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Expression analysis of proline metabolism-related genes in salt-tolerant soybean mutant plants

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Abstract

Salt stress is one of the important abiotic stress factors. Proline is generally thought to play an important role in the improvement of salt tolerance in plants. In the present study, we discussed the relationship between free proline accumulation and the expression patterns of the genes that play roles in proline metabolism (*P5CS*, *P5CR*, *PDH*, *P5CDH*) under 90 mM NaCl stress. We used three salt tolerant M_3 generation soybean mutant plants (Ataem-7/150-68, S04-05/150-2 and S04-05/150-114). The mutants belonging to M_3 generation are determined as tolerant to 90 mM NaCl. The free proline contents of the salt-tolerant mutants were measured at the upper phase of the extract with respect to toluene. We observed 1.96-, 2.43- and 1.14-fold increases in the free proline accumulation of Ataem-7/150-68, S04-05/150-2 and S04-05/150-114 mutant plants after 7 days of salt treatment in accordance with control groups, respectively. The expression analyses were performed using specific primers designed for soybean gene regions. According to the results of the quantitative reverse-transcriptase polymerase chain reaction, all the genes were up-regulated when these mutants were subjected to salt stress. In addition to increased expression levels of these genes in three salt tolerant soybean mutants, the only statistically significant relation was observed between the regulation of *P5CR* and *PDH* gene expressions and proline content in S04-05/150-114 mutant. In further studies, the other possible mechanisms that cause proline accumulation should be evaluated for these salt tolerant soybean mutants.

Keywords: Soybean mutants; proline metabolism-related genes; qRT-PCR.
Abbreviations: *P5CS*_delta-1-pyrroline-5-carboxylate synthase; *P5CR*_pyrroline-5-carboxylate reductase; *PDH*_pyrroline dehydrogenase; *P5CDH*_delta-1-pyrroline-5-carboxylate dehydrogenase; OAT_ornithine-aminotransferase.

Introduction

The molecular responses of plants to environmental stresses have been studied intensively (Hasegawa et al., 2000). Salt stress is one of the major abiotic stresses that limit crop productivity and plant growth due to a reduction in water availability, ion imbalances caused by sodium ion accumulation and hyperosmotic stress, which lead to molecular damage in plants throughout their life cycles (Maggio et al., 2002). General metabolic adaptation, which enables plants to cope with water or osmotic stress, involves the increased synthesis of osmoprotectants. Proline accumulation is used as a selection parameter for salt stress tolerance. Proline is one of the well-known osmoprotectants in plants observed under salinity conditions (Silva-Ortega et al., 2008). Proline can also function as a protein stabilizer, a hydroxyl radical scavenger, a source of carbon and nitrogen and a cell membrane stabilizer. In plants, proline has two synthetic pathways. One pathway utilizes glutamine as the primary precursor through the action of Δ^1-pyrroline-5-carboxylase synthase (*P5CS*), and the other pathway utilizes ornithine through the action of ornithine-aminotransferase (OAT). These pathways participate in the process of stress-induced proline accumulation. However, proline biosynthesis from glutamate is considered to be the predominant pathway, especially under stress conditions (Szabados and Savouré, 2009; Lehmann et al., 2010). The synthesis of proline through the glutamate pathway is regulated by two key enzymes (*P5CS* and Δ^1-pyrroline-5-carboxylase reductase (*P5CR*)) (Delauney and Verma, 1990; Hu et al., 1992; Hu et al., 1999). The catabolic pathway of proline is under the control of two other genes. Proline is oxidized to proline-5-carboxylate (*P5C*) by proline dehydrogenase (*PDH*). P5C is then converted to glutamate both non-enzymatically, by glutamatesemialdehyde, and enzymatically, by P5C dehydrogenase (*P5CDH*) (Szabados and Savouré, 2009; Cecchini et al., 2011; Kim and Nam, 2012; Nishimura et al., 2012). Despite the interest in the role of intracellular organic osmolytes during salt stress, the molecular mechanisms leading to the accumulation of osmolytes are not understood in whole plants. Although the predictable results of proline metabolism in response to salt stress have been identified by several studies, the relationship between salt tolerance and proline-based tolerance processes is unclear. Given the well-known importance of the enzymes *P5CS* and *P5CR* for the synthesis of proline and of *P5CDH* and *PDH* for the catabolism of proline in response to salt stress, we sought to determine how these genes are regulated. qRT-PCR is often used in gene expression analysis to identify biological processes (Jain et al., 2006; Luo et al., 2012). Thus, the aim of the present study was to identify and compare the transcript levels of proline metabolism-related genes in salt-tolerant mutants generated in our laboratories and to determine their effects on tolerance. We evaluated the

relationships between proline accumulation and proline metabolism-related genes (both anabolism- and catabolism-related genes) statistically in salt-tolerant soybean mutants under salt stress conditions.

