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Proline Accumulation and P5CS (Δ^1-pyrroline-5-carboxylate synthetase) Gene Expression in Response to Salt Stress in Zoysiagrasses

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ABSTRACT : Proline is known as an osmotrotectant to enhance tolerance against both salt and dehydration stresses. A P5CS (Δ^1-pyrroline-5-carboxylate synthetase) plays a major role in regulation of synthesis of proline. An overexpression of the mothbean P5CS gene in transgenic tobacco plant increased the levels of proline and osmotolerance. In an attempt to look for the possibility to use content of proline as well as a level of P5CS gene expression as molecular markers for salt tolerance, the amounts of proline and transcript levels of P5CS were measured as functions of either concentration of NaCl or length of treatment period among different species of zoysiagrass. Hybridzoysia showed the highest level of proline (329 μg/g.f.w.) among five different species of zoysiagrass at 250 mM NaCl in 24 hours. The level of P5CS transcript was also the highest in the hybridzoysia at 250 mM NaCl in 24 hours. The transcriptions of P5CS gene were induced at the rates of 1.2, 1.2, 1.8, and 1.8, upon treatment of 250 mM NaCl in *Z. japonica*, *Z. matrella*, *Z. sinica and hybridzoysia* respectively. Based on a correlation between the level of P5CS transcript and the proline content among different species of zoysiagrass, a comparative structural analysis of the gene for P5CS from either *Z. sinica* or hybridzoysia may lead to an understanding of mechanism for salt tolerance shown differently among zoysiagrasses.

Keywords : Zoysiagrass, Proline, P5CS, Salt Stress

It has been shown that plants respond to salt stress by increasing cellular ions such as Na^+ and Cl^- that are compartmentalized into vacuole using Na^+/H^+ exchanger (Maris *et al.*, 1999). The resulting difference in osmotic pressure between cytoplasm and vacuole is relieved by accumulations of proline, betaine, and choline within cytoplasm (Storey & Jones, 1977). Since first observation that proline increases in dehydrated ryegrass was reported, a proline is known to be the general osmoprotectant to protect plants against various environmental stress including low or high temperature, dehydration, as well as high salt stress (Delauney & Verma, 1993). Although the *de novo* synthesis of proline in *Arabiodopsis* upon osmotic stress was reported, both decrease in utilization of proline and degradation of proteins may also contribute the increase in proline concentration (Verbruggen *et al.*, 1993). In facts, the extent of proline concentration has been used as a biochemical marker for salt tolerance in rice since there is a significant difference observed among varieties upon salt treatment (Heo, 1993). The callus of tobacco selected for salt tolerance shows a faster and a higher level of accumulation of proline compared to control callus (Watad *et al.*, 1983).

A Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is known to be a key enzyme in biosynthetic pathway for proline in plants (Hu *et al.*, 1992; Delauney & Verma, 1993). The genes for P5CS were isolated from mothbean, *Arabidopsis,* rice, tobacco, tomato, and grape (Hu *et al.*, 1992; Yoshiba *et al.*, 1995; Igarashi *et al.*, 1997). The transgenic tobacco with a mothbean P5CS gene shows significant increases in P5CS transcript and proline and above all, an increased drought tolerance (Kishor *et al.*, 1995).

Even though there are many demands on study of salt tolerance in zoysiagrass, only a limited number of studies had tried to correlate a proline content to salt tolerance (Lee *et al.*, 1994). Besides, the accuracy in classification of the species used for such studies remained unclear yet. However this study could be performed with well-characterized zoysiagrass plants since classifications of zoysiagrasses had completed carefully based on morphological properties, isozyme pattern, and RAPD analyses (Yang *et al.*, 1995; Choi *et al.*, 1997). The levels of proline and P5CS transcript were measured and compared among five different species of zoysiagrasses.

MATERIALS AND METHODS

Plant materials

Z. sinica, *Z. japonica*, *Z. matrella*, *Z. koreana*, and hybridzoysia (*Z. sinica X Z. japonica*) were donated from the turf laboratory in Dankook University after classifications were done based on morphological properties, isozyme pattern, and RAPD analyses (Choi *et al.*, 1997)

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Salt treatment

The plants were washed with distilled water and transferred into pots filled with solution of a desired concentration of NaCl and treated for a designated time period at 25°C. For an analysis of the concentration course, the hybridzoysia was treated in solution of 0, 50, 150, 250, 300, 400, and 500 mM NaCl for 24 hours. In case of a time course analysis, the hybridzoysia was treated in solution of 250 mM NaCl for 0, 1, 2, 5, 10, 24, 48, 72, and 150 hours. For comparison among Zoysiagrasses the plants of 5 species were treated at 250 mM NaCl for 24 hours.

