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Original scientific paper

THE EXPRESSION OF *GMP5CS*, *GMPAP3*, AND *GMBZIP50* GENES UNDER SALINE CONDITION IN SOYBEAN USING REAL-TIME PCR

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Salinity in higher plants is the cause of toxicity and osmotic stress. To identify the expression pattern of salinity tolerance genes in soybean, it was investigated the relative expression of three genes, *GmPAP3*, *GmBZIP50* and *GmP5CS* at two tissues leaf and root in Williams (as tolerant) and L17 (as susceptible) soybean genotypes under two levels zero (control) and 300 Mm NaCl. For adaptation of plantlets to salinity, salinity was started with 150 mM NaCl for first time and followed by 300 mM NaCl in two times. The housekeeping gene *18SrRNA* was used to normalize data. Data analysis showed that all three genes expression increased under salinity stress. The expression of *GmP5CS* and *GmBZIP50* was two-fold greater in Williams than in L17 genotype, while *GmPAP3* expression was 2.5-fold greater in L17 than in Williams. The expression of *GmP5CS* and *GmBZIP50* was higher in roots than in leaves, while *GmPAP3* expression was higher in leaves than in roots. In conclusion, *GmBZIP50* over-expressed more than two other genes by 375 and 273% in leaves and roots under stressed compared to non-stressed plants. Thus *GmBZIP50* could be more effective candidate gene for producing soybeans with resistance to salinity that may be resulted from transferring and increasing copy number.

Keywords: gene expression, *GmPAP3*, *GmBZIP50*, *GmP5CS*, salinity

Abbreviations: abscisic acid (ABA), ABRE-binding factors (ABF), ABA-responsive elements (ABRE), basic leucine zipper (bZIP), catalase (CAT), coefficient of variation (CV) dehydration-

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responsive element (DRE), oxysterol binding protein (OSBP), Transcription factor (TF), pyrroline-5-carboxylate synthetase (P5CS), purple acid phosphatases (PAP), quantitative reverse transcription polymerase chain reaction (qRT-PCR).

INTRODUCTION

Soybean (*Glycine max* L.) is susceptible to salt stress and thus, soil salinity can severely affect its growth and productivity (ZHANG *et al*., 2015) Salinity also disturbs nutrient relations, transport and partition within plant (HU and SCHMIDHALTER, 2015) Chlorides (Cl^-) associated with sodium (Na^+) are mostly among soluble salts with toxic effect on plants (BALESTRASSE *et al*., 2008). Yield depends on the salinity level and the excess of toxic ions such as Na^+ and Cl^-. The relationship found between K^+/Na^+ ratio and grain yield may be used as a diagnostic tool for estimating soybean yield loss (BUSTINGORRI and LAVADO, 2013). AHMADI and SOLEIMANI (2015) reported that the *GmOSBP*, *CAT*, and *GmBZIP* gene expressions were higher under drought stress in soybean leaves and roots. Under salt stress, plants accumulate several compatible solutes in the cytosol, such as betaine and proline (HASEGAWA *et al*., 2000). The gene *P5CS* (pyrroline-5-carboxylate synthetase) encodes an enzyme that catalyzes the rate limiting reaction in proline biosynthesis in living organisms (RAI and PENNA, 2013). Proline accumulation is an important mechanism for osmotic regulation under salt stress (HUANG *et al*., 2013). Several reports reveal that overexpression of *P5CS* results in an increased proline level as well as osmotic tolerance in transgenic plants (HUR *et al*., 2004; VENDRUSCOLO *et al*., 2007). In a study, AYSIN *et al*., (2015) showed that proline levels were higher under salt stress conditions in both the shoots and the roots in the transgenic tobacco whit *AtNHX1* compared to wild type plants. However, the relationship between proline accumulation and stress tolerance is not always clear (PATTERSON *et al*., 2009). Purple acid phosphatases (PAPs) represent a diverse group of acid phosphatases in animals, micro-organisms and plants (OLCZAK *et al*., 2003). Quantitative PCR analyses indicated isoform and tissue-dependent responses of PAPs isoforms to ABA, salt, and cold stresses (OZTURK *et al*., 2015). The primary biochemical reaction of PAPs is to catalyze hydrolysis of phosphate esters and anhydrides. The physiological role of *GmPAP3* is related to the adaptation of soybean to salt stress, possibly through its involvement in reactive oxygen species forming (LIAO *et al*., 2003). Northern blot analysis revealed that NaCl stress caused a general induction of *GmPAP3* expression in both roots and leaves of various cultivated and wild soybean varieties (LIAO *et al*., 2003). Moreover, *GmPAP3* gene expression is increased under salt (STOLF-MOREIRA *et al*., 2010) and drought AHMADI and SOLEIMANI (2014) stresses in soybean.

