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BOWMAN-BIRK PROTEASE INHIBITOR GENE EXPRESSION IN *PHASEOLUS VULGARIS* – ORGAN SPECIFICITY AND INDUCTION UNDER ABIOTIC STRESSES

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Abstract: Seeds of legume plants are rich in serine protease inhibitors - up to 10% of grain protein content, mainly Bowman-Birk (BBI) and Kunitz types. However, data about the presence of protease inhibitors in tissues other than seeds and their induction under abiotic stress are scarce. We analyzed the organ-specific expression of three *Phaseolus vulgaris* genes for BBI inhibitors with published complete sequences in the NCBI database: PHAVU_004G133900g (ID: 18632939), PHAVU_004G134100g (ID: 18632941), and PHAVU_004G134000g (ID: 18632940) in two Bulgarian common bean cultivars – Ustrem and Blian, testing young, fully expanded and senescing leaves, roots, flowers and pods. Senescing leaves and roots had elevated expression of BBI genes as compared to fully expanded leaves, flowers and pods, especially in cv. Ustrem. The BBI transcript profiling was further assessed in the second trifoliate leaves and roots of cv. Ustrem, subjected to osmotic stress of -0,6 MPa in the nutrient solution, exerted by PEG 6000 or NaCl, with different duration. Relatively weak changes in the expression profiles of the three BBI genes were documented under the applied stresses with different tendencies depending on the gene, the stress type and its duration. Long-term PEG stress upregulated PHAVU_004G133900g and PHAVU_004G134100g but downregulated PHAVU_004G134000g expression in the leaves. The expression of the three genes in the roots was inhibited by the treatment. A slight increase in PHAVU_004G134000g transcript levels was measured in salt-stressed leaves after short-term exposure. The results reveal that abiotic stresses provoke organ-specific dynamic changes in the expression of the studied BBI gene.

INTRODUCTION

Seeds of legume plants are rich in serine protease inhibitors - up to 10% of grain protein content, mainly Bowman-Birk (BBI) and Kunitz types, where they exert both storage and protective functions against phytopathogens and insect predators (McManus et al., 2000). BBIs are members of a small multigene family and have unique structural features as double-headed inhibitors – a small tightly packed molecule of about 8 kDa containing eight cross-linked disulphide bridges and two independent inhibitory sites targeting different proteases (trypsin and chymotrypsin, or trypsin/trypsin), which efficiently prevent both premature germination and seed predation (Grosse-Holz and van der Hoorn, 2016). Regulatory functions of protease inhibitors on endogenous proteases under abiotic stresses are also discussed (Vaseva et al., 2012; Kidrič et al., 2014).

Protease inhibitors in seeds have been extensively studied (Clemente and Domoney, 2006; Piergiovanni et al., 2017). However, data about the presence of protease inhibitors in tissues other than seeds and their involvement in abiotic stress response are limited. The defense function of BBI is evidenced by its up-regulation after wounding (Brown et al., 1985, McGurl et al., 1995 - in alfalfa leaves) and jasmonate induction (Farmer et al., 1992, in tomato and alfalfa leaves). Only a few reports have been published on BBI involvement in salt stress response in wheat (Shan et al., 2008) and drought stress in rice (Huang et al., 2007), peanut (Dramé et al., 2013), and wheat (Vaseva et al., 2016). Abscisic acid-responsive elements (ABRE) and dehydration responsive elements (DRE) were found in the promotor region of the cowpea trypsin inhibitor gene (Anandhan et al., 2010). Possible involvement of BBI up-regulation in stress tolerance was discussed (Shan et al., 2008, Vaseva et al., 2016) but experimental evidence on this point is insufficient.