Results

P5CS, *P5CR*, *PDH* and *P5CDH* transcript levels were investigated in the salt-tolerant soybean mutants (Ataem-7/150-68, S04-05/150-2 and S04-05/150-114) under 90 mM NaCl stress. The expression analyses were performed using specific primers designed for soybean gene regions. The relative fold changes of the genes involved in the anabolic and catabolic proline pathways under salinity stress are shown in Fig. 1 and Fig. 2.

Expression of proline biosynthesis genes in response to salt stress

The expression pattern of the *GmP5CS* transcript under 90 mM NaCl stress was analyzed by quantitative reverse-transcriptase PCR. Q-PCR results showed that the *GmP5CS* transcript was expressed in all mutant plants. According to the results of the qRT-PCR analyses, the transcript level of *GmP5CS* increased greatly (1.6-fold) in response to salt stress in S04-05/150-2 mutant plants compared to control plants. Ataem-7/150-68 and S04-05/150-114 mutants also showed 1.0- and 0.8-fold increases in the mRNA levels of the *GmP5CS* gene compared with control plants, respectively (Fig. 1). *GmP5CR* gene expression levels were also up-regulated in all mutant plants. The highest expression level of *GmP5CR* was observed in Ataem-7/150-68 mutant plant followed by S04-05/150-2 and also given in Fig. 1. The *GmP5CR* gene expression levels were 0.62, 0.47 and 0.28 fold increased during NaCl stress treatment with respect to control groups of Ataem-7/150-58, S04-05/150-2 and S04-05/150-114, respectively.

Expression of proline catabolism genes in response to salt stress

The catabolic pathway genes were also up-regulated in mutant plants. The changes in the transcript levels of the *GmP5CDH* and *GmPDH* genes are shown in Figure 2. *GmP5CDH* gene expression levels in mutant plants showed differences under salt stress treatment. Ataem-7/150-68 mutants showed a 1.38-fold increase, whereas 1.3- and 0.5-fold increases were detected in S04-05/150-114 and S04-05/150-2 plants, in comparison to control plants, respectively. *GmPDH* gene expression levels were also up-regulated by NaCl treatment in salt-tolerant mutant plants compared with control plants. A 0.6-fold increase in Ataem-7/150-68 mutant plants, compared with control plants, was the largest increased observed for *GmPDH* transcript levels. *GmPDH* gene expression analyses demonstrated 0.2-, 0.3- and 0.52-fold increases in expression levels under salt stress for S04-05/150-2, Ataem-7/150-68 and S04-05/150-114 mutant plants, in comparison with control plants, respectively.

Proline content

Proline is an important osmolyte which is accumulated as a final product, in the leaf tissues of salt-tolerant soybean mutants under 90 mM NaCl stress was also evaluated. The proline contents of the salt-tolerant soybean mutants are shown in Figure 3. In Ataem-7 control plants, the proline content was 0.155 µmol proline/g fresh weight, whereas in mutant Ataem-7/150-68 plants, the proline content was increased to 0.305 µmol proline/g fresh weight. For the 14-day-old seedlings of S04-05/150-114 and S04-05/150-2 salt-tolerant mutant plants, the proline contents were increased to 0.252 and 0.537 µmol proline/g fresh weight, respectively, whereas the proline concentration of the S04-05 control group was 0.221 µmol proline/g fresh weight.

Statistical analysis

The relation between proline and proline metabolism-related genes under 90 mM salt stress in salt tolerant soybean mutant plants, were evaluated by Pearson's correlation analysis. The relationships of salt-tolerant soybean mutants Ataem-7/150-68, S04-05/150-2 and S04-05/150-114 were given in Fig. 4. The only statistically significant correlation between proline and proline metabolism-related genes were detected for S04-05/150-114 mutant plant. For proline biosynthesis, the regulation of *PDH* (R^2=0.5432) (Fig.4C) were inversely and for proline degradation, the regulation of *P5CR* (R^2=0.4346) (Fig.4D) were positively correlated in S04-05/150-114 soybean mutant with proline content as well as the regulation of other genes were not connected to proline content. On the other hand, the regulation of proline metabolism-related genes in Ataem-7/150-68 and S04-05/150-2 salt tolerant mutant plants were found unrelated to proline content (Fig.4).