Basic leucine zipper (BZIP) transcription factors are found in all organisms (MUNNS and TESTER, 2008). In plants, *BZIP* regulate processes including pathogen defense, and stress signaling, seed maturation and flower development (JAKOBY *et al*., 2002). Furthermore, overexpression of *BZIP* influenced the expression of some ABA or stress related genes conditions that function in stomata closure in *Arabidopsis* (TANG *et al*., 2012). Four *BZIP* genes were encoded by the genome of the most recent common ancestor of all plants (MUNNS and TESTER, 2008). From soybean, 131 *BZIP* genes were identified and more than one third of these *GmBZIPs* are responsive to at least one of the ABA, salt, drought, and cold stresses (LIAO *et al*., 2008). GAO *et al*. (2011) reported that the expression of *GmBZIP* gene was strongly induced in ABA, drought, salinity, and low temperatures in soybean roots, stems and leaves.

Regarding the expanse of soybean cultivation and its economic importance in the world it would be better to identify the expression pattern of stress tolerance genes in soybean specially

with considering the genetic engineering and biotechnology in agriculture with respect to salty areas. Thus the primary object for further researches will be producing soybeans with resistance to salinity that may be resulted from transferring resistance genes. Therefore, the objective of this research was to study three gene expression patterns under salinity stress in two tolerant and susceptible soybean cultivars.

MATERIALS AND METHODS

Plant materials and treatments

Two soybean cultivars Williams (as tolerant) and L17 (as susceptible) were studied for the expression pattern of *GmPAP3*, *GmBZIP50*, and *GmP5CS* genes in two tissues (root and leaf) under salt stress. Two cultivars were grown based on a completely randomized design with three replications at 30 ±2°C, for 16 h of light and 20 ±2°C, for 8 h of dark in greenhouse conditions. Salt stress treatment was performed at five-leaf stage for 7 days. Non-stress treatment plantlets (as the control) were irrigated every 2 days and in stress treatment for adaptation of plants to salinity, salinity treatment was started with 150 mM NaCl for first time and followed by 300 mM NaCl in two times.

Nineteen days after planting and at the five-leaf stage, leaf and root tissue samples were stored at −80°C for RNA extraction. Sodium and potassium concentrations in tissues leaves and roots were determined using HAMADA and EL-ENANY (1994) method.

RNA extraction and cDNA synthesis

Total RNA was extracted from leaf and root tissues, using the RNX-Plus solution kit (CinnaGen, Iran) according to the manufacturer's recommendations. The RNA was treated with RNase-Free DNase I (Fermentas, Germany) according to the manufacturer's instructions to eliminate remaining genomic DNA. The first-strand cDNA was synthesized from 1 µg total RNA with oligo $(dT)_{18}$ primer in a final reaction volume of 20 µL using the Reverse Transcription Kit (RTPL12, Vivantis, Malaysia) according to the manufacturer's instructions.

Design of QPCR primers

The real-time PCR primers for *GmPAP3*, *GmBZIP50*, *GmP5CS*, and *18SrRNA* (as the housekeeping gene) were designed using Primer BLAST software of the NCBI database (http://www.ncbi.nlm.nih.gov/). Oligo 5 software (National Biosciences Inc., USA) was used to confirm the predicted sequence specificity of the designed primer pairs (Table 1).

