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Original Research

Evaluation of Proline Accumulation and Δ¹-pyrroline-5-carboxylate Synthetase (*P5CS*) Gene Expression during Salinity Stress in Two Soybean (*Glycine max* L. Merr.) Varieties

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Abstract

The aim of our study was to compare the effects of salt stress in two soybean (*Glycine max* L. Merr.) varieties. Two soybean genotypes (Ataem-7 and Üstün-1) were grown under 0, 50, 100, and 150 mM NaCl treatments, and the leaves were harvested for lipid peroxidation analyses, proline content and *P5CS* gene expression levels. According to the results of lipid peroxidation analysis, Ataem-7 variety was found to be more sensitive than Üstün-1 variety for NaCl stress. Proline is an important osmolyte accumulated under environmental stresses. As a response to salinity, we determined their proline levels. Proline accumulation in Ataem-7 variety increased 1.39 fold in accordance with Üstün-1 variety at 150 mM NaCl treatment. *Glycine max* Δ¹-pyrroline-5-carboxylate synthetase (*GmP5CS*) gene expression levels under 50, 100 and 150 mM NaCl stress were determined. When the *GmP5CS* gene expression level was gradually increased in Üstün-1 variety, the highest gene expression level for Ataem-7 was determined at 100 mM NaCl. The *GmP5CS* gene expression in Üstün-1 at 150 mM NaCl increased 2.93 fold compared with 100 mM treatment. When we evaluate the relation between proline accumulation and expression levels of *GmP5CS* gene, it is obvious that accumulations of proline in two soybean varieties are under control of different mechanisms in the presence of salinity.

Keywords: salt stress, proline accumulation, soybean Δ¹-pyrroline-5-carboxylate synthetase (*GmP5CS*), RT-PCR

Introduction

Soil salinity has been an important environmental problem recently. It limits plant growth and crop productivity. It is known that some plants, which are called halophytes, are salt tolerant and they can live under saline conditions, but the exact molecular mechanisms of salinity tolerance is not well understood [1, 2].

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Soybean varieties show a spectrum of salt tolerance ability. The damage of salinity on soybeans depends on the developmental stage of the plant when subjected to salt stress. It has been known that the seedling stage of the soybean plant is more sensitive to salt stress [2].

Osmotic stress caused by salinity has more hazardous effects on the cells. Malondialdehyde (MDA) is an indicator of osmotic stress, the cell subjected. Therefore, cell membrane stability has been used as a differentiation parameter to identify salt-tolerant and salt-sensitive cultivars [3].

Plants use different biochemical and molecular strategies to overcome the detrimental effects of salt stress. One of these defense mechanisms is to accumulate organic solutes. Plant species adjust to high salt concentrations by increasing tissue osmotic potential with the accumulation of both inorganic and organic solutes. One of their cellular responses to short- and long-term salt stress is synthesis and accumulation of osmoprotective compounds [4, 5]. These non-toxic and small molecules are osmotically active compounds and they increase the osmotic potential [6].

Proline is the major compound that has an ability to protect the cells via stabilizing the proteins and cellular membranes. In many plant species, it is observed that accumulation of proline under salinity is widely reported [7].

In plants, proline is synthesized in two pathways. The difference of these pathways is the precursors of proline. Glutamate is the main precursor. Ornithine is also used as a precursor indirectly in proline biosynthesis [8-10]. Under osmotic stress, the glutamate pathway is the main route. Proline accumulation is regulated by the balance between synthesis and catabolism. Δ^1pyrroline-5-carboxylate synthetase (*P5CS*) is the rate limiting key enzyme in the proline mechanism [8, 9, 11-13]. *P5CS* enzyme converted to pyrroline-5-carboxylate (*P5C*) spontaneously and this reaction product is reduced to proline by *P5C* reductase (*P5CR*) enzyme [10]. In *A. thaliana*, the relation between *P5CS* gene expression and proline accumulation during osmotic stress has been reported [10, 14, 15].

In our research, we subjected two soybean genotypes (Ataem-7 and Üstün-1) to 0, 50, 100, and 150 mm NaCl stress. The detrimental levels of salt treatments at different concentrations were determined according to cell membrane lipid peroxidation. The accumulated proline contents were also determined to evaluate salinity tolerance efficiencies of Ataem-7 and Üstün-1 against different NaCl concentrations. The *P5CS* gene expression levels were compared in both varieties, and relations with proline accumulation were discussed.

Materials and Methods

Plant Materials and Growth Conditions

Ataem-7 and Üstün-1 commercial *Glycine max* (L.) Merr. seeds were taken from the Black Sea Agricultural Research Institute, Samsun, Turkey. The seeds were sown into pots containing perlit and grown in growth chamber conditions with 25ºC and 16 h/8 h light/dark regime. The seeds were watered with ¼-strength Hoagland solution.