In the present study, we analyzed the organ-specific expression and the response to drought and salt stress of three *Phaseolus vulgaris* genes for BBI inhibitors with published complete sequences in the NCBI database: PHAVU_004G133900g (ID: 18632939), PHAVU_004G134100g (ID: 18632941), and PHAVU_004G134000g (ID: 18632940). The organ-specific expression of the *BBI* genes in two common bean cultivars (*Phaseolus vulgaris* L., cv. Blian, and cv. Ustrem) differing in seed trypsin inhibitory activity was evaluated. The effects of short and long-term osmotic stress provoked by PEG or NaCl were compared in order to reveal differential gene expression and possible involvement of BBIs in the tolerance to drought and salinity stress.

MATERIALS AND METHODS

Plant material, growth and treatments

Two common bean cultivars cv. Ustrem and cv. Blian (selection of Dobrudja Agricultural Institute in General Toshevo, Bulgaria) were compared in terms of organ-specific expression of *BBI* genes. The preliminary screening revealed higher content of serine protease inhibitors in cv. Ustrem seeds as compared to cv. Blian. The plants used for the transcript profiling were grown for 35 days (reproductive developmental stage) in Hoagland nutrient solution in pots (one plant per pot with a diameter of 9 cm and height of 7 cm) filled with perlite, under controlled conditions (16/8 h photoperiod, 150 $\mu mol.m^{-2}s^{-1}$ light intensity, 24°C and 60% air humidity). The nutrient solution was refreshed every other day. Samples of 0.1 g from young developing, mature and senescing leaves, roots, flowers, and pods were collected, quickly frozen in liquid nitrogen and stored at -65°C until analyses. Osmotic stress of -0.6 MPa in the nutrient solution with different duration was induced by PEG 6000 or NaCl under the same growth conditions. The short term stress was imposed on 20-day old plants for 10 days. Long-term stress began from the very germination and was maintained for 30 days with the constant presence of the stress agent in the nutrient solution. Thus, at the end of both treatment protocols, the plants from the two experimental groups were of the same age. Samples from the second trifoliate leaves and from root tips (0.1 g for pigments, 0.5 g for malondialdehyde, 0.1 g for isolation of total RNA) were collected, quickly frozen in liquid nitrogen and stored at -65°C until analyses.

Biomass measurement and stress parameters

Biomass accumulation in different plant parts and its reduction under stress were estimated gravimetrically. Changes in the relative leaf water content (RWC) were calculated by the formula RWC%= (FW-DW)/(TW-DW), where FW is the fresh weight of the leaves, TW – the weight at full turgidity of the same samples and DW – the dry weight of the sample. TW was measured after keeping the leaf segments at 4°C in distilled water overnight, with subsequent blotting onto filter paper to remove the residual water and weighting. DW was obtained after drying the leaf segments at 80°C until constant weight. Changes in leaf chlorophyll and carotenoid content were analyzed according to Arnon (1949) in 80% acetone extracts. Malondialdehyde content was determined as thiobarbituric acid reactive substances as described by Hodges et al. (1999) in extracts obtained with 0.1% trichloroacetic acid.

Real-time quantitative Reverse Transcription PCR analyses (qRT-PCR)

Total RNA was extracted with GeneJET Plant RNA Purification Kit (Thermo Scientific) and subsequently treated with DNase I (Thermo Scientific) following the manufacturer's protocols. RNA quantification and the quality check were performed with Thermo Scientific Nano Drop 1000 Spectrophotometer. Complementary DNA (cDNA) was synthesized from 100 ng total RNA with iScript cDNA Synthesis Kit (Bio-Rad). The real-time quantitative RT-PCR analyses (qRT-PCR) were performed with LightCycler 480 SYBR Green I Master Mix on three independent biological repeats and the analyses were carried out in three technical replicates of 10.0 µL reaction volumes with 'PikoReal' Real-Time PCR System (Thermo Scientific), at the following conditions: 95°C for 5 min and 40 cycles of 95°C for 10 s followed by 54°C for 20 s and final melting curves analysis with a temperature range of 60° – 95°C in 0.2°C increment for 60 s. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Cq}$ method according to Livak and Schmittgen (2001) with two reference genes (actin – Pv actin, NCBI ID: KF033666 and alfa-Elongation Factor – Pv aEF-1, NCBI ID: KF569517) for normalization of the relative quantification. The organ-specific transcript measurements were calibrated according to the expression levels in the flower sample of cv. Blian (exhibiting the lowest transcript content). The qRT-PCR results on the samples derived from PEG- and NaCl-treated cv. Ustrem plants were calculated based on the levels of the respective gene expression in the control leaf sample which is considered to be equal to 1.