Table 1. Primer pairs used in qRT-PCR reaction for the study of GmPAP3, GmBZIP50, and GmP5CS gene expressions under salt stress in two soybean genotypes

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Segment size
GmPAP3	GTGGCCGGCAGTTGACATCC	GCTGTGCCCTGGCTCTTCTGTG	151
GmBZIP50	CAGTGGCGAGGCGCGGGGCC	GAACCTCTCGAACTCGTTGT	120
GmP5CS	CGAACTGAGCTTGCAGAGGGGC	TCGCTTAGCCTCCTTGCCTCC	165
18SrRNA	TTCGTCTACGTCGCATTT	CGTGGAGCAAGTCGTGTAA	148

Quantitative real-time PCR assay and data analysis

The expression of genes was measured using a real-time PCR Detection System (Bio-Rad, USA). The PCR reaction mixture contained 2 μL of diluted cDNA, 10 μL of SYBR Green qPCR Master Mix (SYBR [Premix ExTagII (TliRNAase Plus), Code RR820L]), 0.5 μL of each gene-specific primer pair and 7 μL distilled water in a final volume of 20 μL. The housekeeping gene *18SrRNA* was used for the normalization of the amount of cDNA in each qPCR reaction. The specificity of amplified segments was checked by melting curve analysis performed from 60°C to 95°C for 60 cycles. Data were processed using the method of $2^{-\Delta\Delta CT}$ according to (LIVAK and SCHMITTGEN, 2001). All data were subjected to one-way analysis of variance using SPSS software and the diagrams were drawn using the Excel.

RESULTS

K^+/Na^+ in response to salt treatment

Analysis of variance for K^+/Na^+ ratio revealed significant differences ($P \leq 0.05$) between genotypes in response to salt stress (Table 2). Also significant interactions were found for the K^+/Na^+ ratio in leaf and root tissues of genotypes Williams and L17 in response to salt stress conditions. Comparing of means for genotypes × tissue × stress interaction, it was revealed that the increase in K^+/Na^+ ratio in salt stress and normal conditions in the leaves of genotype L17 was 6 times higher than in Williams. K^+/Na^+ ratio in the roots of Williams in normal conditions was double more than genotype L17, whereas this ratio in stress condition was equal in both genotypes (Figure 1).

Table 2. Analysis of variance for GmPAP3, GmBZIP50, and GmP5CS gene expressions and K^+/Na^+ ratio under salt stress in two soybean genotypes

Sources of Variation	d.f	Meansquare			
		GmPAP3	*GmP5CS*	*GmBZIP50*	K^+/Na^+
Genotype	1	16.351**	5.467**	14.377**	0.893*
Tissue	1	0.436^{ns}	0.312**	1.429**	0.031*
Stress	1	25.059**	3.831**	43.610**	0.221*
Genotype × Tissue	1	0.011^{ns}	0.757**	3.275**	1.440*
Genotype × Stress	1	16.174**	5.823**	13.971**	0.616*
Stress × Tissue	1	0.726^{ns}	0.347**	1.291**	3.441*
Stress × Tissue × Genotype	1	0.016^{ns}	0.820**	3.470**	0.810*
Error	8	0.213	0.021	0.057	0.001
C.V%		20.03	9.54	8.93	5.49

ns, * and **: Non significant, significant at 5%, and 1% level of probability, respectively.

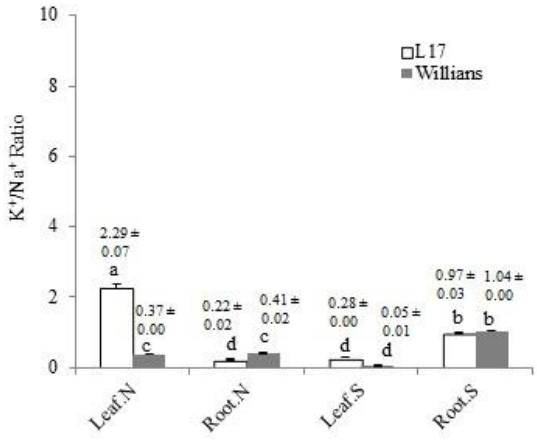

Figure 1. The mean comparisons of K^+/Na^+ ratio in: two tissues, leaf and root in two genotypes in two stress treatments (N = normal, S = stressed). Different letters above columns indicate statistically significant ($P \leq 0.05$) differences. The data above columns are mean ± standard error.

