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Expression profiling of *PAP3, BZIP*, and *P5CS* genes in soybean under drought stress conditions

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Abstract: Under drought stress, a signaling system induces the expression of specific genes to alleviate the harmful effects of drought stress. The *BZIP* gene is a transcription factor in the signaling abiotic stresses and plays a role in the regulation of responses to different stresses in plants. The *P5CS* gene controls the activity of the proline-5-carboxylate synthase enzyme that is involved in proline synthesis in drought stress conditions. In this research, the effect of drought stress was investigated on the expression of *GmPAP3*, *GmBZIP* and *GmP5CS* genes in two soybean cultivars, Williams (tolerant) and L17 (susceptible). Total RNA was isolated from leaves and roots of both nonstressed and stressed plants, and then cDNA was synthesized and used for real-time PCR. The housekeeping gene *18SrRNA* was used to normalize data. Data analysis showed that the expression of *GmPAP3*, *GmBZIP*, and *GmP5CS* genes increased under drought stress. *GmPAP3* and *GmBZIP* expressions were two-fold while *GmP5CS* expression was seven-fold greater in Williams than in L17. *GmPAP3* and *GmP5CS* gene expressions were similar in leaves and roots, while *GmBZIP* expression was higher in roots than in leaves. In conclusion, the increased expression of these genes could be attributed to higher drought tolerance in cultivar Williams and it seems that transferring these genes into susceptible cultivars may enhance drought tolerance in soybean.

Key words: *GmPAP3*, *GmBZIP*, *GmP5CS*, *Glycine max*, water stress

1. Introduction

Drought stress can negatively influence various aspects of physiobiochemical characters of soybean that result in yield reduction (Ashraf et al., 2011). Therefore, genetic improvement for drought tolerance could increase stability in soybean yield (Nevo and Chen, 2010). In order to alleviate drought stress, plants enhance the osmotic potential of their cells by synthesizing and storing osmolytes such as proline (Hu et al., 1992; Kavi Kishor et al., 1995). Proline plays a major role as a protective osmolyte by scavenging free radicals and altering redox potential by increasing the nicotinamide adenine dinucleotide phosphate supply (Hare et al., 1999; Hasegawa et al., 2000). The first two steps of proline biosynthesis are catalyzed by Δ1-pyrroline-5-carboxylate synthetase (*P5CS*) and the activity of glutamyl kinase and glutamic-g-semialdehyde dehydrogenase. Then P5C reductase (P5CR) reduces the Δ1-pyrroline -5-carboxylate (P5C) form to proline (Hu et al., 1992). The rate-limiting step in this pathway is enforced by the gamma-glutamyl kinase activity of *P5CS*, which is sensitive to feedback inhibition by relatively low levels of proline (Smith et al., 1984). In addition, in *Arabidopsis*, the gene encoding *P5CS* is induced under drought and salt stresses and abscisic acid (ABA) treatment. However, *P5CR* is not induced in drought and salt stress conditions (Yoshiba et al., 1995). The overexpression of the gene encoding *P5CS* could increase proline and confer tolerance to osmotic stress in transgenic tobacco (*Nicotiana tabacum* L.) plants (Kavi Kishor et al., 1995). The higher seed yield and quality has been closely attributed to the physiological characteristics of the plants, resulting in higher leaf proline, carbohydrates, and relative water content (RWC) under low water conditions (Amini et al., 2014). Thus, the gene encoding *P5CS* plays an important role in the biosynthesis of proline (Abrahám et al., 2003).

Basic leucine zipper (BZIP) transcription factors are found in all organisms. Four *BZIP* genes were encoded by the genome of the most recent common ancestor of all plants (Ashraf, 2010). Gao et al. (2011) reported that *GmBZIP1* was highly expressed in soybean roots, stems, and leaves under ABA, drought, high-salt, and low-temperature treatments. Furthermore, overexpression