The primers used for the qRT-PCR analyses were selected with the NCBI "Pick Primers" tool on the published mRNA sequences for PHAVU_004G133900g, PHAVU_004G134100g, and PHAVU_004G134000g:

Statistics

Measurements were performed in biological triplicates (3 plants for each treatment group). Values are presented as means and standard error. Statistically significant differences between all variants were estimated by ANOVA multiple range test (Stat Graphics Plus) or t-test (Excel, qRT-PCR data) at the significance levels $P < 0.05$.

Table 1. Primer pairs, used in the qRT-PCR analyses.

Pvbbi 134100 F	GGTGTGCTTCGTGCTACTCT
Pvbbi 134100 R	CAGCGGCATTGAGGAGGTAT
Pvbbi 134000 F	GGATGAAGCCCTTTCAGGCT
Pvbbi 134000 R	ACACAGAAACTTGCATCAGAACA
Pvbbi 133900 F	GTGTGCACGGCTTCAATACC
Pvbbi 133900 R	TTTCTCAGTCATCATCTTCACCAC
Pv aEF-1 KF569517 F	CAAGGCTGAGCGTGAAAGAGGA
Pv aEF-1 KF569517 R	CCAAGGGTGAAAGCAAGAAGAGC
Pv actin KF033666 F	CAACCCAAAAGCTAACCGTGAGAA
Pv actin KF033666 R	GAGATCGCGTCCTGCCAAGTC

RESULTS AND DISCUSSION

The two cultivars were selected for the BBI transcript analyses as a result of preliminary screening studies of the antitrypsin activity in seeds of different Bulgarian bean cultivars (unpublished data). The aim of the performed gene expression analyses was to check whether the difference in the content of protease inhibitors found in the seeds of the two cultivars will persist in the different plant organs. Both cultivars were characterized with similar developmental patterns and growth parameters at the reproductive stage (Fig. 1) with slight but statistically significant differences in fresh weight, number of trifoliate leaves, number and weight of pods (Table 2). One marked distinction between the cultivars was the earlier and more abundant flowering of cv. Blian, while cv. Ustrem had fewer flowers and pods but produced a higher amount of leaf biomass.

cv. Blian cv.Ustrem

Figure1. The common bean cultivars at the reproductive stage

Table 2. Growth and development parameters of cv. Blian and cv. Ustrem (47 days- old plants). Fresh weight (FW) was given as mean ± sd of three individual plants. Different letters following values denote statistically significant differences at p≤0.05.

cultivar	Root FW (g.plant^{-1})	Shoot FW without pods (g.plant^{-1})	Number of trifoliate leaves	Number of pods per plant	FW of pods (g)
Blian	5.19±0.73a	13.96±1.26a	6.5±1.29a	6.5±1.3b	27.26
Ustrem	7.15±0.69b	17.56±1.05b	9.5±0.71b	2±1a	4.95

The gene expression of BBIs at the reproductive stage was tested in the following organs: senescing leaf (S), fully expanded active leaf (A), young developing leaf (D), root, flower, and green pod (Fig. 2). The comparative analyses showed similar dynamics, however cv. Ustrem consistently revealed higher transcript levels of BBIs in the vegetative organs, confirming the trend observed in the seeds of the two tested cultivars in the preliminary screen for protease inhibitor activity.

