryam Parvini Kohneh Shahri1,*, Reza Darvishzadeh2

1 Department of Biology, Urmia Branch, Islamic Azad University, Urmia, Iran
2 Department of Plant Production and Genetics, Faculty of Agriculture and Natural Resources, Urmia University, Urmia, Iran

*Corresponding author: Maryam Parvini Kohneh Shahri, Department of Biology, Urmia Branch, Islamic Azad University, Urmia, Iran. Tel: +98-4435225346, Fax: +98-4432722738, E-mail: m.parvini@iaurmia.ac.ir

Background: Salinity is one of the most important environmental stresses which reduces the nutrient uptake, growth and yield of crops including sunflower.

Objectives: The aim of this study was evaluating the expression pattern of telomerase gene, TERT, in sunflower plants under salinity stress.

Materials and Methods: Sunflower plants of both sensitive and resistant lines were grown in greenhouse and treated with different levels of NaCl (2, 5 and 8 dSm⁻¹). The expression pattern of TERT gene was evaluated at 8th leaf stage 6, 12 and 24 hours post salt treatment using real time-PCR, since the effects of salt stress are eventually manifested in the leaves.

Results: In both lines, salt-subjected plants showed reduced size and dried leaves, due to breakthrough of the growth. Compared to the control group, treated groups tended to indicate downregulated pattern of TERT gene expression.

Conclusions: This study offers TERT as a new gene affected by salt stress when growth is arrested.

Keywords: Salt stress, Sunflower, TERT gene expression

1. Background
Sunflower (Helianthus annuus L.), a plant with a Northwestern origin, is one of the most important crops in the world, which has high amounts of unsaturated fatty acids (1, 2). Therefore, it is a nutritious oily seeds with high quality for population. Economically, the unique features of this plant are its short growth period and adaptation to various weather conditions making it suitable for cultivation in dry and low rainfall areas (3).

It is also an ideal species to study of the crop tolerance against environmental stresses, including salinity. Salinity is one of the adverse abiotic stress factors that negatively affects plant growth and productivity (4-6). It is known as limiting environmental factor which diminishes the yield of crop in arid and semi-arid regions (7-9). Plants present complex responses to salt stress for adaptation which depends on the duration of salinity stress (10, 11). Crops have developed several mechanisms to resist against salt stress. The first category of mechanisms is mechanical, indicated with changes in the dimension or deformation of the physical body of the plant in response to stress. The second category is biological, manifested with effects on the growth rate and productivity (12-14). Over the past years, several studies have clarified the resistant mechanisms against salinity based on physiological, morphological and biochemical aspects (15, 16).

Telomerase is a ribonucleoprotein with reverse transcriptase activity, whose role is to insert the tandem telomeric sequence motif at the end of DNA (17, 18). Telomeres usually contain short and repetitive G-rich nucleoprotein structures at the ends of linear eukaryotic chromosomes, including 5’(TTAGGG)3’ in mammals, and 5’(TTTAGGG)3’ in plants which was first characterized in Arabidopsis thaliana in plants (19). Its appropriate elongation is necessary to maintain the integrity and vital stability of the genome. The telomerase compensates telomere shortening using its catalytic protein subunit TERT, and an RNA subunit TER (20). Thus telomerase leads the primary mechanism of replenishing the lost terminal sequences in eukaryotic telomeres, which is
essential for self-renewing of the cells. Inappropriate telomerase expression provokes domino-like effects, finally causing abnormal proliferation (21). In plants, this enzyme is present at the embryonic and primary developmental stages, associated with reproduction. It is also detectable in dividing meristem cells (22, 23). It has been shown that \textit{TERT} gene acts directly as the main factor determining the telomerase activity in plant tissues (22, 24).

2. Objectives
The aim of this study was to explore the changes of \textit{TERT} gene expression in sunflower when exposed to the different salt concentrations. Changes in \textit{TERT} gene expression through plant stresses have not apparently been detected in the previous studies.

3. Materials and Methods

3.1. Plant material and Salt Stress Treatment
Two genotypes of sunflower including resistant (AS5305) and sensitive (9CSA3) (21) with different sensitivity to salt stress were selected and obtained from INRA, France. The seeds were planted in 3-cm depth of 30 × 25 cm pots containing farm soil and sand mixture with the 2:1 ratio (Fig. 1A). The plants were grown under controlled conditions at 25 ± 3 °C, 65% relative humidity and 12 h dark-light photoperiod for six weeks (17). The crops were irrigated every 3 days in a week. Salinity responses of the two different sunflower genotypes were investigated at 8\textsuperscript{th} leaf stage applying 0 (control group), 20, 40 and 90 mM NaCl (treated groups), where electrical conductivities of the solutions were 0.65 dSm\textsuperscript{-1}, 2 dSm\textsuperscript{-1}, 5 dSm\textsuperscript{-1} and 8 dSm\textsuperscript{-1} respectively (1, 25). The factorial experiment was conducted in completely randomized design with three replicates. Leaf sampling was done 6, 12 and 24 hours post-exposure to salinity. The leaves close to the apical meristem were selected for sampling at the 8\textsuperscript{th} leaf stage. The harvested leaves were covered gently with aluminum foil, numbered, and finally transferred to liquid nitrogen for storage and RNA extraction later (26).

