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Screening of *PvLEA3* gene mRNA expression levels with qRT-PCR in different bean varieties (*Phaseolus vulgaris* L.) subjected to salt and drought stress

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Abstract: Bean is an edible grain legume species with the greatest selectivity in terms of ecological conditions. Increasing the yield per unit area depends on identification and breeding of species resistant to different ecological conditions. In this regard, we aimed to evaluate mRNA levels of the *PvLEA3* gene and lipid peroxidation levels in 7 different bean varieties subjected to NaCl and PEG stresses. Lipid peroxidation analysis (MDA) and qRT-PCR analysis were performed with root and leaf tissues sampled at 3 and 27 h of stress conditions. All results showed that the *PvLEA3* gene plays a key role in the defense mechanism against both salt and drought stress. According to the analysis results, Göynük-98 and Yunus-90 varieties were more resistant to stress conditions than the others. Our findings revealed that *PvLEA3* gene expression might be used as a marker for early detection of the tolerance capability of bean varieties against salt and drought stress conditions.

Key words: Abiotic stress, qRT-PCR, *LEA* genes, MDA

1. Introduction

Legumes (*Leguminosae*), which have a market value of $40 billion in the world economy and 60 million tons production per year, are the third largest family in the plant kingdom (Gepts et al., 2005; Ashraf et al., 2012). Since the earliest civilizations, legumes have been used for human nutrition and particularly for a source of protein. According to field studies, 22% of vegetable proteins and 7% of carbohydrates needed in human nutrition and 38% of proteins and 5% of carbohydrates consumed by animals are provided by edible grain legumes (Wery and Grinac, 1983; Gepts et al., 2005).

Over the years, the world has experienced a significant increase in legume production. At the beginning of the 2000s, while 55 million tons of legumes were produced in the world, nowadays it is seen that the total production of the legumes has reached around 60 million tons per year. Legumes also have great importance among the agricultural products in Turkey, which is in the top 10 legumes producers in the world. However, today, decreasing cultivation areas and total production levels are becoming an important threat in Turkey. As a result of the production decrement, large volumes of imported dry beans, chickpeas, and lentils have been reported every year (Ashraf et al., 2012).

Biotic and abiotic stress factors are thought to play a major role in decreased quality and quantity of crops (Atkinson and Urwin, 2012). Among all stress factors, soil salinity and drought are the most important ones that limit productivity in agriculture. Thus, understanding the fundamentals of salt and drought tolerance in bean and generating more adaptive varieties to environmental stress conditions accordingly are imperative issues in the agriculture of developing countries. However, the development of stress-tolerant plants still awaits accumulation of more knowledge about defining the genetic components involved in the stress response mechanism (Hiz, 2014). In this respect, understanding environmental stress responses in plants is a challenging topic in plant research.

Environmental stress factors such as salinity and drought produce extensive changes in the regulation of gene expression, gene activation or suppression, and signal transduction pathways that can affect the life cycle of plants. Two main stress inducible gene groups have been identified in *Arabidopsis* that are thought to play an important role in stress response (Shinozaki and Yamaguchi-Shinozaki, 2007; Cramer et al., 2011). The first group includes regulatory proteins such as transcription factors, protein kinases, enzymes related to phospholipid metabolism, and other signaling molecules. The second

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group contains functional genes that encode protective proteins against abiotic stress tolerance such as late embryogenesis abundant (LEA) proteins, chaperones, osmotin, mRNA-binding proteins, water channel proteins, and detoxification enzymes (Cramer et al., 2011).

LEA proteins are thought to be associated with desiccation tolerance in plants. These proteins were first discovered in the late stages of embryo development in cotton seeds and they have also been shown to accumulate in vegetative tissues of plants subjected to abiotic stress conditions (Dure et al., 1981; Galau et al., 1987; Bies-Etheve et al., 2008; Hundertmark and Hincha, 2008). Biotechnological approaches have shown that overexpression of LEA genes in different plant species such as Arabidopsis, tobacco, rice, wheat, and lettuce produces increased abiotic stress resistant phenotypes (Leprince and Buitink, 2010).