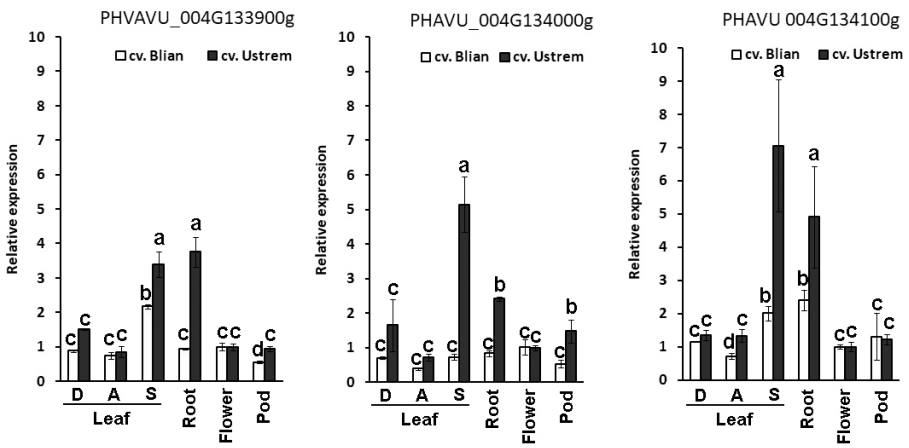

Figure 2. Organ-specific *BBI* gene expression in cv. Blian and cv. Ustrem. D – young developing leaf, A – fully expanded active leaf, and S – senescing leaf. The relative expression of the genes is presented according to the levels detected in cv. Blian flower sample. The small letters above the bars designate statistically significant differences among the samples (n=3, p≤0.05, t-test).

The highest *BBI* transcript abundance in this cultivar was found in senescing leaves and roots and the lowest – in mature fully developed leaves and flowers. Based on the hypothesis that PIs are involved in the control of the endogenous proteases, the results suggest that the upregulation of *BBI* in the senescing leaves could be linked to the active proteolysis and nutrient remobilization towards other plant parts, which is a typical feature of the senescing plant organs (Hortensteiner and Feller, 2002). The perlite substrate used in the experiments was not sterile; therefore, a possible contact of the roots with microorganisms driving the BBI expression therein could not be excluded. Mycorrhizae stimulatory effect on the expression of protease inhibitors has been previously reported (McGurl et al., 1995). Earlier studies point at the potential role of PIs in developmental control (Anandhan et al., 2010). Our data on the faster development of cv Blian and consistently lower transcript level of BBI in this cultivar support but do not provide sufficient evidence for such a direct link.

The diverse expression profiles of the BBIs in the different organs of the two cultivars, although exhibiting level variations, remained consistent, and the two cultivars appeared to respond similarly to the applied osmotic stresses (Fig. 3). The most pronounced growth inhibition was observed in the shoots of the plants subjected to a long-term PEG 6000 treatment (Fig. 4, relative FW as percentage of that in control plants). The absolute values in control plants were: shoots – 11.65±1.29 g and roots – 5.19±1.27 g for cv. Blian; shoots – 11.41±1.84 g and roots - 4.79±0.67g for cv. Ustrem. The short-term PEG stress had a relatively mild effect on the shoot growth and it did not provoke root growth inhibition. However, the high salinity stress inhibited the root growth even after a short duration (Fig. 4).

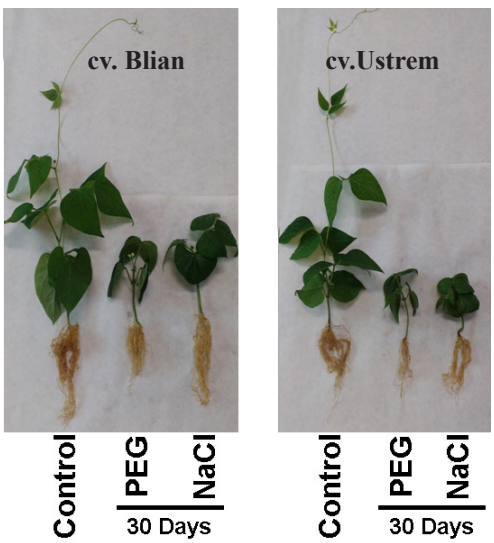

Figure 3. Plants of cvs. Blian and Ustrem at
long term osmotic stress of -0.6 MPa in the nutrient solutio