Discussion

Proline is an important multifunctional amino acid and plays a role in carbon and nitrogen metabolism, cell signaling, nutrient adaptation and protection against osmotic and oxidative stresses (Khedr et al., 2003; Claussen et al., 2005; Claussen et al., 2006; Tripathi et al., 2007; Lehmann et al., 2010). Proline accumulation in response to drought or salinity stress has been reported to occur in the cytosol to adjust the osmotic balance. Transgenic plants overexpressing *P5CS* have been shown to have increased proline concentrations that paralleled increased tolerance to drought and salinity stresses (Hmida-Sayari et al., 2005). It has been reported that, under salt stress, proline accumulation was greater in sensitive rice cultivars than in salt-tolerant genotypes. Although, in some plant species, proline concentrations can be used as a measure of stress, the exact mechanisms and relationships between proline accumulation and abiotic stress tolerance have not been clearly defined (Kishor et al., 2005; Ashraf and Foolad, 2007; Szabados and Savoure, 2009; Wang and Han, 2009; Dobra et al., 2011; Ku et al., 2011).To investigate the role of proline metabolism in salt-tolerant soybean mutants, 14-day-old seedlings belonging to M_3 generations of Ataem-7/150-68, S04-05/150-2 and S04-05/150-114 salt-tolerant mutant plants were subjected to 90 mM salt stress, and their proline concentrations and gene expression patterns of proline metabolism-related genes were compared. Free proline contents were 1.96-, 2.43- and 1.14-fold increased after one week of salt treatment in Ataem-7/150-68, S04-05/150-2 and S04-05/150-114 mutant plants, with respect to control, respectively. The maximum proline accumulation levels were obtained for the mutant S04-05/150-2. All of the mutant plants showed increased proline content but at different levels. The level of proline in S04-05/150-114 mutant plants was not significantly different from that in S04-05/150-114 control plants. For S04-05/150-114 plants, it appears that salt tolerance is independent of proline accumulation. We observed varying responses in terms of the expression levels of proline-related genes following 90 mM NaCl stress. In all salt-tolerant soybean mutants, the proline

Table 1. Primer sequences for proline metabolism-related genes used in qRT-PCR analyses.

Name	Accession Number	Forward Primer (5'→3')	Reverse Primer (5'→3')
GmP5CS	NM_001251224.1	ATTCCTGTCCTGGGTCATGCAGAT	AAGAGTTTCCATGGCATTGCAGCC
GmP5CR	NM_001248985.1	TGACAGTAATCCCACCCAGCTCAA	ACTTCAGAACCAGGTTGGGTCCAT
GmPDH	NM_001250359.1	TCAACTTGCCAACCAGAGACTCCT	ATCGATAGCCGGTTAACTGTGGT
GmP5CDH	XM_003549616.1	TAGGGCGACTATGGTAATTGCGGT	TGCCCACAGTGTCGAAACGGAATA