In the present study, we aimed to evaluate mRNA levels of the *PvLEA3* gene (GenBank: DQ196430.1) in the leaf and root tissues of seven different bean varieties (Yunus-90, Akman-98, Şehirali-90, Karacaşehir-90, Eskişehir-855, Göynük-98, Önceler-98) subjected to 3 and 27 h of 100 mM salt (NaCl) and drought (PEG) stress. Because it is an appropriate system to study drought and salinity problems we chose *Phaseolus vulgaris* L. for our study. *PvLEA3* mRNA levels were measured by qPCR, also called quantitative PCR or real-time PCR. The role of *PvLEA3* in stress response and the usefulness of this gene for the detection of tolerance capability of bean varieties against salt and drought stress conditions were investigated.

2. Materials and methods

2.1. Plant material, growth conditions, and stress treatment

Seven different bean varieties (*Phaseolus vulgaris* L.) obtained from the Transitional Zone Agricultural Research Institute, Eskişehir, Turkey, were used for the study. These bean seeds were germinated and grown hydroponically in pots containing 0.2 L of modified 1/10 Hoagland solution. The Hoagland solution included macronutrients (K_2SO_4, KH_2PO_4, $MgSO_4.7H_2O$, Ca $(NO_3)_2.4H_2O$, and KCl) and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4.5H_2O$, NH_4Mo, and $ZnSO_4.7H_2O$) with the following final concentrations of these ions: 2 mM Ca, 10^{-6} M Mn, 4 mM NO_3, 2.10^{-7} M Cu, 1 mM Mg, 10^{-8} M NH_4, 2 mM K, 10^{-6} M Zn, 0.2 mM P, 10^{-4} M Fe, and 10^{-6} M B.

Seven bean varieties with three biological replicates each placed on one pot that contained five plants were grown in a controlled environmental growth chamber with light of 250 mmol m^{-2} s^{-1} photosynthetic photon flux at 25 °C, and with 70% relative humidity. Twenty-five-day-old plants grown in controlled media were used for stress treatments. The plants were treated with salt by the addition of NaCl to the nutrient solution, to a final concentration of 100 mM. Osmotic pressures of the NaCl solutions were determined by Vapor Pressure Osmometer 5520. The average osmotic pressure of the 100 mM NaCl solution was estimated as 190 mmol/kg. For the drought treatment (Verslues et al., 2006), polyethylene glycol 6000 (PEG 6000) was added to the nutrient solution until the osmolality of the solution measured by a vapor pressure osmometer was the same as that of the 100 mM NaCl solutions. In this way, an isoosmotic level of stress was applied to both NaCl- and PEG-treated plants, with the goal of matching the stem water potentials of the plants exposed to these treatments.

Five plants per biological replicate were harvested after 0, 3, and 27 h of stress exposure and harvested plant tissues were then pooled. They (leaves and roots) were ground in liquid nitrogen and used for RNA extraction.

2.2. Estimation of lipid peroxidation

Malondialdehyde (MDA) is a marker of oxidative lipid injury that changes in response to environmental factors leading to stress in plants. TBA-MDA content was determined as described by Hodges et al. (1999). The experiment contained three biological replicates, consisting of three technical replicates each for both stressed and nonstressed plant samples. Plant tissue samples were homogenized with liquid nitrogen and with 80:20 (v:v) ethanol:water, followed by centrifugation at 3000 × *g* for 10 min. A 1-mL aliquot of appropriately diluted sample was added to a test tube with 1 mL of either (i)) TBA solution composed of 20.0% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (ii) +TBA solution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95 °C on a hot plate (neoBlock1, 2-2503) for 25 min, cooled, and centrifuged at 3000 × *g* for 10 min. Absorbance values were measured at 440 nm, 532 nm, and 600 nm by ELISA microplate reader (SpectraMax M2). The equivalents of malondialdehyde were calculated by the following equations:

1) $[(\text{Abs } 532_{+TBA}) - (\text{Abs } 600_{+TBA}) - (\text{Abs } 532_{-TBA} - \text{Abs } 600_{-TBA})] = A$
2) $[(\text{Abs } 440_{+TBA} - \text{Abs } 600_{+TBA}) \, 0.0571] = B$
3) MDA equivalents (nmol mL^{-1}) = $(A - B/157{,}000) \, 10^6$

2.3. RNA extraction and qRT-PCR analysis

RNA extraction was performed using the TRIzol protocol followed by RNeasy mini kit (Qiagen, Cat no: 74104) cleanup (Chomczynski and Mackey, 1995). The quantity and quality of RNA were determined by Nanodrop ND-Spectrometer 1000 and also confirmed by gel electrophoresis containing 1.5% agarose and formaldehyde. A two-step procedure was used for real-time reverse

transcriptase-polymerase chain reaction (RT-PCR). Reverse transcription reactions were performed with 2 µg of RNA, 2.5 µM Anchored-oligo(dT)18, 1X Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 20 U Protector Rnase Inhibitor, 1 mM deoxynucleotide Mix, 5 mM DTT, and 10 U Transcriptor High Fidelity Reverse Transcriptase using the High Fidelity cDNA Synthesis Kit (Roche).

Real-time PCR was performed using a Light Cycler Nano System (Roche). The sequences of primers (presented in the Table) of the target gene *PvLEA3* (GenBank: DQ196430.1) and actin (*ACT*) were designed based on sequences information of bean genes available in the databank (http://www.ncbi.nlm. nih.gov/). Amplifications of PCR product were monitored via SYBR Green I dye. Copy numbers of genes (*PvLEA3*, *ACT*) under stress treatments were determined by using standard curves. The qRT-PCR analysis contained three biological replicates, consisting of three technical replicates each for both stressed and nonstressed plant samples.

2.4. Statistical analysis

The abundance of target gene transcripts was normalized to *ACT* and set relative to control plants according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Changes in relative expression levels (REL) of the gene were checked for statistical significance according to one-way ANOVA. The results were considered statistically significant if the P-value was <0.05 in the Dunnett test.

3. Results

We performed qRT-PCR for comparative analysis of the *PvLEA3* gene expression in different bean varieties and in different plant tissues (root and leaf). Primarily, we evaluated malondialdehyde (MDA) levels as lipid peroxidation can be considered a marker under stress conditions. Increased reactive oxygen species (ROS) lead to overproduction of MDA and it is a final product of polyunsaturated fatty acid peroxidation in plant cells (Rao et al., 2005).

In the current study, 3 and 27 h of stress conditions (NaCl and PEG) led to increased MDA levels in root and leaf tissues in all bean varieties except for Göynük-98 and Yunus-90 compared to their untreated controls. MDA levels were gradually decreased in these bean varieties under 3 and 27 h of NaCl and PEG stresses. Figures 1 and 2 show the comparative evaluation of MDA levels in Göynük-98 and Yunus-90 bean varieties (Supplementary data are available for the MDA levels of other bean varieties).

PvLEA3 mRNA levels were significantly altered in different bean varieties subjected to salt or drought stress. The *PvLEA3* mRNA levels normalized to the expression levels of control samples and to the most suitable housekeeping gene (*ACT*) in all bean varieties under all stress conditions are presented in Figures 2 and 3.

Significantly increased mRNA levels of the *PvLEA3* gene compared to the control samples were seen in the roots of almost all bean varieties subjected to 3 h of PEG stress. The highest increments were seen in Göynük-98 (almost 4000-fold) and Yunus-90 (almost 245-fold) (Figure 3). Gene expression levels of *PvLEA3* in the leaves of the bean varieties were similar to those obtained from the roots. Göynük-98 and Yunus-90 were the bean varieties with the highest mRNA levels of the *PvLEA3* gene in their leaves (Figure 3).