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of *BZIP* influenced the expression of some ABA or stress-related genes that function in stomata closure in *Arabidopsis* (Gao et al., 2011). Transferring the *Poncirus trifoliata BZIP* transcription factor to tobacco increased *PtrABF* gene expression under drought stress relative to the wild type through the inhibition of reactive oxygen species (Huang et al., 2010). On the other hand, the expression of stress-responsive genes and the production of antioxidant enzymes could increase water deficiency tolerance in tobacco (Huang et al., 2010). *OsBZIP46* is a member of the third subfamily of *BZIP* transcription factors in rice. It has been found to be highly similar to the ABA-responsive element binding factor (ABF/AREB). *ABI5* and *OsBZIP23* are two transcriptional activators that harbor stress tolerance to *Arabidopsis thaliana* and rice (*Oryza sativa* L.), respectively. *OsBZIP46* was strongly expressed in drought, heat, hydrogen peroxide, and ABA treatments. However, it was not induced under salt or cold stresses (Tang et al., 2012). Purple acid phosphatases (PAPs) are members of the metallophosphoesterase family (Li et al., 2011). Phosphorus (P) is an important macronutrient for plant growth and development (Kong et al., 2014). Drought and low phosphorus availability are major limiting factors for plant growth, especially in tropical and subtropical areas, because terrestrial plants prefer to uptake P in its inorganic form, phosphate (Pfaffl, 2001; Valentovic et al., 2006). PAP can hydrolyze organic phosphorus in the soil to release inorganic phosphate and enhance plant P utilization (Kong et al., 2014). Overexpressing *GmPAP4* in *Arabidopsis* resulted in significant rises in P uptake and utilization in comparison to the wild type (Kong et al., 2014). Northern blot analysis revealed that NaCl stress caused a general induction of *GmPAP3* expression in both roots and leaves of various cultivated (*Glycine max*) and wild (*Glycine soja*) soybean varieties (Liao et al., 2003). In another study, *GmPAP3* gene expression was reported to have increased under drought stress in soybean (Zhu et al., 1998; Stolf-Moreira et al., 2010).

Taking into account the area under soybean cultivation and its economic importance around the world, it is important to investigate the function and the expression pattern of the genes controlling plant tolerance to common stresses such as drought and salinity. In this research, we investigated the differences in the expression pattern of *GmPAP3, GmBZIP*, and *GmP5CS* genes in response to drought stress between tolerant and susceptible soybean cultivars using real-time PCR.

2. Materials and methods

2.1. Plant materials and treatments

Two soybean cultivars (Williams as a tolerant cultivar and L17 as a nontolerant cultivar) were studied for the expression of *GmPAP3*, *GmBZIP*, and *GmP5CS* genes in two different tissues (root and leaf) under drought 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. The pots (20 × 20 cm) were filled with soil and five seeds were planted per pot. Drought stress treatment was performed on the two soybean cultivars at two-leaf stages for 7 days, whereas nonstressed plantlets (as the control) were irrigated every 2 days. Nineteen days after planting and at the five-leaf stage, RNA was extracted from leaf and root tissues. All tissue samples were stored at –80 °C for RNA extraction.

2.2. Measurement of relative water content

RWC was measured 19 days after sowing at the 5-leaf stage by the method described by Schonfeld et al. (1988). Fresh weight (Wf) was determined from the three youngest fully expanded leaves immediately after excision. Turgid weight (Wt) was obtained by soaking the leaves for 16 to 18 h in distilled water. After soaking, leaves were quickly and carefully dried with tissue paper prior to determining Wt. Dry weight (Wd) was determined after drying the leaf samples for 72 h at 70 °C (Zhu et al., 1998). RWC was calculated by the following equation: RWC = [(Wf – Wd) / (Wt – Wd)] × 100.

2.3. 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 and then RNA was dissolved in 50 µL of diethylpyrocarbonate (DEPC)-treated distilled water. The RNA samples were treated with RNase-Free DNase I (Fermentas, Germany) according to the manufacturer's instructions to eliminate remaining genomic DNA. The concentration of RNA and its purity was determined using a NanoDrop 2000 spectrophotometer (Thermo, USA). RNA quality was evaluated by electrophoresis on 1.5% (w/v) agarose gel (Figure 1). The first-strand cDNA was synthesized from 1 µg of total RNA with Oligo $d(T)_{18}$ primer in a final

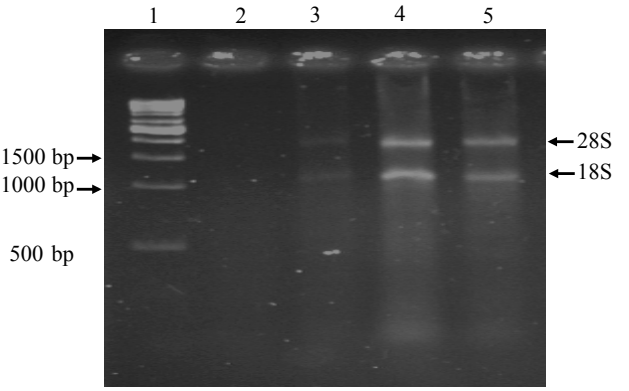

Figure 1. The quality of extracted RNA from soybean on 1.5% agarose gel (1: Ladder DNA, 1 kb, 4: leaf RNA, 5: root RNA).