Salt Stress Treatment

Salt treatments with 10-day-old seedlings grown in perlit were started by adding the ¼-strength Hoagland solutions containing 0, 50, 100, and 150 mM NaCl. The experiment was designed in a complete randomized parcel with three replications. This treatment was continued through 7 days. Morphological damages were observed and photographed after 7 days. After each treatment, the leaves were harvested and stored at -20ºC until used for analyses.

Lipid Peroxidation

Frozen leaf samples were ground in liquid nitrogen, and 1 ml extraction medium containing 0.5% (w/v) thiobarbituric acid and 20% (w/v) trichloroacetic acid was added. The mixture was heated for 30 min at 95ºC. The samples were cooled to 4ºC and the reaction was stopped. After centrifugation for 10 minutes at 3000 g, absorbances of the supernatants were determined at 532 and 600 nm. Extinction coefficient for MDA is 155 $mM^{-1} \cdot cm^{-1}$. Results were expressed as μmol MDA$\cdot g^{-1}$ FW [3].

Proline Level

The free proline content was determined according to Bates et al. [16]. 0.5 g frozen leaf samples were homogenized with 10 ml 3% sulphosalicylic acid at 4ºC. The extract was filtered with Whattman No. 2 filter paper. In a test tube, 2 ml filtrate, 2 ml acid-ninhydrin, and 2 ml glacial acetic acid were added and left at 100ºC for 1 hour. Reaction was terminated on ice. Reaction mixture was extracted with 4 ml toluene. The chromophor containing toluene was seperated from the hydrated phase. The absorbances at 520 nm were spectrophotometrically determined using toluene as a blank. Proline concentrations were calculated according to standard curve and expressed as μmol proline$\cdot g^{-1}$ FW.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from the soybean leaves of control and salt-stressed plants with Trizol (Invitrogen, Carlsbad, USA). First-strand cDNA synthesis was performed in a total volume of 20 μl with iScript cDNA synthesis kit for real-time polymer chain reaction (RT-PCR) (Bio-Rad, USA). Each reaction mixture contained 1 μg of total RNA, 4 μl iScript reaction mix. Reaction mixtures were incubated at 25ºC for 5 minutes, 42ºC for 30 minutes, and then 85ºC for 5 minutes, then stored at 4ºC. The cDNA concentrations were determined spectrophotometrically [17].

Primer Design and RT-PCR Analyses

Specific primer pairs were designed for *Glycine max* (L.) Merr. *P5CS* gene with IDT (Integrated DNA Technologies, USA) primer design software. Primer pairs of *Glycine max* delta-pyrroline-5-carboxylate synthetase (*GmP5CS*) gene (GenBank accession No. AY492005.1) used for the amplification were: 5'-GGCTGCAATGCCATGGAAACTCTT-3' and 5'-ACTTGCCTTGGGTCCTCCATACAA-3'. We used *Glycine max* forrest beta-tubulin (tubB3) (GenBank accession No. U12286.1) as a housekeeping gene. The designed primer pairs for beta-tubulin gene were 5'-AGCGTGTGTGACATTGCTCCTAGA-3' and 5'-TCGTTCATGTTGCTCTCTGCC TCT-3'.

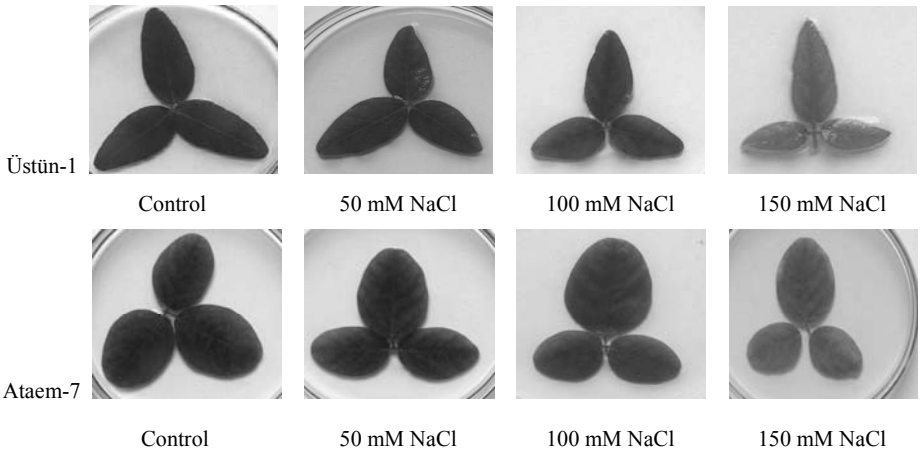

Fig. 1. The morphological changes of the leaves of Üstün-1 and Ataem-7 soybean cultivars.