synthesis metabolism-related genes (*P5CS* and *P5CR*) were up-regulated. The S04-05/150-2 soybean mutant showed the highest *GmP5CS* transcript levels. During salt stress, the accumulation of *GmP5CS* transcripts was followed by the increased proline concentrations in all mutants. Although the increase in proline levels was not found to be statistically significant in S04-05/150-114 mutants, the *Gm*P5CS expression level was increased in these mutants. It has been reported that free proline concentrations are correlated with the expression patterns of proline-related genes. Proline is known to self-regulate its metabolism (Kishor et al., 2005). Despite being the rate-limiting enzyme for proline biosynthesis, the *P5CS* gene is known to be the primary regulator of proline levels under salt stress. Increased levels of *P5CS* mRNA have been reported in several plants, such as *Arabidopsis*, soybean, tobacco and *Medicago truncatula* (Ma et al., 2008; Kim and Nam, 2012). Dobrá et al. (2011) demonstrated the up-regulation of *P5CS* gene expression in the leaves of tobacco plants following 6-day-long drought stress. Hien et al. (2003) indicated that *P5CS* activity is not responsible for the differential proline accumulation in plants that have different levels of abiotic stress tolerance. Ma et al. (2008) suggested that the overexpression of *P5CS* enhanced the reduction of P5C to proline. This conversion decreases the stress injury to membranes and provides more energy for use in the recovery processes. In our salt-tolerant soybean mutants, the *GmP5CR* transcript levels increased following stress application, and the greatest increases was recorded in Ataem-7/150-68 mutants. Other proline metabolism-related gene expression levels have previously been investigated in other plants in response to abiotic stresses, such as salt stress and drought. In Arabidopsis plants, it has been shown that *P5CR* transcript levels were unaffected by stress treatment. Willett and Burton (2002) indicated that hyperosmotic stress did not result in an increase in the transcript level of the *P5CR* gene. Stein et al. (2011) also reported that *P5CR* over-producing transgenic plants did not appear to have increased levels of proline. There is some evidence that the levels of *P5CR* mRNA are increased in soybean seedlings, *Arabidopsis* and pea plants by salt treatments (Ma et al., 2008). Ma et al. (2008) also observed increases in the levels of *P5CR* transcripts in salt-tolerant wheat varieties in response to 250 mM NaCl stress. Sripinyowanich et al. (2013) indicated that, in *Oryza sativa*, *P5CR* transcript levels were increased after 4 weeks of salt stress treatment. *P5CR* has also been suggested to be the rate-limiting factor in proline synthesis from glutamate under salinity conditions (Silva-Ortega et al., 2008). Yooyongwech et al. (2012) observed that positive correlation between proline content and regulation of *P5CS* gene in contrast to *P5CR* gene in rice genotypes. Understanding the regulation mechanisms and the genes involved in these processes is important for evaluating the protective effects following stress treatment. The catabolic pathways and their regulatory mechanisms are also important. The *P5CDH* and *PDH* genes are the primary regulators of the proline oxidation that is required to maintain the cellular ROS balance (Szabados and Savouré, 2009).

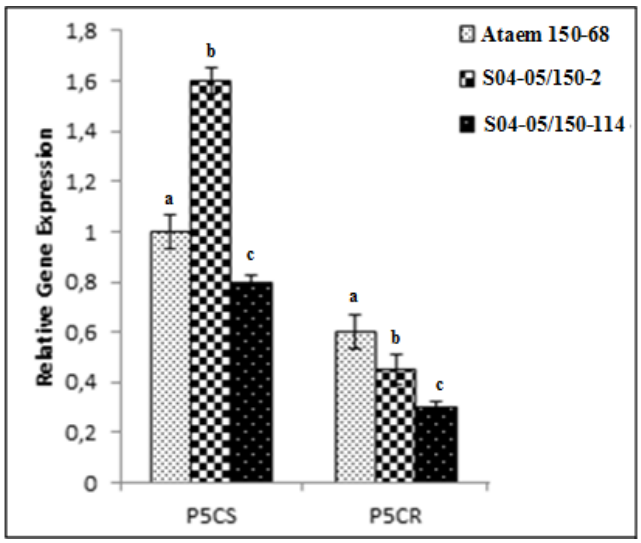

Fig 1. The expression levels of proline synthesis-related genes (*GmP5CS* and *GmP5CR*) in salt-tolerant soybean mutants treated with 90 mM NaCl for 7 days. The data are represented as the means ± SD and are derived from 3 replicates. The differentially given letters represent significance at the 0.05 level.

The S04-05/150-2 mutant showed the smallest increase in the transcript level of *GmP5CDH* among the mutants. A 4.61-fold increases in the expression level of *GmP5CDH* was observed compared to the expression level of the *GmPDH* gene in the Ataem-7/150-68 mutant, suggesting that the mitochondrial electron transport chain was activated and may be responsible for stabilizing mitochondrial respiration. This proline catabolic pathway is important for regulating cellular reactive oxygen species in mitochondria. This mechanism may be responsible for influencing additional regulatory pathways in the Ataem-7/150-68 mutant (Szabados and Savouré, 2009; Kim and Nam, 2012). Cvikrová et al. (2012) suggested that the relationship between increased proline degradation and the production of reducing agents to provide ATP may be required for mitochondrial oxidative phosphorylation and recovery processes. Illumination has been known to have the opposite effect on *PDH* gene expression. Transcriptional repression of the *PDH* gene during daylight has been reported by Szabados and Savouré (2009). Stein et al. (2011) suggested that the increased levels of *P5CS* and *PDH* transcripts were necessary to control proline levels under abiotic stress. As described by Lehmann et al. (2010), no positive correlation was observed in the present study between the rate of proline accumulation and increased *GmPDH* gene expression levels. Yooyongwech et al. (2012) indicated that there is no relation between the regulation of *PDH* and proline in rice genotypes. Our results indicated that there was no repression effect on transcript levels, as described by Lehmann et al. (2010).