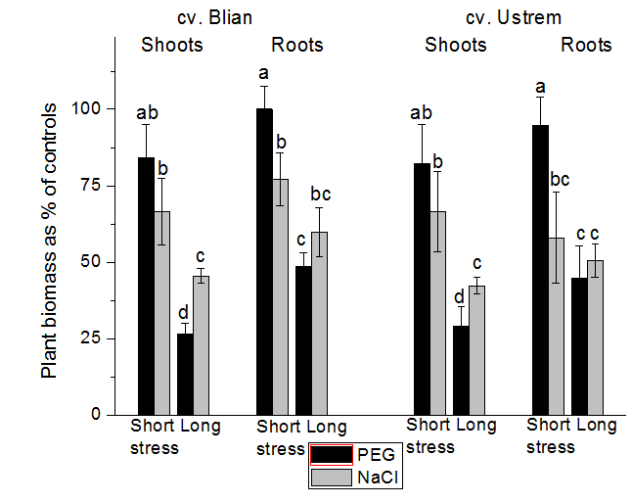

Figure 4. Fresh weight of shoots and roots of the stressed plants presented as a percentage of the controls. Black columns - PEG 6000 stress, grey columns – NaCl stress. Values are the mean of three replicates with standard errors. Small letters above the bars designate statistically significant differences among samples at $p \leq 0.05$.

The measured leaf water status confirmed that the applied stress was moderate (Table 3). A tendency of increase in chlorophyll content (Chl a+b) was observed in the PEG stress experimental group whereas the opposite trend was detected in plants subjected to salt stress (Table 3), pointing at differential targets affected by the two stressors. Oxidative damage to the cellular membranes (Fig. 5, values relative to that of controls) was registered in both cultivars, especially under long-term osmotic stress. The absolute MDA values in control plants were 12.35±0.43 nmol.g-1 FW in leaves and 2.65±0.45nmol.g-1 FW in roots for cv. Blian; 12.95±0.42 nmol.g-1 FW in leaves and 2.71±0.18 nmol.g-1 FW in roots for cv. Ustrem.

Table 3. Leaf relative water (RWC) and pigment content in control plants and plants subjected to short term or long-term osmotic stress of intensity -0.6 MPa. Means and standard errors of three replicates are presented. Different letters following values denote statistically significant differences at p≤0.05.

Parameter	cv. Blian					cv. Ustrem				
	Control	Short stress		Long stress		Control	Short stress		Long stress	
		PEG	NaCl	PEG	NaCl		PEG	NaCl	PEG	NaCl
RWC%	92,54±1,36a	77,98±2,69bc	80,76±2,02b	73,46±1,33c	74,14±6,48c	91,58±0,69a	81,92±5,95b	80,46±1,26b	74,97±1,75c	73,22±0,66c
Chl a+b mg/g FW	1,103±0,15b	1,211±0,10a	0,781±0,02c	1,246±0,06a	1,016±0,12b	1,194±0,05ab	1,211±0,1a	0,973±0,19bc	1,149±0,12ab	0,787±0,01c
Carot. mg/g FW	0,391±0,09ab	0,297±0,01b	0,272±0,01b	0,468±0,02a	0,365±0,02ab	0,399±0,14ab	0,441±0,04a	0,349±0,06ab	0,429±0,04a	0,287±0,01b