Measurement of proline

The concentration of proline was measured according to the method of Bates *et al.* (1973) as following. A 0.5 g of leaf was frozen with liquid nitrogen and pulverized with mortar and pestle and 1.5 μl of 0.2 N perchloric acid was added to the powdered tissue and pulverized to make a fine suspension. The suspension was centrifuged at 4°C and 10,000 g for 20 minutes. To the supernatant $KHCO_3$ was added to adjust pH 4 and centrifuged again at 4°C, 10,000 g for 20 minutes and the supernatant was saved. To 1 ml of the supernatant, 1 ml of acid reagent (acetic acid 60 ml+ phosphoric acid 20 ml+ H_2O 20 ml+ninhydrin 1.25 g) were added and 1 ml of acetic acid and put in boiling water bath for 1 hour before an absorption was measured at 520 nm after cooling on ice for 10 minutes.

Northern blot hybridization

Three grams of leaf was ground with mortar and pestle after freezing in liquid nitrogen. Total RNA was extracted according to the hot phenol methods and separated in 1.2% formaldehyde gel at 70 V for 5 hours, and northern analysis was done according to methods by Sambrook *et al.* (1989). The random priming method was used to make ^{32}P-labeled probes using the mothbean P5CS gene (accession number: M92276) to detect the P5CS transcripts and soybean 5S rRNA gene(accession number: X15199) to confirm equal loadings of RNAs. After the hybridization at 42°C was completed, washings with a solution of 0.2X SSPE and 0.1% SDS at room temperature were done followed by an additional washing with the same solution at 60°C. The signals on autoradiogram were analyzed densitometrically by NIH Image 1.62 and the signals for P5CS were normalized by those for 5S rRNA.

RESULTS AND DISCUSSIONS

Proline contents among Zoysiagrasses

When hybridzoysia was treated with 250 mM of NaCl, proline contents in leaf started to increase significantly in 10 hours after the treatment and reached a peak (329 μg/g.f.w.) at 24 hours followed by a gradual decrease as shown in Fig. 1. The overall patterns of proline accumulation with the time of treatment were similar between rice and zoysiagrass only with an exception of the faster induction in zoysiagrass (Igarashi *et al.*, 1997).

When proline contents in control and 24 hour treatment at 250 mM of NaCl were compared, hybridzoysia showed an increase of 22 times that is higher compared to *Nicotiana tabacum, Glycine max, and Arabidopsis thaliana* showing 20, 11, and 8 times of increase rate, respectively (Delauney & Verma, 1993). In the light of that the hybridzoysia shares the genetic background of *Z. sinica* known to grow in seashore, it may adapt better to salt stress by accumulating the proline as osmoprotectant faster than other plants. In addi-

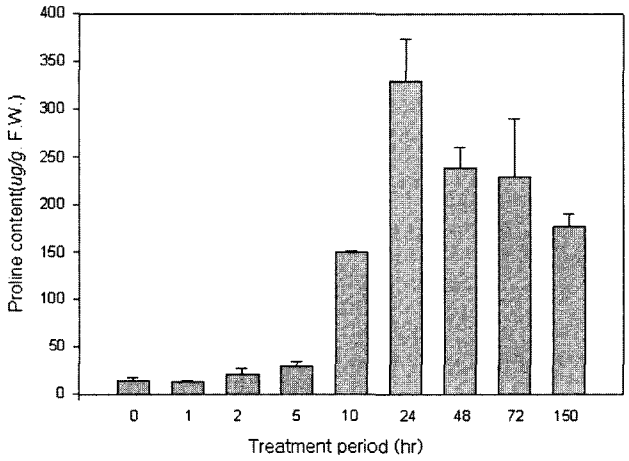

Fig. 1. Proline content of hybrizoysia according to treatment period at 250 mM Nacl (n=3).

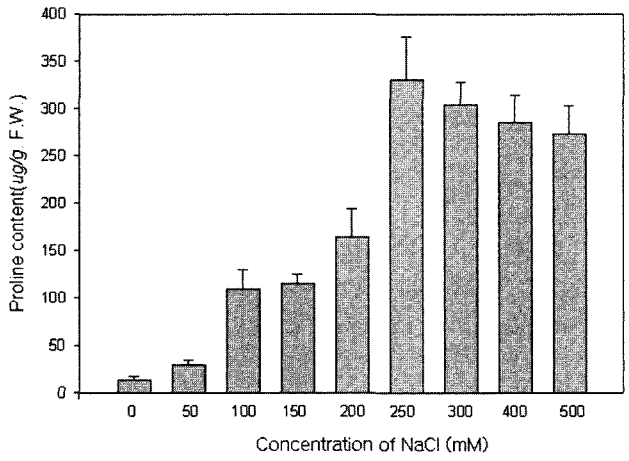

Fig. 2. Proline content of hybrizoysia treated with different NaCl concentrations for 24 hours (n=3).

tion, when the proline contents of hybridzoysia under different salt concentrations were measured a small difference was found between control and 50 mM NaCl condition but the proline contents gradually increased at over 100 mM of NaCl with a peak (331 μg/g.f.w.) at 250 mM of NaCl. The proline contents decreased at over 250 mM of NaCl as shown in Fig. 2.