RT-PCR amplifications were performed in a 25 µl reaction mixture containing 500 ng cDNA template and 12.5 µl iQ SYBR green Supermix (Bio-Rad, USA). The PCR amplification conditions for *GmP5CS* and beta-tubulin genes were as follows: 10 min at 94ºC followed by 41 cycles of 30 s at 94ºC, 30 s at 62ºC, and finally 60 s at 72ºC, followed by 72ºC 5 min. The polymerase chain reaction product sizes were corrected by electrophoresis on 2% agarose gels [18].

Relative Quantification

Comparison of relative expression results between the salinity treatments in both soybean cultivars were determined according to "delta-delta CP method" in real time PCR [19].

Statistical Analysis

The results are presented as mean values ±standard errors. Statistical significance between mean values was assessed by analysis of variance and Duncan's multiple range tests. A probability of (0.05) was considered significant.

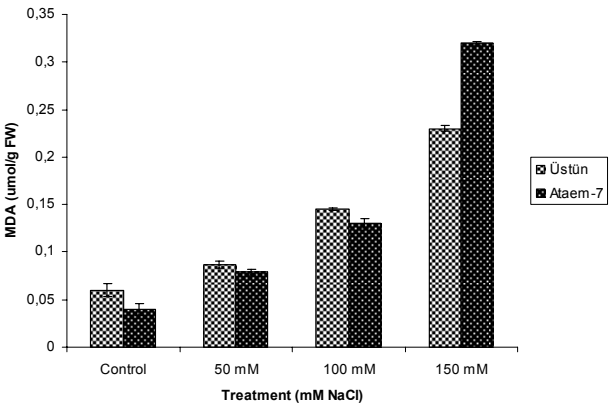

Fig. 2. MDA content in leaves of control and NaCl-stressed soybean cultivars. Values are means of 3 replicates. Vertical bars indicate ±S.D.

Results

To evaluate the detrimental effects of salinity on soybean plants, morphological changes were detected, and these changes were supported with biochemical parameters. The leaf growth of soybean plants was observed to be affected by salinity stress. The effects of three different salt concentrations were first detected due to visual symptoms of damages on the leaves of two commercial soybean varieties. The yellowing-browning areas on the leaves were observed as the visual symptoms of salt stress response after 7 days of irrigation with 150 mM NaCl in both varieties (Fig. 1).

Lipid Peroxidation

Lipid peroxidation is an important paremeter to determine the oxidative damage level caused by environmental stress. Salinity induced increases in lipid peroxidation as estimated through MDA production in leaves of soybean cultivars (Fig. 2). The increase in lipid peroxidation of Üstün-1 variety was observed higher than Ataem-7 variety at 50 and 100 mM NaCl stress. At 150 mM NaCl stress, while MDA levels for control plants of Üstün-1 and Ataem-7 were 0.05 and 0.04 µmol/g FW, lipid peroxidation rates were increased to 0.25 and 0.34 µmol/g FW after 150 mM NaCl treatment, respectively ($p<0.05$). In this study, we defined the salt sensitivities of soybean varieties due to lipid peroxidation rates.

Proline Accumulation

Free proline contents in leaves of control and NaCl-treated plants of two soybean varieties were determined on the day seven of stress (Fig. 3). In control groups, the proline contents were nearly non-detectable. In both varieties, proline accumulation increased due to increased NaCl concentration subjected. The results showed that increases in proline levels between cultivars were not statistically different under 50 and 100 mM NaCl concentrations. A great difference was found in proline accumulation between two

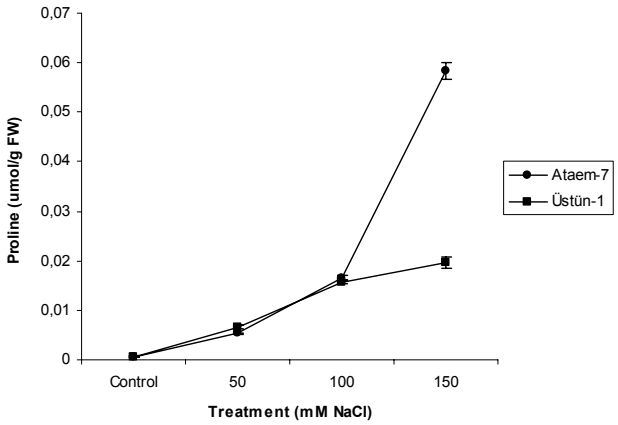

Fig. 3. Free proline content in leaves of control and NaCl-stressed soybean cultivars. Values are means of 3 replicates. Vertical bars indicate ±S.D.

soybean cultivars at 150 mM NaCl stress. In a particular Ataem-7 variety, proline levels were significantly stimulated; being aproximately 1.39-fold more in comparison to Üstün-1 variety ($p<0.05$).