When the impacts of NaCl stress on mRNA levels of the *PvLEA3* gene were evaluated, increased mRNA levels of *PvLEA3* were seen in the roots of all bean varieties except for Akman-98 at 3 h of stress treatment. Similar changes were observed in the leaves of all bean varieties subjected to NaCl stress as seen in Figure 3. The highest mRNA levels of the *PvLEA3* gene were again observed in Göynük-98 and Yunus-90 varieties (Figure 3).

Moreover, 27 h of PEG stress led to an increase in mRNA levels of the *PvLEA3* gene in a statistically significant manner only in the roots of Göynük-98 and Yunus-90. The highest increments were seen in Göynük-98 and Yunus-90 in both root and leaf tissues as observed at 3 h of PEG stress (Figure 4).

With regard to the evaluation of all the data obtained at 27 h of NaCl stress, only Göynük-98 and Yunus-90 showed increased mRNA levels of the *PvLEA3* gene. Other bean varieties showed lower mRNA levels than their untreated control samples (Figure 4).

Table. The sequences of primers of the target gene *PvLEA3* (GenBank: DQ196430.1) and actin (*ACT*).

Primers	Sequences (5'→3')	Primer length (bp)	Annealing temperature (Tm)
LEA-3 F	CAC AGA GGT GAT TCA TGA TGT T	22	58 °C
LEA-3 R	ACC CTT CTC CAG AGT CTT	18	58 °C
ACT F	TGA GCA AGG AGA TTA CAG CAT TGG	24	56 °C
ACT R	CAT ACT CTG CCT TCG CAA TCC AC	23	56 °C