Expression of the GmP5CS gene

Analysis of variance for the expression of *GmP5CS* gene revealed significant differences ($P \leq 0.01$) between genotypes and tissues in response to salt stress (Table 2). Williams showed a higher expression compared to L17 with considering both normal and stressed conditions (Figure 2a). The relative expression of *GmP5CS* was higher ($P \leq 0.01$) in roots than leaves by 20% (Figure 2b). The level of *GmP5CS* expression in Williams was two fold higher than L17 (Figure 2c). In salt stress *GmP5CS* gene expression increased in both leaf and root compared to the normal conditions, however, the increase was significantly higher in roots than in leaves (Figure 2d). A higher *GmP5CS* gene expression was detected in the roots than in leaves of Williams (40%), but the increasing in the leaves was more than in roots of L17 (18%). However, the difference in the expression level between roots and leaves was larger in genotype Williams compared to L17 (184%, 71%), respectively. A similar result was reported in *Vigna* root under salt stress (ZHANG *et al*., 1995). Mean comparison for genotype × stress interaction resulted three distinct classes a, b and c (Figure 2e). Mean comparison for the stress × tissue interaction indicated a similar expression level for *GmP5CS* in leaves and roots under normal condition, but the expression was significantly higher in roots (2.3 fold) compared to the leaves (1.7 fold) in response to the salt stress conditions (Figure 2f). Means comparing for genotype × tissue × stress interaction showed that the increase in *GmP5CS* gene expression was 2.5 and 5.8 times higher in the leaves and roots of Williams than in L17 at salt stress condition (Figure 2g).

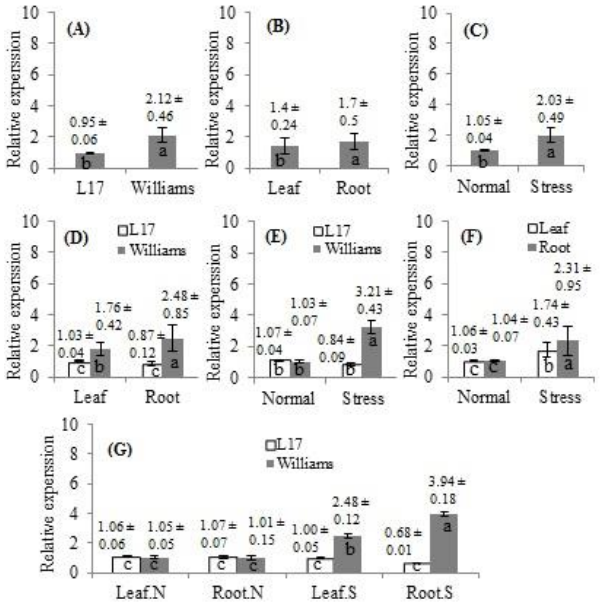

Figure 2. The mean comparisons of *GmP5CS* gene expression in: (**A**) two genotypes, Williams and L17; (**B**) two tissues, leaf and root; (**C**) two stress levels, salt stress and normal condition; (**D**) two genotypes, in two tissues; (**E**) two genotypes, in two stress treatments; (**F**) two tissues, leaf and root in two stress treatments, and (**G**) two tissues, leaf and root in two genotypes in two stress treatments (N = normal, S = stressed). Different letters above columns indicate statistically significant (P≤ 0.01) differences. The data above columns are mean ± standard error.

Expression of the GmPAP3 gene

Analysis of variance for *GmPAP3* gene expression revealed significant differences (P≤ 0.01) between genotypes, stress conditions, and their interactions (Table 2). Mean comparisons revealed that in both normal and a stress condition, the level of *GmPAP3* gene expression was significantly higher in the genotype L17 compared to Williams (Figure 3a).