reaction volume of 20 µL using the Reverse Transcription Kit (Product No. RTPL12, Vivantis, Malaysia) according to the manufacturer's instructions. The synthesized cDNA was diluted 20-fold to be used as the template for real-time PCR. The cDNAs were stored immediately at –20 °C.

2.4. Design of PCR primers

The real-time PCR primers for genes *GmPAP3*, *GmBZIP*, *GmP5CS*, and *18SrRNA* (as the housekeeping gene) were designed using the Softberry website (http://www.softberry.com/) and Primer BLAST software of the NCBI database (http://www.ncbi.nlm.nih.gov/). Oligo 5.0 primer analysis software (National Biosciences Inc., USA) was used to confirm the predicted sequence specificity of the designed primer pairs (Table 1).

2.5. Quantitative real-time PCR (qPCR) assay and data analysis

The expression of *GmPAP3*, *GmBZIP*, *GmP5CS*, and *18SrRNA* 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 Ex TagII (Tli RNAase Plus), Code RR820L]), 0.3 µL of each gene-specific primer pair, and 7.4 µL of distilled water in a final volume of 20 µL. Thermal cycling conditions for the qRT-PCR were: first denaturation at 94 °C for 2 min, and then 40 cycles of denaturation at 94 °C for 10 s and annealing and extension at 60 °C for 45 s. The housekeeping gene *18SrRNA* was used for the normalization of the amount of cDNA in each qPCR reaction. Control PCR reactions with no templates were also performed for each primer pairs. The specificity of amplified segments was checked by melting curve analysis performed from 60 °C to 95 °C for 60 cycles. Since the deviation error of amplification efficiency between target genes and the reference gene was less than 10% according to our trial experiments, 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 16.0 and the diagrams were drawn using Excel software. The comparisons of treatments' mean differences by Duncan multiple range test are shown with columns.

3. Results

To investigate a possible link of *GmP5CS*, *GmPAP3*, and *GmBZIP* genes with physiological processes during plant responses to drought stress, the relative transcript levels of each gene were studied in root and leaf tissues at the seedling stage under drought stress and nonstress conditions.

3.1. Effect of drought stress on plant–water relations

Data analysis revealed a significant difference ($P \leq 0.05$) between Williams and L17 soybean cultivars in RWC after 7 days of water stress conditions. Higher RWC in Williams compared to L17 under stressed conditions indicated a higher level of drought tolerance in cultivar Williams (Figure 2). It was shown that RWC may be considered as an index for the amount of plant damage due to drought stress (Farooq et al., 2009). Studying the effect of drought stress in wheat under a controlled environment, it was reported that RWC had a significant positive correlation with grain yield in wheat under stressed conditions (Khakwani et al., 2011; Amini et al., 2014). Therefore, as an effective criterion of water balance in stressed plants, RWC may be utilized in selecting drought-tolerant cultivars

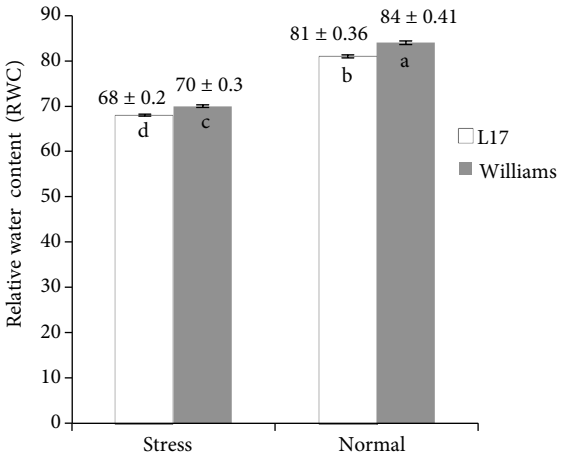

Figure 2. The RWC content in two soybean cultivars (Williams and L17) under drought stress and normal conditions. The data above columns are mean ± standard error.