3.2. Total RNA Extraction and cDNA Synthesis
RNA extraction kit RNX-plusTM (Sinoclon Co., Iran) was used according to the manufacturer’s protocol. Briefly, leaf samples in amounts of 50-100 mg were homogenized and purified. Then, 600 μL of RNX-plus buffer was added to the tube containing sample powder.
and vortex for 10-15 s. The tube was placed at room temperature for 5 min. Then 200 μL of chloroform was added to the solution and placed on ice for 15 min. The solution was centrifuged at 13000 rpm for 15 min. After removing the supernatant phase, isopropanol was added and the mixed sample was placed at -20 °C for 30 min. The final pellet was dissolved in nuclease-free water. One-percent agarose gel electrophoresis and spectrophotometer were used to verify the quality and quantity of the extracted RNA respectively.

Complementary DNA (cDNA) synthesis Kit (Fermentas LIFE SCIENCE # K1621) was used according to the manufacturer’s instructions to perform reverse transcription reaction, using 6 μg of total RNA with the oligo-dT primers.

### 3.3. Real-Time Polymerase Chain Reaction (PCR)

Quantitative reverse transcription-PCR (qRT-PCR) was performed in duplicate using 6.25 μL of Maxima SYBR Green/ Fluorescein qPCR Master Mix (2X) (Thermo Fisher Scientific, Germany), 5 pM of forward and reverse primers and 50 ng of cDNA for each reaction in a final volume of 12.5μL. The sequences of the primers given in Table 1, including TERT and ACTIN as an internal control were designed by Oligo 7 software. Relative gene expression was analyzed by comparative

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**Table 1.** List of primers and real-time PCR conditions

| Genes | Primer sequence | Annealing temperature (°C) | Cycle number |
|-------|-----------------|-----------------------------|--------------|
| TERT  | F: TTGCTCGCATGTATATGGTG | 59 | 40 |
|       | R: TGCGCTTCCCTCCGATCAG | 59 | 40 |
| ACTIN | F: GCAGGGATGACCAAGTG | 57 | 40 |
|       | R: CCCACCCTGACCAACAATGT | 57 | 40 |

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**Figure 2.** Relative expression pattern of TERT gene in the sensitive genotype of sunflower. Comparison of the main effects of different doses of salinity include 2 dSm⁻¹, 5 dSm⁻¹ and 8 dSm⁻¹ (A), Comparison of the main effects of the times include 6, 12 and 24 hours after salinity (B). *** Differences are highly significant (p ≤ 0.001) compared to corresponding control.
Ct method, $2^{-\Delta \Delta C}$. The target gene was normalized by the reference gene, ACTIN and calibrated for each sample against the control (27).

3.4. Statistical Analysis
Data from real-time PCR were expressed as mean±SD, and the differences of the mean values were statistically analyzed by SPSS software (Version 20) using one-way ANOVA followed by Tukey’s HSD test. P values less than 0.05 were considered statistically significant.

4. Results

4.1. Morphology of the Leaves
Apparently, in both sunflower genotypes, control groups without any salinity treatment were healthier and vigorous (Fig. 1B). When plants were subjected to the salinity, their leaves showed lower expansion or a smaller size (Fig. 1C) and dried due to salt stress (Fig. 1D).

4.2. TERT Gene Expression Assay by qRT-PCR
To evaluate the efficiency of our experimental groups versus control group in declaring how TERT gene expression is affected by salt stress, we used qRT-PCR. The effects of salt stress on TERT gene expression in the leaves of both sunflower genotypes have presented in the Figures 2 and 3. Generally speaking, the levels of TERT gene expression decreased in all salt treatments, but the rate of this reduction was different between experimental groups which were exposed to the different doses of salinity. At the first doses of salinity includes 2 dSm$^{-1}$, the expression of TERT gene showed severe and statistically significant reduction (P≤0.001) in both genotypes of sunflower, in comparison with control. This reduction was efficient enough to prove the direct effects of salt stress on the TERT gene expression. Interestingly, ***

![Figure 3](image-url) Relative expression pattern of TERT gene in the resistant genotype of sunflower. Comparison of the main effects of different doses of salinity including 2 dSm$^{-1}$, 5 dSm$^{-1}$ and 8 dSm$^{-1}$ (A), Comparison of the main effects of the times including 6, 12 and 24 hours after salinity (B). *** Differences are highly significant (p ≤ 0.001) compared to corresponding control.
applying the 5 dSm⁻¹ and 8 dSm⁻¹ doses showed slight depletion in the TERT gene expression when compared with the 2 dSm⁻¹ dose of salinity treatment (Fig. 2A and 3A). Such slight depletion in the sensitive genotype was remarkable in comparison with the resistant one (Fig. 4).