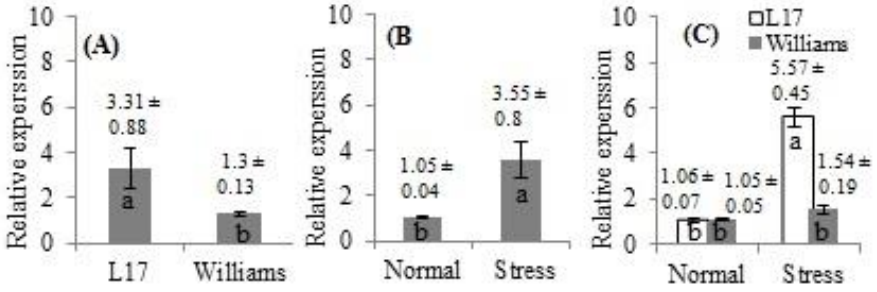

Figure 3. The mean comparison of GmPAP3 gene expression in: (**A**) two genotypes, Williams and L17; (**B**) two stress levels, salt stress and normal condition, and (**C**) two genotypes, in two stress treatments. Different letters above columns indicate statistically significant (P≤ 0.01) differences. The data above columns are mean ± standard error.

Moreover, the expression level of *GmPAP3* showed a fourfold increase in response to salt stress (Figure 3b). Different responses of genotypes to salt stress resulted in a significant genotype × salt stress interaction. Under salt stress the expression level of *GmPAP3* in genotype L17 increased by 262% which was significantly higher compared to Williams (Figure 3c).

Expression of the GmBZIP50 gene

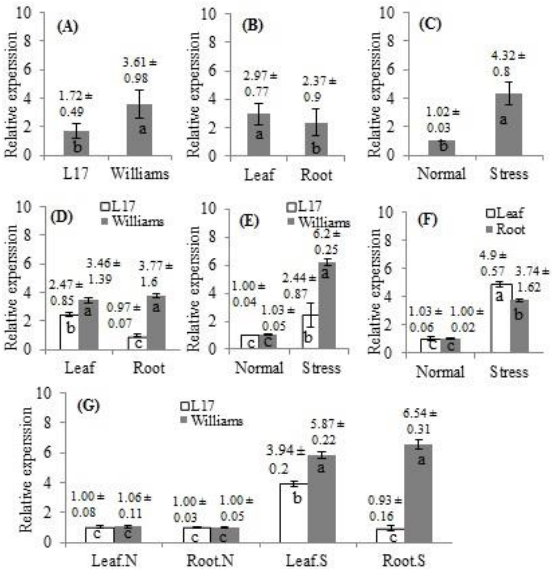

Figure 4. The mean comparison of GmBZIP50 gene expression in: (**A**) two genotypes, Williams and L17; (**B**) two tissues, leaf and root; (**C**) two stress levels, salt stress and normal condition; (**D**) two genotypes in two tissues; (**E**) two genotypes in two stress treatments; (**F**) two tissues, leaf and root, in two stress treatments; (**G**) two tissues, leaf and root, in two genotypes, in two stress treatments (N = normal, S = stressed). Different letters above columns indicate statistically significant ($P \leq 0.01$) differences. The data above columns are mean ± standard error.

Analysis of variance for *GmBZIP50* gene expression showed significant differences ($P \leq 0.01$) between genotypes and tissues (Table 2). In addition, significant differences were found for genotype × stress, and tissue × stress interactions, indicating significantly different responses of genotypes and tissues to salt stress. Mean comparison revealed that the level of *GmBZIP50* gene expression was significantly higher in Williams compared to L17 (Figure 4a).

The relative expression of *GmBZIP50* was higher in leaves than roots by 25% (Figure 4b). *GmBZIP50* expression increased by 4.3 fold in salt stressed plants compared to normal condition, showing a significant difference between two conditions (Figure 4c). A significant difference was also observed between two plant tissues and higher level of expression was detected in the roots and leaves of Williams that was four and one times larger than in L17 (Figure 4d). In response to the salt stress, *GmBZIP50* expression in Williams was 2.5 times higher than in L17 that resulted in a significant genotype × stress interaction (Figure 4e). In salt stress, the level of expression

increased in both leaf and root tissues compared to the normal condition. However, the increase was significantly higher in leaves (30%) than in roots (Figure 4f). By comparing means for genotype × tissue × stress interaction, it was observed that the increase at salt stress in the leaves and the roots of Williams was 1.5 and 7 times larger than L17 (Figure 4g).