Table 1. Primer pairs used in qRT-PCR reaction for the study of *GmPAP3*, *GmBZIP*, and *GmP5CS* gene expressions under drought stress in two soybean cultivars.

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

under stressed conditions (Vaezi et al., 2010; Amini et al., 2014). Our findings on the reduction of RWC in plants under drought stress are in agreement with previous reports (Lecoeur and Sinclair, 1996; Altinkut et al., 2001; Vaezi et al., 2010; Amini et al., 2014). Comparison of the level of RWC between susceptible and tolerant cultivars under stressed conditions was done in previous studies on soybean (*Glycine max* L.), maize (*Zea mays* L.), and safflower (*Carthamus tinctorius* L.), showing a higher level of RWC in the drought-tolerant cultivars compared to the susceptible ones (An et al., 2002; Valentovic et al., 2006, Amini et al., 2014).

3.2. Expression of the *GmP5CS* gene

Analysis of variance of the expression level of the *GmP5CS* gene revealed significant differences ($P \leq 0.01$) between cultivars in response to drought stress conditions (Table 2). In general, cultivar Williams showed a higher level of *GmP5CS* gene expression compared to L17, when considering both normal and stressed conditions and two tissues (Figure 3a). Totally, the expression of *GmP5CS* showed a seven-fold increase in response to drought stress compared to the normal condition (Figure 3b). A significant difference was also observed in the expression level of *GmP5CS* between two plant tissues (Figure 3c). A higher level of *GmP5CS* gene expression was detected in the roots than in the leaves of both Williams and L17. However, the difference in the expression level of *GmP5CS* between roots and leaves was slightly larger in cultivar Williams compared to L17. A similar result was reported in soybeans infected by arbuscular mycorrhizae (Porcel et al., 2004). Mean comparison of cultivars by stress interaction resulted in three distinct classes, a, b, and c (Figure 3d). The relative expression of *GmP5CS* under drought stress was three-fold greater in Williams (class a) compared to cultivar L17 (class b). However, under normal conditions, both cultivars showed no significant differences in expression level of *GmP5CS*. Mean comparison of the stress by tissue interaction indicated a similar expression level for *GmP5CS* in leaves and roots under normal conditions, but the level of *GmP5CS* expression was significantly higher in leaves (7.3-fold) compared to the roots (6.5-fold) in response to drought stress (Figure 3e). Comparing the means for cultivar × tissue × stress three-way interactions, it was revealed that the increase in the level of *GmP5CS* gene expression in response to drought stress in the leaves and roots of cultivar Williams was 2 and 4.5 times bigger than that in cultivar L17 (Figure 3f). In agreement with our results, several studies examined the expression level of the *P5CS* gene in different tissues of susceptible and tolerant cultivars of crops such as soybean and rice in response to drought stress (Igarashi et al., 1997; Zhu et al., 1998; Porcel et al., 2004; Stolf-Moreira et al., 2010; Xu et al., 2013), and Amini et al. (2014) found that proline, soluble carbohydrates, and protein content increased in response to water stress.

3.3. Expression of the *GmPAP3* gene

Analysis of variance showed significant differences ($P \leq 0.01$) in the level of *GmPAP3* gene expression between cultivars, stress conditions, and cultivar × stress interactions in response to drought stress treatment (Table 2). Mean comparisons considering the total expression of *GmPAP3* under both normal and drought stress conditions

Table 2. Analysis of variance for *GmPAP3*, *GmBZIP*, and *GmP5CS* gene expressions under drought stress in two soybean cultivars.

Sources of variation	d.f	Mean square		
		GmPAP3	*GmP5CS*	*GmBZIP*
Genotype	1	24.466**	44.348**	5.322**
Tissue	1	^{ns} 0.072	0.489**	2.081**
Stress	1	110.510**	134.550**	33.942**
Genotype × tissue	1	^{ns} 0.440	2.768**	^{ns} 0.103
Genotype × stress	1	24.267**	44.501**	5.719**
Stress × tissue	1	^{ns} 1.002	0.966**	2.053**
Stress × tissue × genotype	1	^{ns} 0.458	2.949**	1.095^{ns}
Error	8	0.173	0.068	0.052
C.V%		10.85	6.49	9.06

^{ns} and **: Nonsignificant and significant at 1% level of probability, respectively.