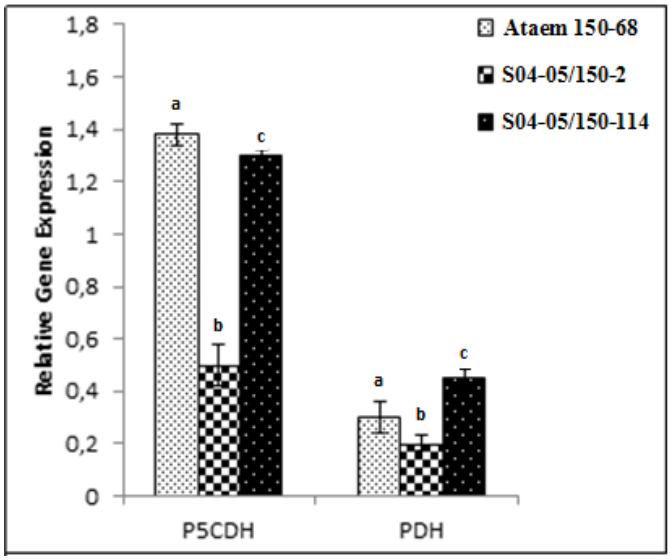

Fig 2. The expression levels of proline catabolism-related genes (*GmP5CDH* and *GmPDH*) genes in salt-tolerant soybean mutants treated with 90 mM NaCl for 7 days. The data are represented as the means ± SD and are derived from 3 replicates. The differentially given letters represent significance at the 0.05 level.

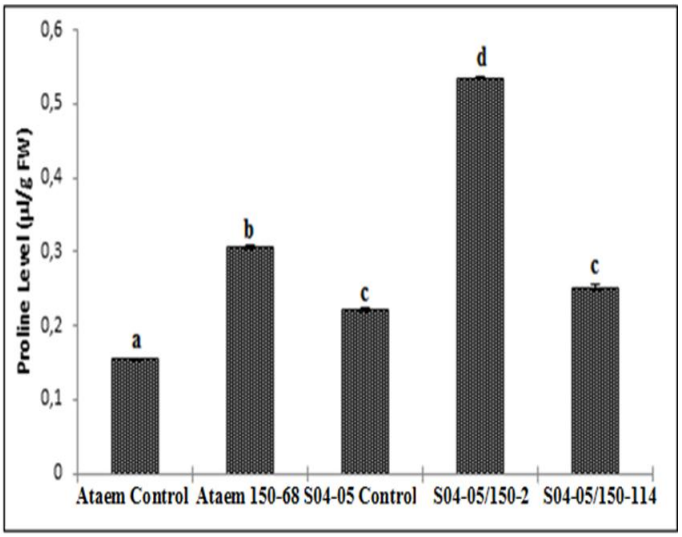

Fig 3. Proline content in salt-tolerant soybean mutants belonging to Ataem-7 and S04-05 soybean varieties under 90 mM NaCl stress. The proline levels of the mutants were evaluated and compared with the levels in the control group. The differentially given letters represent significance at the 0.05 level.

We observed positive correlation between *PDH* transcript level and proline content. Alternative regulation pathways of for proline synthesis in these salt-tolerant mutants have been considered. The differences between the mutants have been assessed at the transcriptional level, but translational control is another potential model for regulation. This is one possible scenario for the rate-limiting enzymes under salt stress. According to this model, plant tissues accumulate proline independent of increases in *P5CS* mRNA levels, which occur only under hyperosmotic stresses (Willet and Burton, 2002; Mazzucotelli et al., 2008). In conclusion, we have shown that the level of proline in plants plays a role in facilitating the rapid response to salt stress that is necessary to survive under extreme conditions. Proline concentration levels act like a signal that activates the responses to salt stress. The correlation analysis showed that the regulations of proline metabolism-related genes and proline content are differentially related in salt tolerant soybean mutants. Compared with control plants, proline over-producing mutants were able to respond rapidly in terms of the coordination of the differentially regulated proline metabolism-related genes. These salt-tolerant mutants should be evaluated in further studies to determine whether other genes that are involved in different mechanisms could serve as tools to improve salt tolerance.