Differential Expression of *Glycine max* delta-pyrroline-5-carboxylate Synthetase Gene (*GmP5CS*) under Different Salinity Conditions

GmP5CS gene expression level was determined at control and salt-treated groups in both soybean varieties by RT-PCR. Beta-tubulin (tub B3) cDNA fragment of 195 bp was used as a loading control gene. For *GmP5CS* gene expression analyses, gene-specific primers for 123 bp region of *GmP5CS* gene were used. The results of *GmP5CS* gene expression analysis studies were given in Fig. 4. The data given in the figure represent the changes in fold ratio of expression levels of *GmP5CS* gene in leaves of two soybean varieties with respect to control under three different NaCl concentrations. We found that in Üstün-1 variety, according to increasing NaCl concentrations, the expression levels were found 1.05, 1.5, and 4.45 fold more expressed than control, respectively. At Ataem-7 variety 0.55, 5, and 0.53 fold increases in expression level of *GmP5CS* gene were recorded in response to 50, 100, and 150 mM NaCl treatments, respectively (Fig. 4). The highest expression level for Ataem-7 variety was observed at 100 mM NaCl treatment. The amount of expression increased by 9.09 fold at 100 mM NaCl when compared to 50 mM NaCl. When the seedlings were subjected to 150 mM NaCl, the expression level of *GmP5CS* gene was found nearly to 50 mM NaCl level (Fig. 4). In spite of these findings in Ataem-7, the expression level for delta-pyrroline-5-carboxylate synthetase gene was gradually increased in Üstün-1 cultivar. The *GmP5CS* gene expression in soybean plants subjected to 150 mM NaCl increased by 4.24 and 2.93 fold in Üstün-1 cultivar when compared to 50 and 100 mM NaCl, respectively (Fig. 4). *GmP5CS* transcripts were detected (screened) in the leaves of salt-stressed plants and are given in Fig. 5. The transcripts in Ataem-7 soybean variety increased in response to 100 mM NaCl. The accumulation of *GmP5CS* mRNA was observed in response to 150 mM salt stress in Üstün-1 variety (Fig. 5).

Discussion

In this study, we evaluated the effects of salinity on two soybean cultivars and the relations between proline accumulation and *GmP5CS* gene expression levels.

Salinity is an important environmental stress and the plants show different salt stress sensitivites. MDA content is reported to increased due to increasing salinity stress level subjected [3, 4, 9, 20]. We examined the lipid peroxidation rates to determine the sensitivity differences between the soybean cultivars under salinity conditions. In both

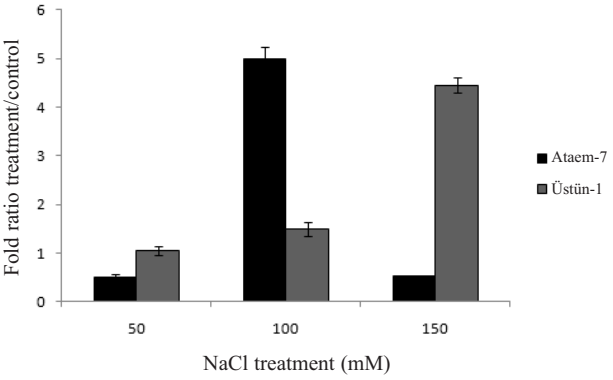

Fig. 4. Effects of different concentrations of salt stress on *P5CS* expression in Ataem-7 and Üstün-1 varieties (Means±SD).

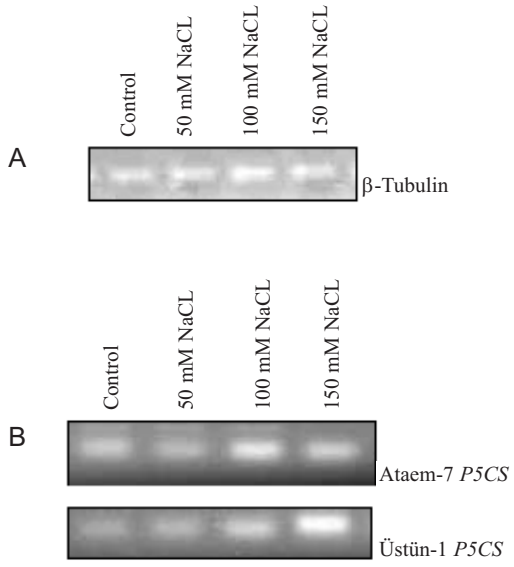

Fig. 5. Expression of P5CS in Ataem-7 and Üstün-1 variety. (A) β-tubulin expression in response to various treatments as housekeeping gene. (B) *P5CS* expression in two soybean cultivars in response to 50, 100, and 150 mM NaCl. Expressions in the leaves were detected by RT-PCR. The *P5CS* specific fragment was amplified by RT-PCR with 41 cycles.