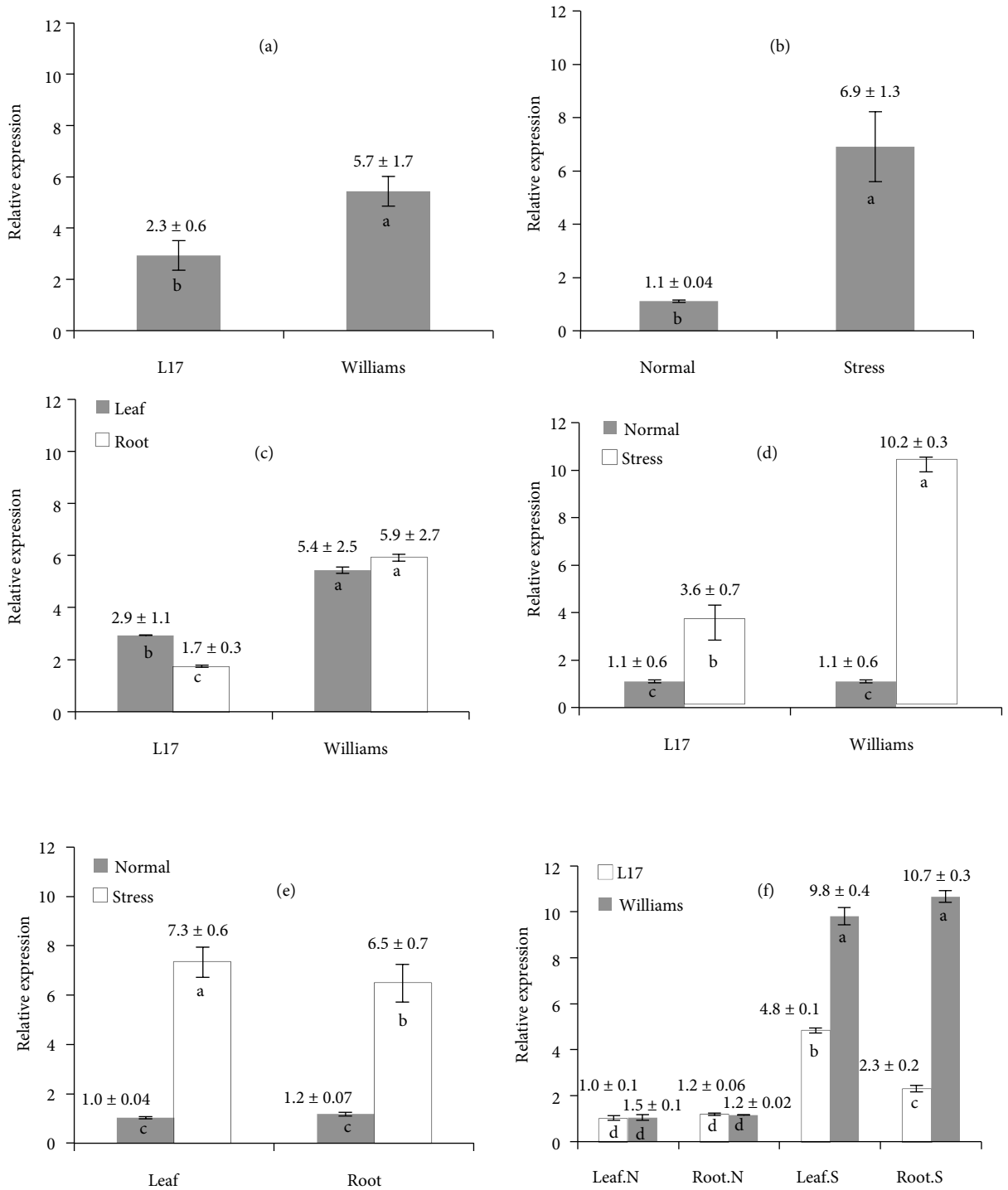

Figure 3. The mean comparisons of *GmP5CS* gene expression in: (a) two cultivars, Williams and L17; (b) two stress levels, drought stress and normal condition; (c) two tissues, leaf and root, in two cultivars; (d) two cultivars in two stress treatments, (e) two tissues, leaf and root, in two stress treatments, and (f) two tissues, leaf and root in two cultivars 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.