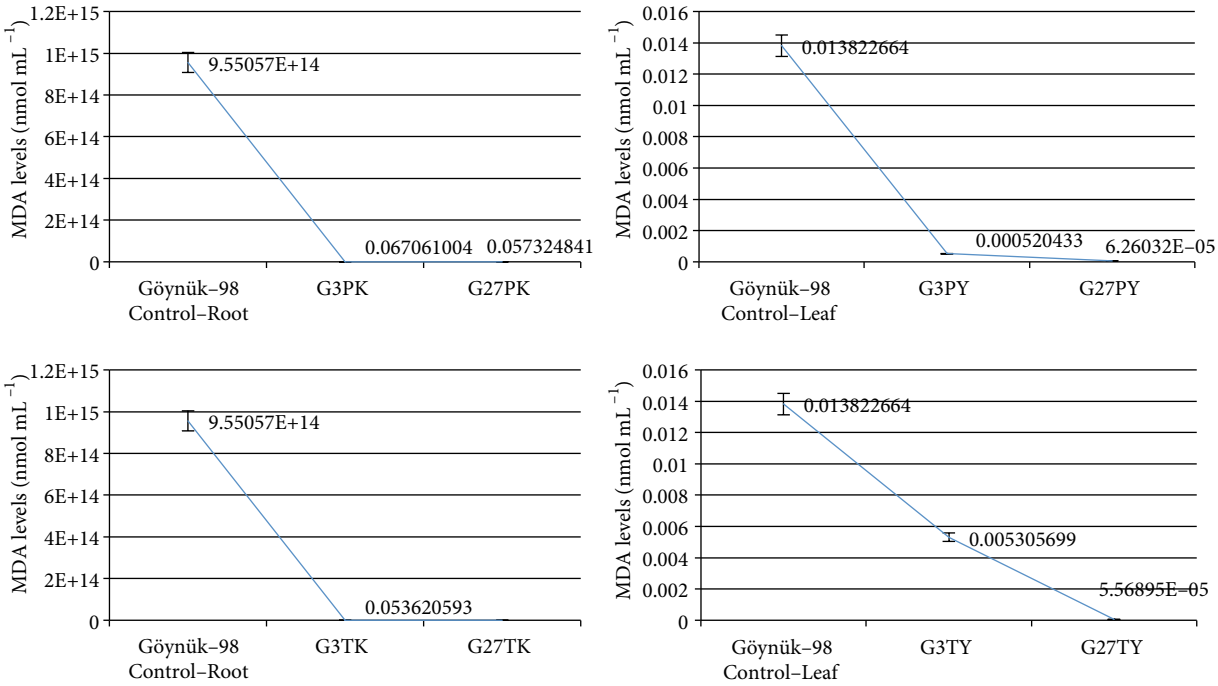

Figure 1. Comparative evaluation of MDA levels in leaf and root tissues of Göynük-98 subjected to NaCl and PEG stresses. G3PK: 3 h PEG stressed roots of Göynük-98; G27PK: 27 h PEG stressed roots of Göynük-98; G3PY: 3 h PEG stressed leaves of Göynük-98; G27PY: 27 h PEG stressed leaves of Göynük-98; G3TK: 3 h NaCl stressed roots of Göynük-98; G27PK: 27 h NaCl stressed roots of Göynük-98; G3TY: 3 h NaCl stressed leaves of Göynük-98; G27TY: 27 h NaCl stressed leaves of Göynük-98. Error bars represent standard error.