DISCUSSION

Plant growth is greatly affected by environmental abiotic stresses such as drought and high salinity. The responses of plants to abiotic stress such as salinity are complicated processes that need analysis using genomics and physiological methods. Therefore, there is an increasing interest in studying the physiological and molecular mechanisms involved in the response of plants such as soybean to salinity. Available phosphorus deficiency is a major limitation for the growth and development of soybean and *PAPs* have an important role in uptaking and recycling phosphorus (LI *et al*., 2012). Comparative study in the expression of *PAP* gene family and the response to phosphorus deficiency in soybean has facilitated investigation into the physiological role of *GmPAPs* (LI *et al*., 2012). Studies have shown that *GmPAP15* and *GmPAP23* had an increased expression in roots (LI *et al*., 2012) and leaves of soybean under phosphor deficit conditions (LIAO *et al*., 2003). Also, in the present study, *PAP3* gene was expressed in leaves and roots of soybean with 292% and 189% increase in expression at salt stress condition compared to the non-stress, respectively. This result is in accordance with the findings of LIAO *et al*. (2003) and LI *et al*. (2012). Also ZHU *et al*. (1998), LIAO *et al*. (2003) and STOLF-MOREIRA *et al*. (2010) similarly reported a significant increase in the level of *PAP3* gene expression in soybean in response to salt stress condition. The intracellular determination of *GmBZIP* showed that it is a nuclear encoded defense protein in relation to abiotic responses in tomato (ORELLANA *et al*., 2010). The *BZIP* gene is a transcription factor in signal transduction during abiotic stresses and its expression has been reported in soybean (GAO *et al*., 2011), maize and transformed *Arabidopsis* in response to salinity, chilling, ABA, and pathogens (YING *et al*., 2012), *Arabidopsis thaliana* L. and *Oryza sativa* L. in response to heat and hydrogen peroxide (TANG *et al*., 2012), transgenic soybean for *GmBZIP44*, *GmBZIP62*, and *GmBZIP78* in response to salt and freezing stresses (LIAO *et al*., 2008), rice in response to salinity, drought, cold, oxidative stress, and ABA (HOSSAINA *et al*., 2010). The results of *BZIP* gene expressions in soybean roots and leaves in two genotypes, Williams and L17, under drought stress showed that the *BZIP* and *CAT* gene expressions in Williams was two-fold greater than L17 (AHMADI and SOLEIMANI, 2014). In the present study, the *GmBZIP50* gene expression in soybean leaf and root under salt stress was increased by 375% and 273%, respectively, compared to the same tissues in the non-stressed plants. These results are in agreement with those of XIANG *et al*. (2008), HOSSAINA *et al*. (2010) in various tissues in rice under salt stress, those of AHMADI and SOLEIMANI (2014) in leaves and roots in soybean under drought stress and those of GAO *et al*. (2011) in roots, shoots and leaves of transformed soybean under different conditions. Also, GAO *et al*. (2011) and TANG *et al*. (2012) studied gene expressions in rice under salt stress condition and similarly reported an increase in the expression of the *BZIP* gene in different tissues such as roots, stems, and leaves. Proline has a role in osmotic adjustment, membrane protection and membrane processes, the inhibition of free radicals, oxidation, and division and cell developments (KISHOR *et al*., 2005). The accumulation of a higher content of proline by increasing *P5CS* gene expression could protect plants against oxidative and osmotic stresses (HAN and HWANG, 2003). In the present study, the increase in *GmP5CS* gene expression in soybean leaf and root tissues under salt stress was 65% and 123%, respectively, compared to the same tissues in the non-stressed plants.