revealed that the expression level of *GmPAP3* in cultivar Williams was significantly higher than that of cultivar L17 (Figure 4a). Moreover, the expression level of *GmPAP3* in the two cultivars showed a six-fold increase in response to drought stress (Figure 4b). Different responses of cultivars to drought stress resulted in a significant cultivar × drought

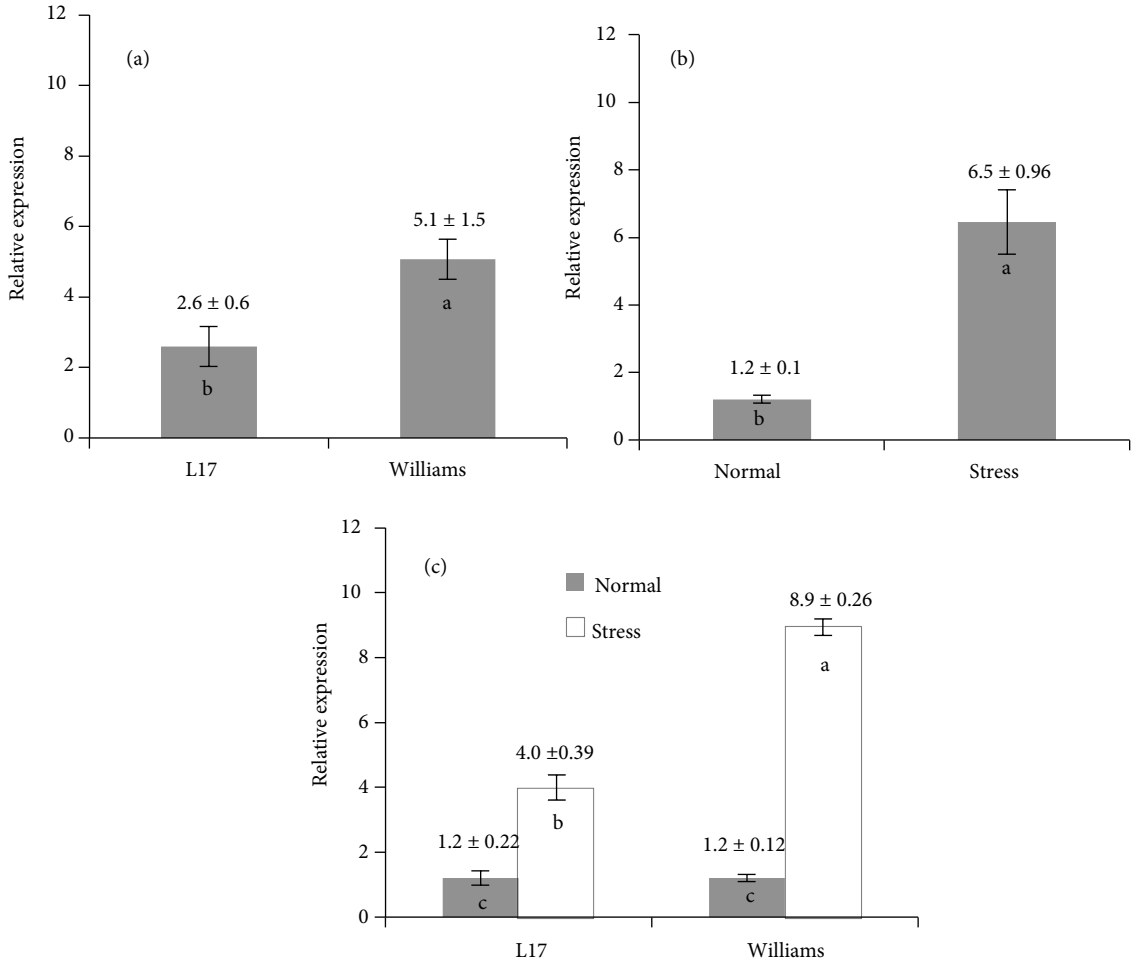

Figure 4. The mean comparison of *GmPAP3* gene expression in: (a) two cultivars, Williams and L17; (b) two stress levels, drought stress and normal condition; and (c) two cultivars in two stress treatments. Different letters above columns indicate statistically significant ($P \leq 0.01$) differences. The data above columns are mean ± standard error.

stress interaction. Under drought stress the expression level of *GmPAP3* in cultivar Williams increased by 123%, which was significantly higher compared to cultivar L17 (Figure 4c).