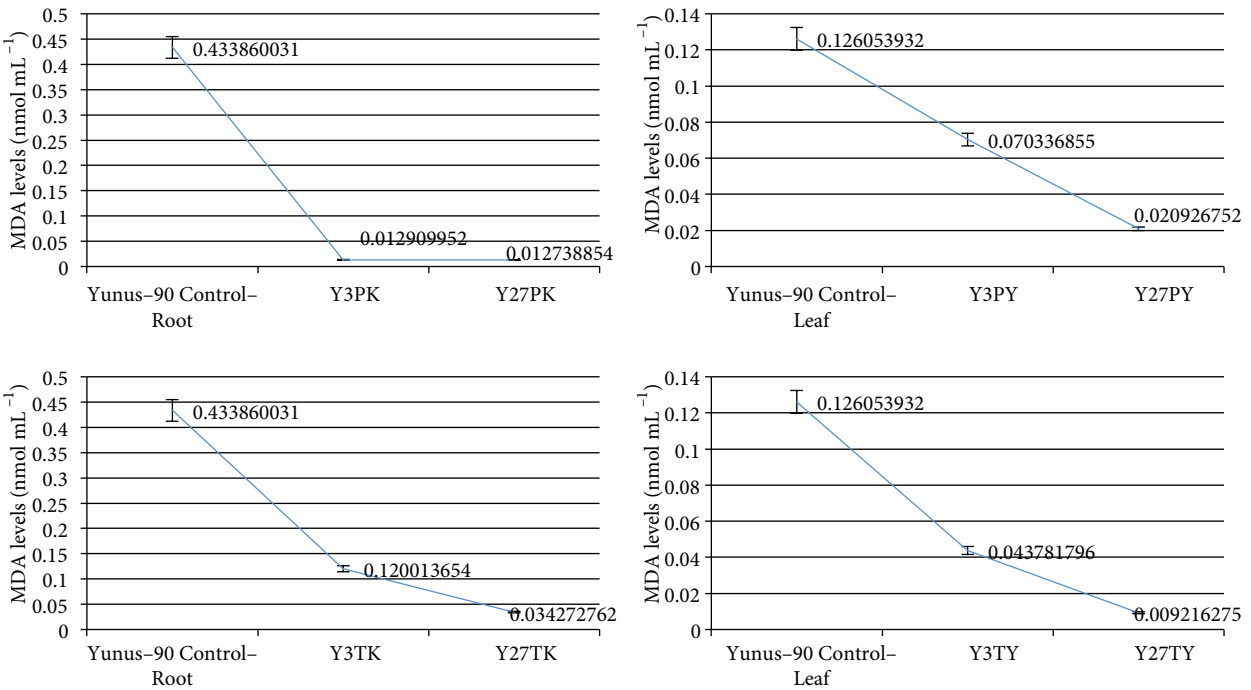

Figure 2. Comparative evaluation of MDA levels in leaf and root tissues of Yunus-90 subjected to NaCl and PEG stresses. Y3PK: 3 h PEG stressed roots of Yunus-90; Y27PK: 27 h PEG stressed roots of Yunus-90; Y3PY: 3 h PEG stressed leaves of Yunus-90; Y27PY: 27 h PEG stressed leaves of Yunus-90; Y3TK: 3 h NaCl stressed roots of Yunus-90; Y27PK: 27 h NaCl stressed roots of Yunus-90; Y3TY: 3 h NaCl stressed leaves of Yunus-90; Y27TY: 27 h NaCl stressed leaves of Yunus-90. Error bars represent standard error.

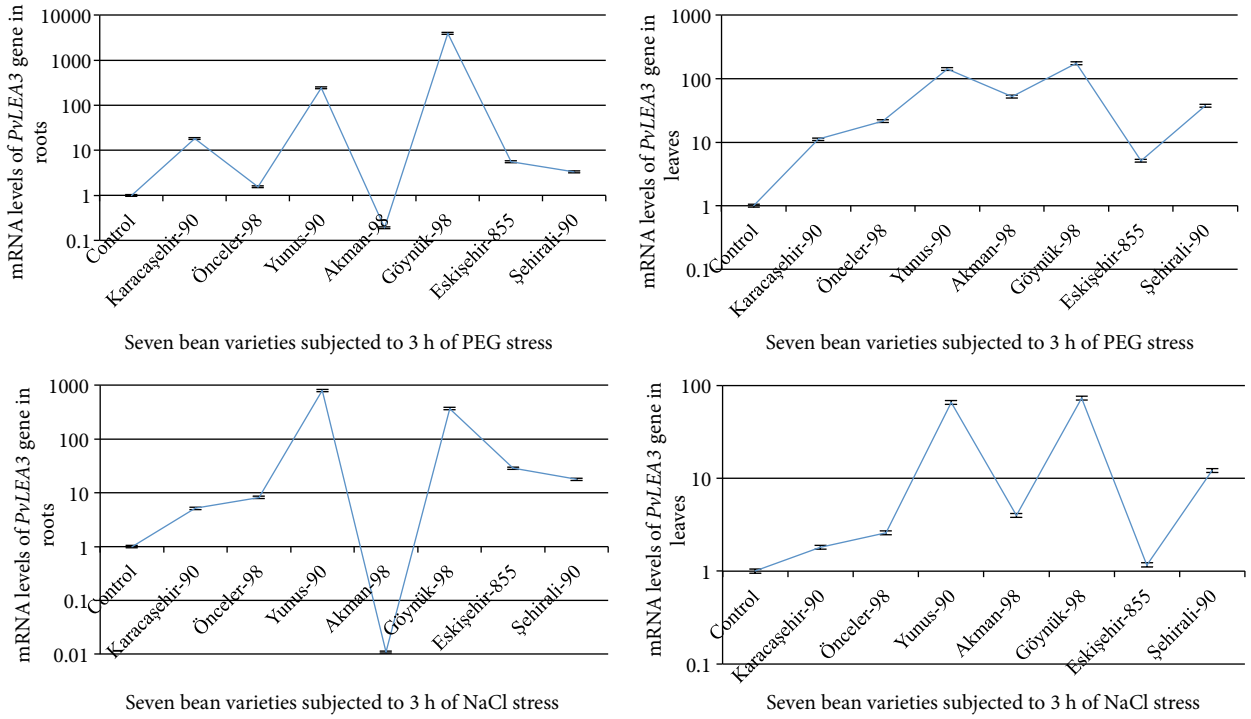

Figure 3. mRNA levels of the *PvLEA3* gene in the roots and leaves of seven bean varieties subjected to 3 h of NaCl and PEG stresses. qRT-PCR data obtained from all bean varieties were normalized to their own untreated control. Error bars represent standard error.