In both genotypes, the rate of TERT gene expression was evaluated at different salt concentrations and time lapses of applied concentrations likewise. According to the results, no relationship between the time lapses of salt concentrations used was significant (Fig. 2B and 3B).

5. Discussion
Abiotic environmental stresses such as drought and salinity are important factors in reducing crop production in the world. Salinity is one of the most important stresses generally disrupts the growth and stimulates the necrosis of the leaf (28, 29). Similar to other crops, the growth and yield parameters of sunflower are extremely affected by salinity (30). Previous studies have demonstrated that various facets of plants such as morphology and physiology, including tissue proliferation (31), growth traits (25, 32), induction of reactive oxygen species (ROS) (33), deregulation of photosynthesis (34), antioxidant enzyme activity in sunflower include catalase (CAT) and peroxidase (POX) (35, 36), lipid metabolism and protein synthesis (37), ionic related channels perturbations (38) and ionic toxicity (39) are affected during salt stress. It also reduces the size of leaves and fruits and generally, plant growth shows a decline under salinity (40,41). It is necessary to understand the molecular mechanisms and gene expression patterns affecting the growth and development of the crops to improve resistance to salt stress. It has been demonstrated that transcription of an endogenous gene, ABA is upregulated through high salinity stress (42), which can induces the expression of Lea (43), rd29A and rd29B genes (44). Elsewhere, the genes involved in enzymatic mechanisms showed different expression patterns through different salinity treatments (45). Ionic transporter genes, SOS1, NHX and HKT are also induced by salt stress (46). In addition, several genes and their relation with salinity tolerance have been detected today (47,48). Despite the results of many studies indicating the genes are affected by salt stress (49), there is no study considering TERT as a new gene affected by salt stress when growth arrest. This is the first study evaluating the TERT expression pattern in both susceptible and resistant genotypes of sunflower which have different salt tolerance capacities.

Overall, it has been shown that salinity causes cell death, with affected tissues fail to reproduce as a result. According to the results obtained from animal cells, the expression of telomerase enzyme decreases during cell death, but increases upon cell reproduction (50). In striking contrast to animals, one interesting finding in plants is that plant tissues also show lower levels of telomerase gene expression when subjected to salinity, due to loss of reproductive capacity in dividing

Figure 4. Comparing the TERT gene expression pattern between sensitive (Genotype A) and resistant (Genotype B) genotypes of sunflower at the same doses of salinity, 2 dSm⁻¹, 5 dSm⁻¹ and 8 dSm⁻¹.
meristematic cells (51). The results presented here demonstrate for the first time that TERT expression is disturbed in plants when facing environmental stress. According to our results, it is clear that both of genotypes show diminished rate of TERT expression through salinity. On the other hand, laboratory trials conducted to assess the variation of TERT expression at different doses of salt indicated no significant relationship between the time lapses of all subjected salt concentrations.

In the sensitive genotype, immediate and significant reduction of TERT expression was seen through the first dose of salt treatment as 2 dSm⁻¹. Thereafter, gradual and reduction of expression was seen through the first TERT

In the sensitive genotype, immediate and significant reduction of TERT expression was seen through the first dose of salt treatment as 2 dSm⁻¹. Thereafter, gradual and mild depletion in the rate of gene expression appeared at 5 dSm⁻¹ and 8 dSm⁻¹ doses of treatment. It means that the rate of TERT gene expression at the upper doses of treatment was still higher than the lowest one. It suggests struggle of the plant to be survived when exposed to the salinity, especially at higher doses. Indeed, parallel increase in TERT gene expression with the rise of salt doses, may be a result of adaptation of the plant to salt stress, which may resume the growth and proliferation processes. Regarding the resistant genotype, a relationship seems to exist between the depletion of TERT gene expression and resistance genes, since the intensity of TERT expression in resistant genotype was more than that in the sensitive type, which suggests a likely relationship between TERT and resistant genes.

Being the resistant genes silent in the sensitive genotypes, it seems that changes in the pattern of TERT gene expression constitute one of the main mechanisms for adaptation of the plant to salt stress. However, in resistant genotypes, this adaptation needs cooperatively expression of TERT and resistant genes. It is a subject that needs further investigations to indicate the exact association between the expression of TERT and resistant genes.

Totally, these results confirm that salinity directly affects the TERT gene expression.

6. Conclusion

The main role of TERT gene is argued to be beyond maintaining the integrity and vital stability of the genome through cell cycle. It is suggested to have a role through salt stress.

In this study, the rate of TERT gene expression significantly diminished in sunflower when exposed to the salt stress. Interestingly, the responses of two different sunflower genotypes were found as similar, since both genotypes showed diminished rates of TERT expression during salinity. It can be suggested that resistant genes have a correlation with TERT expression, a subject which evokes further investigations in the future. Furthermore, understanding the relationship between TERT and necrotic genes warrants further consideration.

Acknowledgements

The authors appreciate Urmia Institute of Biotechnology and Islamic Azad University, Urmia branch for the lab facilities.

Conflict of interest

The authors indicate no potential conflict of interest.

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