It was reported that the Itallian ryegrass showed a similar pattern of induction such as a gradual increase of proline up to 100 mM NaCl treatment and a large increase from 150 mM to 300 mM (Lee & Choi., 1995). Compared to the maximum level of proline (23 μg/g.f.w.) in rice at 250 mM NaCl treatment (Igarashi *et al.* 1997), hybridzoysia was shown to accumulate 14 times higher level of proline. Taken all together, a maximum induction of proline in zoysiagrasses was occurred after 24 hours at 250 mM NaCl.

Comparing the amounts of proline accumulated upon treatment of 250 mM NaCl for 24 hours among five species of zoysiagrass, significant increases were observed in all of the species examined (Fig. 3). In facts, there were some degrees of variation in the amount of proline under non-stress condition depending on species of zoysiagrasses. The *Z. matrella* showing 38 μg/g.f.w. was the highest in proline contents and both *Z. sinica* and hybridzoysia showed lower levels of proline contents than other species.

Considering the level of proline accumulated upon the stress, *Z. japonica* showed the lowest (85 μg/g.f.w). and hybridzoysia did significantly higher level (331 μg/g.f.w.) and the *Z. sinica* was second to the highest. A heterotic improvement may explain the enhanced ability to accumulate proline in hybridzoysia.

Z. matrella, Z. japonica, and Z. koreana, showed 2, 3, and 5 times higher levels of induction, respectively upon the salt treatment, *Z. sinica* and hybridzoysia, however, showed the higher levels of induction such as 14 and 22 times of increase, respectively. These results are similar to the report showing that hybridzoysia accumulates the highest level of proline among zoysiagrasses (Lee *et. al.*, 1994). The higher levels of proline accumulation may be explained in the light of the fact that *Z. sinica* and hybridzoysia share parts of the same genetic background to adapt for habituation at seashore by increasing the synthesis of proline. This enhanced synthesis of proline may be due to a higher level of P5CS gene expression or an enzymatic activity. In order to understand the molecular mechanism underlying the higher level of proline accumulation, the level of mRNA for P5CS was measured.

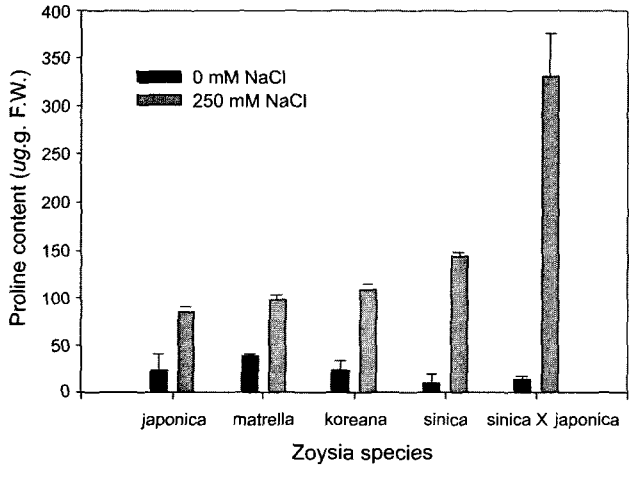

Fig. 3. Comparison of proline content among five species of Zoysiagrass in either control or at 250mM NaCl condition during 24 hours. Values are the means of three replications.

Northern analysis of P5CS transcripts in zoysiagrasses

The transgenic tobacco with a mothbean P5CS gene showed a higher level of transcript and an enhanced salt tolerance compared to the nontransgenic plant (Kishor *et al.*, 1995). This may support a close correlation between the level of P5CS gene expression and salt tolerance. Based on this observation the mothbean P5CS gene was used to probe the levels of the transcript for P5CS genes among different species of zoysiagrass as increase time of treatment at 250 mM NaCl (Fig. 4).

The transcript for P5CS gene increased gradually and reached a peak in 24 hours and then decreased. Rice showed a similar pattern such as a peak of P5CS gene expression at a level of transcript in 24 hours but *Arabidopsis* showed the faster response showing a peak in level of P5CS transcript at 2 hours after an exposure to 250 mM NaCl (Yoshiba *et al.*, 1995; Igarashi *et al.*, 1997). In case of northern analysis as a function of salt concentration, a gradual increase of P5CS

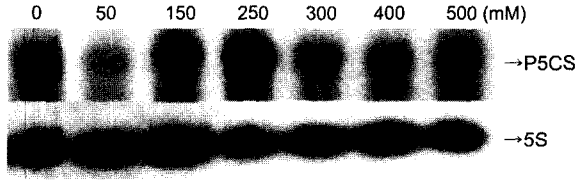

Fig. 4. Northern analysis of mRNA for P5CS accumulated in hybridzoysia as a function of NaCl concentration.