3.4. Expression of the *GmBZIP* gene

Analysis of variance in *GmBZIP* gene expression showed significant differences ($P \leq 0.01$) between cultivars and tissues (Table 2). In addition, significant differences were found for cultivar × stress and tissue × stress interactions, indicating significantly different responses of Williams and L17 cultivars and leaves and root tissues to drought stress conditions. Mean comparisons revealed that in both normal and stressed conditions in general, the level of *GmBZIP* gene expression was significantly higher in cultivar Williams compared to L17 (Figure 5a). The relative expression of *GmBZIP* was found to be significantly higher ($P \leq 0.01$) in roots than leaves by 33% (Figure 5b). Comparing total expression in the two tissues and both cultivars, the expression of the *GmBZIP* gene was increased by four-fold in response to drought stress, causing a significant difference in the expression level of *GmBZIP* between normal and stressed conditions (Figure 5c). In response to the drought stress, the level of *GmBZIP* gene expression in cultivar Williams was two times higher than in L17, which resulted in a significant cultivar × stress interaction (Figure 5d). In drought stress, the level of *GmBZIP* gene expression increased in both leaf and root tissues compared to the normal conditions. However, the amount of increase was significantly higher in roots than in leaves (Figure 5e). In this research, we detected two-fold higher expression of *GmBZIP* in the drought-tolerant cultivar, Williams, compared to the susceptible one, L17, and a higher level of *GmBZIP* gene expression in roots than leaves. In agreement with our findings several studies on