However, the tolerant genotype (Williams) showed a much higher expression than the susceptible genotype (L17) (128%). Therefore, it was deduced that the expression of the *P5CS* gene in tolerant plants should have a considerable role in the synthesis and accumulation of proline. Our results coincide with the reports of HUANG *et al*. (2012) on the proline increased under salt treatments in the roots of Jerusalem artichoke (*Helianthus tuberosus* L.). Also, HMIDA-SAYARI *et al*. (2005) reported the overexpression of Δ1-pyrroline-5-carboxylate synthetase increases proline production and confers salt (100 mMNaCl) stresses tolerance in transgenic potato plants. KIM and NAM (2013) reported that the transgenic potato for *P5CS* from *Arabidopsis thaliana*, showed an improved tolerance to salinity through a much less altered tuber yield and weight compared to the non-transgenic ones. Also, our results were similar to the research conducted by ZHANG *et al*. (2015) in overexpression of a novel Δ1-pyrroline-5-carboxylate synthetase gene from *Solanum torvum*, CHEN *et al*. (2009) on the expression patterns of *PvP5CS* in common bean treated with drought, cold and salt (200 mM NaCl) stresses, STOLF-MOREIRA *et al*. (2010) on two tolerant (MG/BR46) and susceptible (BR16) soybean genotypes in salt stress, CELIK and ATAK (2012) for increased *GmP5CS* gene expression under 150 mM NaCl 2.93 fold compared with 100 mM treatment, RUIZ-LOZANO *et al*. (2006) and PORCEL *et al*. (2004) on transformed lettuce plants carrying *LSP5CS* and transformed soybean plants carrying *GmP5CS*, and with a study on transformed tobacco plants carrying *P5CS* (KISHOR *et al*., 2005). Thus, *P5CS* gene plays a critical role in regulating stress-induced proline accumulation during abiotic stresses such as salt and drought and is actively serve as osmotic protection. In agreement with our results, several studies examined the different expression level of *P5CS* gene under salinity in various tissues of susceptible and tolerant genotypes of some plants such as *Arabidopsis thaliana* L. (YOSHIBA *et al*., 1995), *Vigna* (ZHANG *et al*., 1995), *Oryza sativa* L. (IGARASHI *et al*., 1997; ZHU *et al*. 1998), *Glycine max* L. and *Lactuca sativa* L. (PORCEL *et al*., 2004), Potato (HMIDA-SAYARI *et al*., 2005), *Phaseolus vulgaris* L. (CHEN *et al*., 2009), *Glycine max* L. (CHEN *et al*., 2007; CELIK and ATAK, 2012; ZHANG *et al*., 2015) and *Medicago truncatula* L. (KIM and NAM, 2013). In the studies of MUNNS *et al*. (2006), RAI and PENNA (2013) and HUANG *et al*. (2013) the relationship between proline accumulation and salt stress tolerance reported.

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EKSPRESIJA *GMP5CS, GMPAP3,* I *GMBZIP50* GENA U USLOVIMA SALINITETA KOD SOJE KORIŠĆENJEM REAL-TIME PCR

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Izvod

Salinitet u viših biljaka je uzrok toksičnosti i osmotskog stresa. Da bi identifikovali obrazac ekspresije gena tolerantnosti na salinitet kod soje, ispitivana je relativna ekspresija tri gena, *GmPAP3*, *GmBZIP50* i *GmP5CS* na dve tkiva listova i korena u Williams (kao tolerantnog) i L17 (kao osetljivog) genotipa soje u dva nivoa NaCI, nula (kontrola) i 300 mM. Za adaptaciju biljčice do saliniteta, salinitet je počeo sa 150 mM NaCl prvi put i zatim 300 mM NaCl u dva navrata. Gen *18SrRNA* se koristio za normalizaciju podataka. Analiza podataka je pokazala da je ekspresija sva tri gena povećana pod stresom saliniteta. Ekspresija *GmP5CS* i *GmBZIP50* je bila dvostruko veća u Williams u odnosu na L17, dok je *GmPAP3* bio 2,5 puta veći u L17 u odnosu na Williams. Ekspresija *GmP5CS* i *GmBZIP50* je bila veća u korenu nego u lišću, dok je *GmPAP3* ekspresija bila veća u listovima nego u korenu. U zaključku, *GmBZIP50* overekspresija u odnosu na druga dva gena od 375 i 273% u lišću i korenu pod stresom u poređenju sa biljkama u odsustvu stresa. Tako *GmBZIP50* može biti efikasan kandidat gen za proizvodnju soje sa toleratnošću na salinitet što može proisteći iz prenosa i povećanja broja kopija.

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