Materials and Methods

Plant materials

In the present study, three M_3 generation soybean mutant plants (Ataem-7/150-68, S04-05/150-2 and S04-05/150-114) were generated by irradiation with a gamma radiation dose of 150 Gy using a Cs-137 gamma source from Our Leukemia Children Foundation (Istanbul). These mutants had been selected as tolerant to 90 mM NaCl under *in vivo* and *in vitro* salt stress treatments compared to control genotypes (Çelik and Atak, 2012). The seeds belonging to each salt-tolerant mutant were grown into perlite under greenhouse conditions using a 16 h light/8 h dark photoperiod at a day/night temperature of 24/18°C. The seeds were watered with Hoagland solution on a regular basis.

Salt stress treatment

To determine the relationship between salt tolerance, proline accumulation and proline metabolism-related gene expression patterns, seeds belonging to *in vitro* and *in vivo* selected salt-tolerant three M_2 soybean mutant plants were planted into perlite and regenerated fourteen-day-old plants (M_3) were irrigated with Hoagland solution containing 90 mM NaCl for 7 days. NaCl-free Hoagland treatment was used for the control group. After 7 days of treatment, the leaves were harvested and used for analyses.

Proline analysis/free proline content

The levels of free proline were measured according to Bates et al. (1973). Briefly, 0.25 g of salt-tolerant mutant leaves were homogenized with 3% (w/v) sulfosalicylic acid, and the homogenate was filtered through Whatman filter paper No 2. The homogenate was combined with 1 ml of ninhydrin acid and 1 ml of glacial acetic acid, and the mixture was then heated for 1 h at 100°C. The reaction was terminated in an ice bath. The mixture was combined with 2 ml of toluene using a vortex for 20-30 s. The upper toluene layer was measured on a spectrophotometer at 520 nm. Toluene was used as the blank. The calculations were given as µmol proline/g fresh weight.

Total RNA isolation and cDNA synthesis

Total RNA from individual leaves was extracted using the UltraClean™ Plant RNA Isolation Kit (MoBio, Carlsbad, USA). First-strand cDNA was synthesized in a total volume of 25 µl using the iScript cDNA Synthesis Kit (BioRad, USA). Reaction mixtures were incubated at 25°C for 5 min, 42°C for 30 s, and 85°C for 5 min and stored at 4°C, as instructed in the manufacturer's protocol.