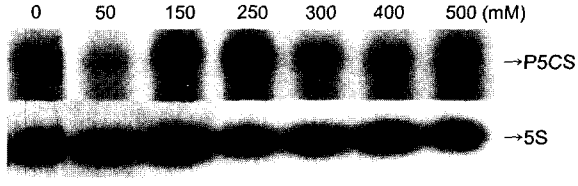

Fig. 5. Northern analysis of mRNA for P5CS accumulated in hybridzoysia as a function of NaCl concentration.

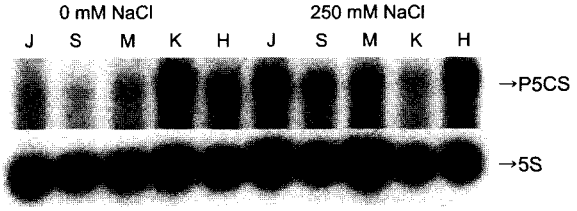

Fig. 6. Northern analysis of mRNA for P5CS accumulated in five species of zoysiagrass treated with 250 mM NaCl for 24 hours, J: *Z. japonica*, S: *Z. sinica*, M: *Z. matrella*, K: *Z. koreana*, H: Hybridzoysia.

transcripts with a peak of two times higher level at 250 mM NaCl compared to the control was observed (Fig. 5). The Arabidopsis also showed a gradual increase but an earlier peak at 150 mM NaCl and then decrease at 200 mM NaCl (Jiping & Zhu, 1997).

The similar pattern in P5CS transcript accumulated in both rice and zoysiagrass may be reasonable in the light of that they belong to the same family, Poaceae. Besides, it appears that the amounts of proline accumulated positively correlated to those of the P5CS transcripts as a function of incubation time as well as concentration of NaCl treated. This may indicated the level of proline accumulated is determined mainly at the level of transcription of P5CS gene. Based on the previous reports that the P5CS is a key enzyme in biosynthetic pathway of proline in plants, it is possible to conclude that the level of P5CS transcript upon salt stress can be used as a good molecular marker for tolerance against the stress.

In order to trace any clue to explain a molecular mechanism to distinguish the difference shown in the levels of proline accumulated in response to 250 mM of NaCl among different species of zoysiagrass, the levels of the transcript were measured (Fig. 6). Depending on the environmental conditions, the levels of the P5CS transcript among zoysiagrasses were different such that *Z. koreana* showed the highest level of P5CS transcript under non-stress condition but *Z. sinica* did the highest upon salt stress. In terms of the induction rate comparing the level of the P5CS transcript at 250 mM NaCl to that at 0 mM NaCl, *Z. sinica* and hybridzoysia showed 1.8 and 1.9 times increases, respectively in contrast to both *Z. japonica* and *Z. koreana* showing 1.2.

As the treatment time period or concentration of salt varied, parallel trends were found in levels of between proline and P5CS transcripts. Besides, both *Z. sinica and* hybridzoysia sharing the genetic background of *Z. sinica* accumulate the higher levels of both P5CS transcripts and proline. This may be somewhat expected in the light of that the *Z. sinica* is able to grow in seashore of high salt condition.

Upon the salt treatment, proline was increased 22.5 times and P5CS mRNA did 1.9 times in hybridzoysia. This may indicate that one of the factors determining the level of proline is transcription rate of P5CS gene but others such as rate of translation or the enzymatic activity may plays another role. In facts, the transgenic tobacco with mothbean P5CS gene expressed under 35S promoter showed an enhanced level of proline (Kishor *et al.*, 1995). Hybridzoysia may undergo alteration during recombination to have a stronger promoter for P5CS gene that induces an accumulation of the higher level of P5CS transcript and then the increased amount of proline. Besides, it was reported that a salt tolerant tobacco accumulating a higher level of proline showed depressed activity of P5CS enzyme in 10 mM proline containing media (Watad *et al.*, 1983; Hu *et al.*, 1992; Fujita *et al.*, 1998). This may suggest another possible mechanism to explain the highest level of proline accumulated in hybridzoysia by the assumption that the P5CS enzyme of hybridzoysia is modified not to be regulated in feed-back mode by proline. In facts it was reported that a salt tolerant mutant of *Nicotiana plumbaginifoliana* showed a lack of feed-back inhibition on the conversion of [5-^{13}C] glutamate to [5-^{13}C] proline operated by wild type plants (Roosens *et al.*, 1999). These assumptions may support the necessity of cloning of the P5CS gene from the hybridzoysia or *Z. sinica* to examine any uniqueness in either promoter or amino acid sequence of the P5CS gene or protein.

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