varieties, lipid peroxidation levels were increased according to the increasing NaCl concentrations. We observed that the Ataem-7 variety is more sensitive to NaCl stress than the Üstün-1 variety.

Plants have different ways of respond to salinity as they have different tolerance capacities. In this study, we determined the proline accumulation as a result of salt stress. Proline accumulation is affected by different factors like genetic differences [7, 21]. Proline accumulation is defined by some scientists as a tolerance parameter. Some researchers reported that there is a positive correlation between stress tolerance and accumulation of proline content [7, 22-25]. Wang and Han [25] observed proline accumulation with increased salt concentration. Giannakoula et al. [26], reported higher proline accumulation under aluminium stress in maize in tolerant lines than stress-sensitive plants. But still it is not clear that if proline accumulation is a stress effect or it is a cause of stress tolerance [21, 23, 25]. Cha-um and Kirdmane [27] observed that the proline content is increased in the leaf tissues of sensitive lines of sugarcane under salinity and water deficit conditions than tolerant genotypes. Jampeetong and Brix [28] studied effects of salinity on Salvinia natans at 0, 50, 100, and 150 mM NaCl concentrations. They reported that proline accumulation increased according to the subjected salinity stress. Lacerda et al. [29] and Demiral and Türkan [30] also reported that proline accumulation is seen in the sensitive cultivars under salt stress. Lutts et al. [31] observed more proline accumulation in the leaves of salt-sensitive rice cultivar after 10 days of stress.

In contrast, in our study we observed a lack of relationship between salinity tolerance and proline accumulation. We found more proline content in salt-sensitive cultivar Ataem-7. All these results support that tolerance mechanisms in plants are highly affected by morphological and physiological modifications specific to those plants [31, 32].

P5CS has been reported as the main responsible gene in proline biosynthesis [31]. Also, proline decomposition by proline dehydrogenase enzyme, which is a mitochondrial enzyme, is another mechanism that increases the proline concentration. Transgenic approaches to improve tolerance by inducing proline accumulation is studied in tobacco and *A. thaliana* [24, 33]. The expression level of this gene is very important to understand the mechanisms of accumulation. In our study, the proline concentration results at 100 mM NaCl showed that Ataem-7 and Üstün-1 varieties had nearly the same level of proline in leaves, but transcripts of the gene didn't show the same behavior. These results show that the accumulation of proline in two soybean varieties are under control of different mechanisms in the presence of salinity. The gradual increases of *GmP5CS* expression in Üstün-1 variety seem to be related to salt tolerance. But the increment level of *GmP5CS* expression ratio in Ataem-7 at 100 mM NaCl is found more than Üstün-1 variety. *GmP5CS* gene expression levels showed 8.7 fold increase at Üstün-1 variety when compared to Ataem-7 after 150 mM NaCl treatment. The interesting point is the differences between high proline content and low expression levels of *GmP5CS* in sensitive cultivar Ataem-7 at 150 mM NaCl. This finding indicates that the proline accumulation can not be only a result of increased stress-inducible expression of delta-pyrroline-5-carboxylate synthetase gene.

It is known that proline production may vary among cultivars. Proline concentration is under control of synthesis and catabolism mechanisms of proline [31]. The leaves of Ataem-7 have pigment degradation as a result of high osmotic stress. Lipid peroxidation results confirm these morphological findings. The highest proline level of Ataem-7 was determined at 150 mM NaCl. But the *GmP5CS* gene expression level responsible to accumulate proline decreased nearly to control level. In this situation, proline degradation is expectative. In many plants, feedback inhibition of the *P5CS* enzyme is studied [17, 32, 33]. The reported result of this mechanism is to block the proline synthesis and to effect the redox potential in plastids and accelerate chlorophyll damage [13]. As a result, in the Ataem-7 cultivar it is obvious that *GmP5CS* gene expression is not the only responsible mechanism of proline accumulation. Therefore, beyond *GmP5CS*, other targets should be considered in further studies.

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