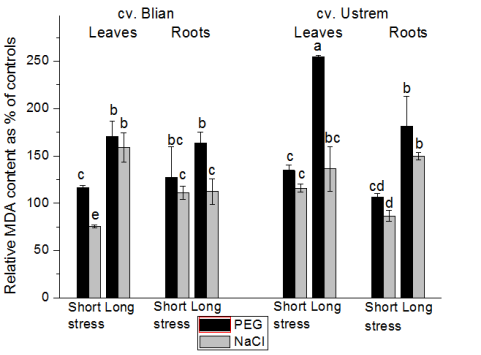

Figure 5. Relative MDA content (as a percent of controls) in the organs of the stressed plants. Black columns - PEG 6000 stress, grey columns – NaCl stress. Values are the mean of three replicates with standard errors. The small letters above the bars designate statistically significant differences among the samples at p≤0.05.

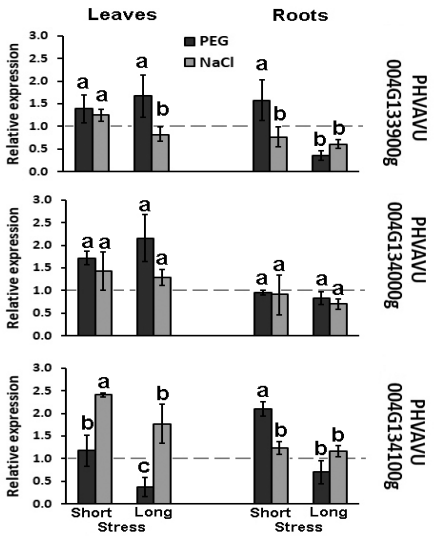

Figure 6.
Changes in the expression of selected *BBI* genes (PHAVU_004G133900g, PHAVU_004G134100g and PHAVU_004G134000g) in cv. Ustrem, subjected to short- and long-term osmotic stress. Dark gray columns present PEG 6000 stress, and light gray columns – NaCl stress. The relative expression of each transcript is based on the levels detected in the control leaf sample (basal level equal to 1, marked with a dashed line on the graph). The letters designate statistically significant differences in the samples derived from the respective organ (n=3, p≤0.05, t-test).

Taking into account the lack of any distinct differences in the stress parameters of the two tested cultivars, we proceed with the monitoring of the *BBI* gene expression in the second trifoliate leaves and the roots of cv. Ustrem plants, focusing mainly on the comparative analyses between the short- and the long-term exposure to PEG and NaCl (Fig. 6). The changes in the expression profiles as a result of the imposed stresses were relatively weak. A slight upregulation of PHAVU_004G134000g expression was documented in the salt-stressed leaves after short-term exposure to NaCl. Short-term PEG stress also provoked a weak but statistically significant increase of the transcript levels in the roots. Long-term PEG stress inhibited the expression of PHAVU_004G134000g in the leaves and PHAVU_004G133900g in the roots of the treated plants. The constant exposure to NaCl also had a negative impact on the PHAVU_004G133900g transcript presence in the roots. The fluctuations of the transcript levels upon the different stress treatments in the other experimental groups were not statistically significant. The results demonstrate that dynamic changes in the *BBI* gene expression occurred in response to abiotic stresses.

In conclusion, the comparative expression analyses revealed differential BBI accumulation in the vegetative and the reproductive organs of the two cultivars, with cv. Ustrem presenting consistently higher transcript levels than cv. Blian. Further experiments are necessary to clarify if this observation is related to some differences in development between the two cultivars. The physiological responses of the cultivars towards the imposed stresses were fairly similar. However, we registered some differences in the measured parameters which were dependent on the type of the osmotic stressor (PEG or NaCl) and the duration of the treatment (short-term versus prolonged stress). The relatively weak changes in the expression profiles of the three *BBI* genes provoked by the applied stresses show distinct trends in the different treatment groups.

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Conflicts of Interest: The authors declare no conflict of interest.

Authorship statement: LS and IV designed the experiments. LS and KI performed the experiments and analysed growth and stress parameters. IV and KI performed the transcript profiling. IV and LS wrote the manuscript.

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