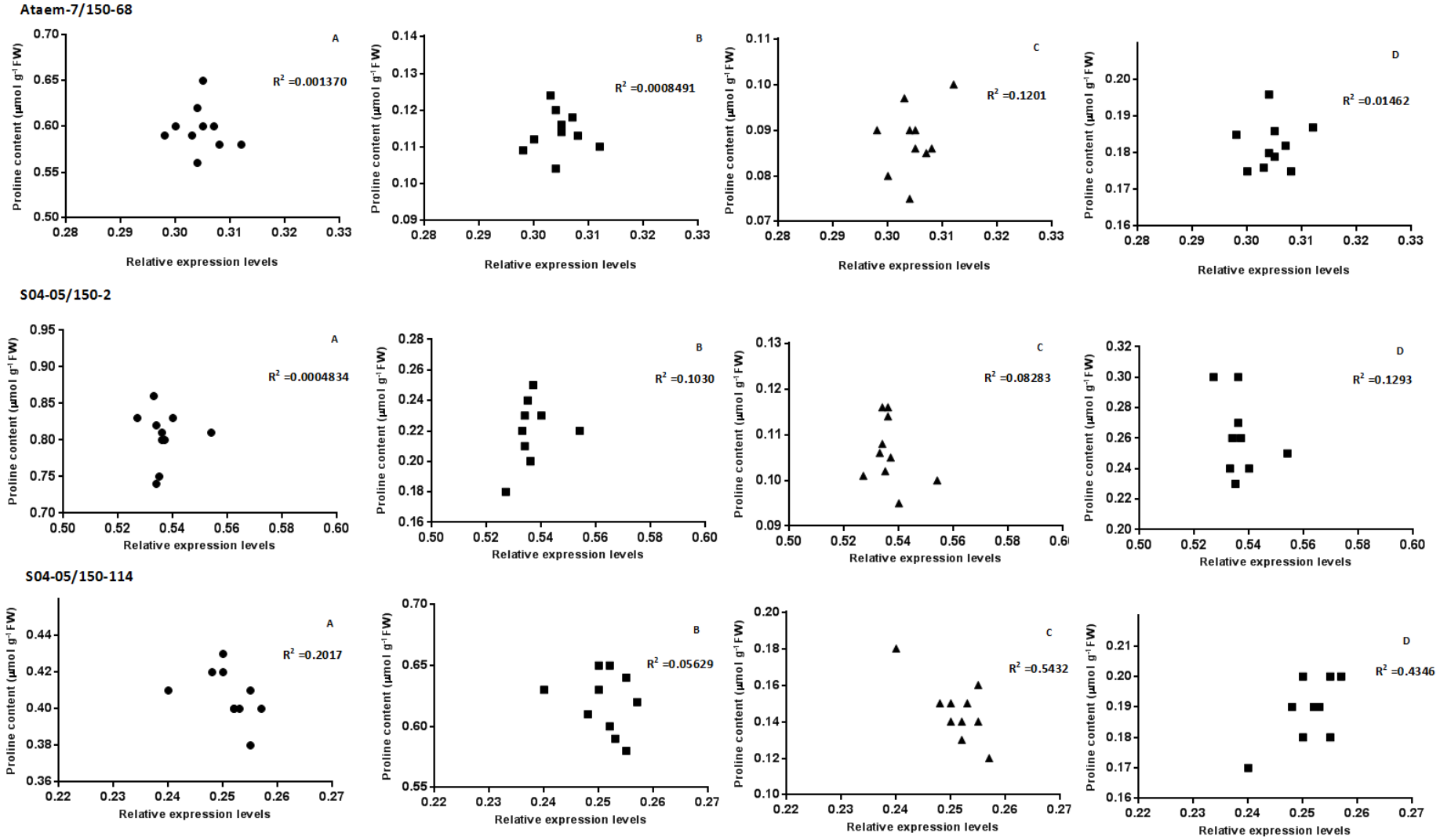

Fig 4. Relationships between proline and proline metabolism-related genes of salt-tolerant soybean mutants, Ataem-7/150-68, S04-05/150-2 and S04-05/150-114 which exposed to 90 mM NaCl stress. Relations between *P5CS* expression and proline content (A), *P5CDH* expression and proline content (B), *PDH* expression and proline content (C), *P5CR* expression and proline content (D).

Quantitative RT-PCR

The first strand cDNAs were diluted 20X with RNase-free water, and qRT-PCR analyses were performed using the IQ SYBR Green Master Mix (BioRad, USA) in a final volume of 25 µl with 500 ng of cDNA. *Glycine max* forrest b-tubulin (GenBank accession No. U12286.1) was used as a housekeeping gene using the specific primers F5'-AGCGTGTGTGACATTGCTCCTAGA-3' and R5'-TCGTTCATGTTGCTCTCTGCCTCT-3'. The primers used for *GmP5CS*, *GmP5CR*, *GmP5CDH*, and *GmPDH* cDNAs can be found in Table 1. Quantitative RT-PCR of all samples was repeated using a Real Time PCR Detection System (Mini Opticon^{TM}, USA). The amplification reaction was programmed as follows: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 30 s at 60°C, , and 30 s at 72°C and, finally, an extension step of 10 s at 72°C. The level of transcripts was determined in comparison with b-tubulin gene expression on the 7th day of the treatment. In gene expression studies, at least 3 independent real-time PCR reactions were performed on the same cDNA preparation.

Data analysis

An arbitrary threshold was set at the midpoint of the log DRn versus cycle-number plot. The threshold cycle (C_T) value is defined as the cycle number at which the DRn crosses this threshold. The C_T values of the triplicate PCRs were averaged and used for the quantification of transcript levels. The quantification of the relative transcript levels was performed using the $\Delta\Delta C_T$ method (Pfaffl, 2001). Raw expression values were calculated in Microsoft Excel using the average C_T values and PCR efficiencies.

Statistical analysis

The significance of differences between mean values was compared using Duncan's multiple range test. Differences at $P<0.05$ were considered significant (Duncan, 1955; Mize and Chun, 1988). Relationships between proline content and proline metabolism-related gene expression levels of salt-tolerant soybean mutants which were subjected to 90 mM salt stress, were determined by Pearson's correlation analysis. For each of variables, relationship between proline content and mean relative difference and associated variance is evaluated using Pearson's correlation coefficient.

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