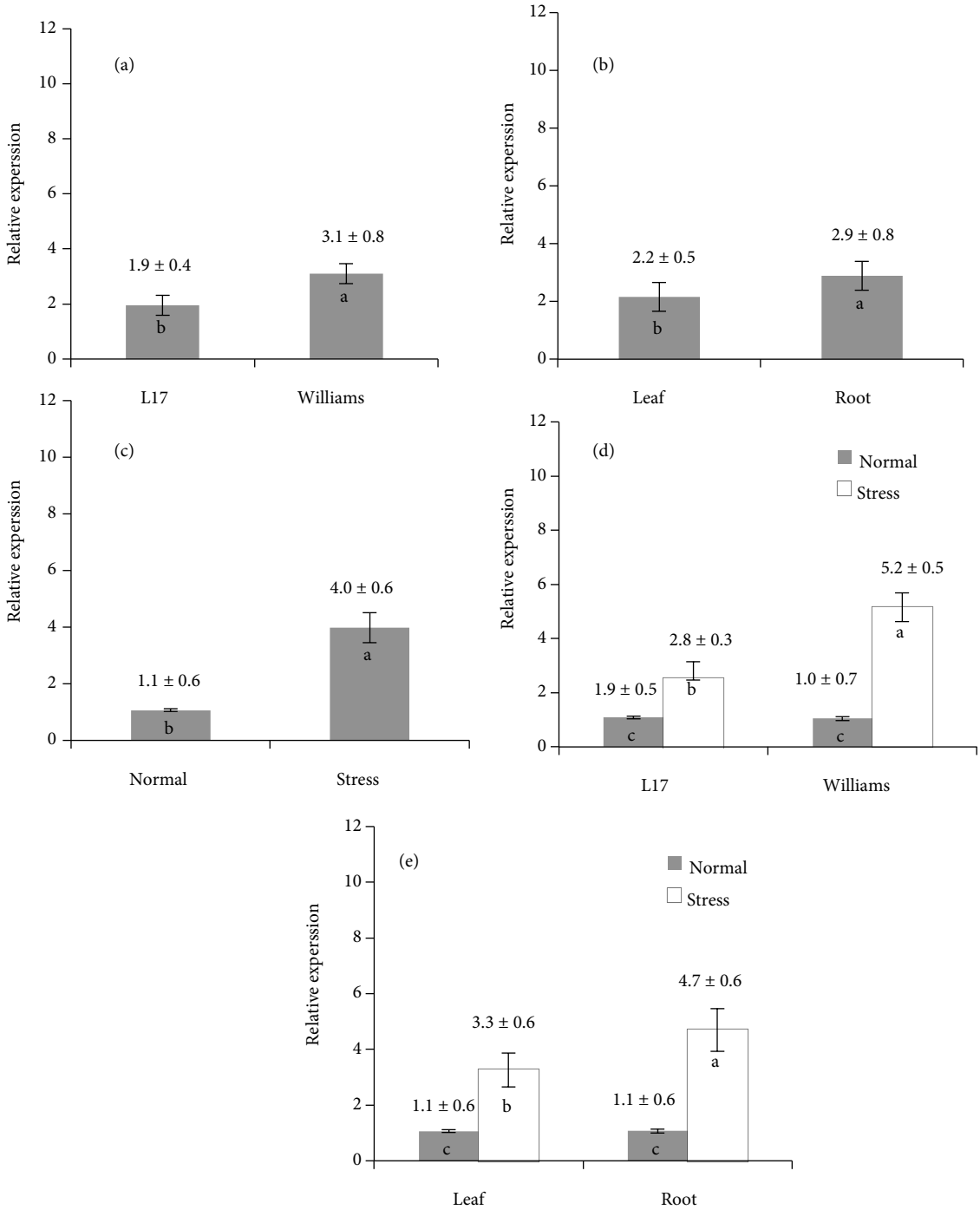

Figure 5. The mean comparisons of *GmBZIP* gene expression in: (a) two cultivars, Williams and L17; (b) two tissues, leaf and root, (c) two stress levels, drought stress and normal condition; (d) two cultivars in two stress treatments; and (e) two tissues, leaf and root, in two stress treatments. Different letters above columns indicate statistically significant ($P \leq 0.01$) differences. The data above columns are mean ± standard error.

transgenic plants carrying the *BZIP* gene, such as tobacco, wheat, and *Arabidopsis*, reported significant increases in the *BZIP* gene expression level in response to drought stress conditions (Hong et al., 2000; Ashraf, 2010; Gao et al., 2011). Tang et al. (2012) also studied gene expressions in rice under drought stress conditions and similarly reported an increase in the expression of the *BZIP* gene in different tissues such as roots, stems, and leaves.

4. Discussion

The response and tolerance of plants to water deficit are complicated processes that need analysis using genomics and physiological methods (Harb et al., 2010). Therefore, there is an increasing interest in studying the physiological and molecular mechanisms involved in the response of soybean to drought. 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 the *PAP* gene family and the response to phosphorus deficiency in soybean has facilitated investigation into the physiological role of *GmPAP*s (Li et al., 2012). At present, 35 *GmPAP* genes have been recognized in the soybean genome by Libault et al. (2010) and Severin et al. (2010). Libault et al. (2010) reported that the node, flower, and sheath of soybean have the highest *PAP* expression. Studies have shown that *GmPAP15* and *GmPAP23* had increased expression in roots (Li et al., 2012) and leaves of soybean under phosphorus-deficit conditions (Liao et al., 2003). Li et al. (2012) also reported the expression of some *PAP* genes in leaves and seeds. In the present study, the *PAP3* gene was expressed in leaves and roots of soybean with 85% and 78% increase in expression compared to the control, respectively. This result is in accordance with the findings of Zhu et al. (1998), Liao et al. (2003), Stolf-Moreira et al. (2010), Libault et al. (2010), Severin et al. (2010), and Li et al. (2012). By microarray analysis two sets of *BZIP* genes in *Arabidopsis* (Kang et al., 2010) and by Southern blotting only one copy of *BZIP* in the soybean genome (Gao et al., 2011) have been identified. 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* by *ZmBZIP72* in response to drought, salinity, chilling, ABA, and pathogens (Ying et al., 2012), and in *Arabidopsis thaliana* and rice in response to heat and hydrogen peroxide (Tang et al., 2012). 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). In this research, the increase in *GmBZIP* gene expression in soybean leaf and root tissues under drought stress was 76% and 70%, respectively, in compared to the same tissues in the nonstressed plants. The results are in agreement with those of Gao et al. (2010) in roots, shoots, and leaves of transformed soybean under different conditions and those of Orellana et al. (2010) about the increase in the expression in leaves and roots of tomato. 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; Verbruggen and Hermanz, 2008). The accumulation of a high content of proline by increasing *P5CS* gene expression could protect plants against oxidative and osmotic stresses (Han and Hwang, 2003). In this research, the increase in *P5CS* gene expression in stressed conditions was observed in both leaf and root tissues. However, the tolerant cultivar (Williams) showed a much higher expression than the susceptible cultivar (L17). 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 Stolf-Moreira et al. (2010) on two tolerant MG/BR46 and susceptible (BR16) soybean cultivars in drought stress, with the reports of 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).

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