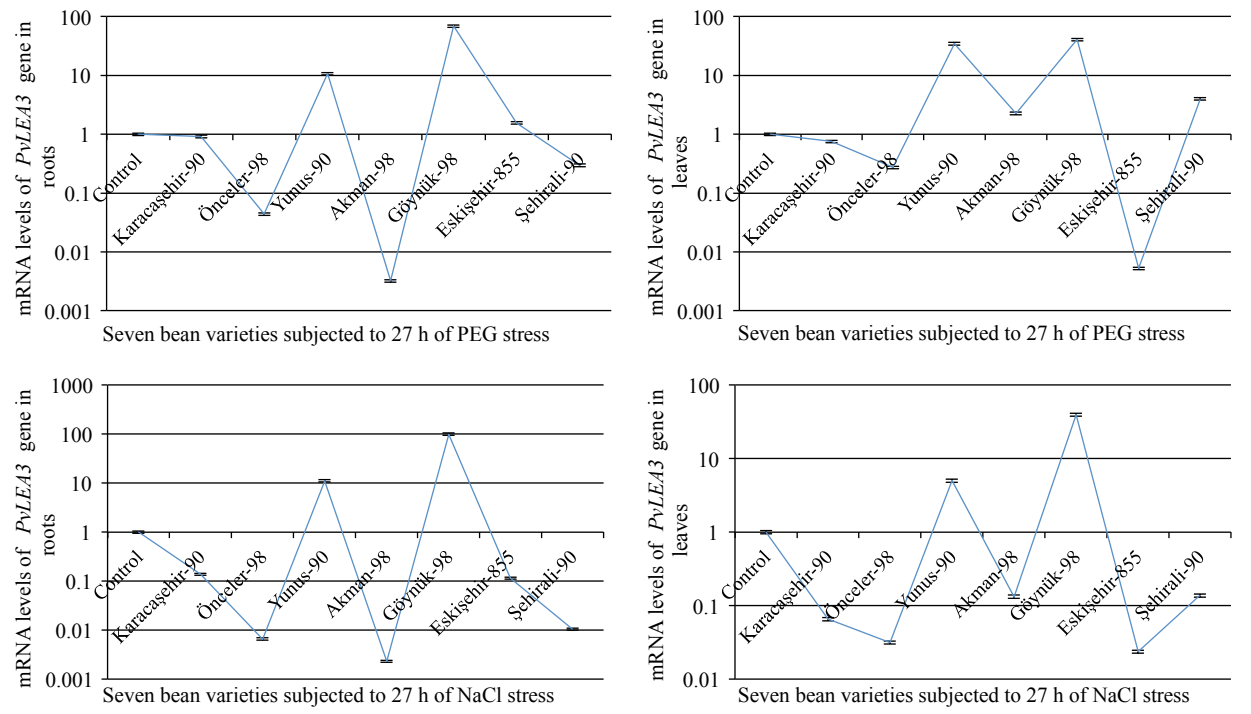

Figure 4. mRNA levels of the *PvLEA3* gene in the roots and leaves of seven bean varieties subjected to 27 h of NaCl and PEG stresses. qRT-PCR data obtained from all bean varieties were normalized to their own untreated control. Error bars represent standard error.

4. Discussion

Abiotic stress, such as drought and salinity, are critical threats to agriculture and to the environment by leading to deviation in the normal physiology, development, and function of plants, which can cause irreversible damage to the plant system. Crop losses caused by abiotic stresses can be reduced by manipulating the genes and plant metabolism with the use of genetically engineered plants. Hence a better understanding of the effects of abiotic stress on the plant genome is needed for the accumulation of more knowledge about the genetic components involved in the stress response mechanism (Aydın et al., 2014; Hiz Can Mahmut, 2014).

Drought and salinity stresses produce water deficit that leads to the accumulation of the LEA protein group, also associated with water limitation produced during plant development under normal environmental conditions (Colmenero-Flores et al., 1999; Vicient et al., 2000; Sheoran et al., 2006). In the current study, we evaluated mRNA levels of the *PvLEA3* gene against NaCl and PEG stress conditions in different bean varieties. *PvLEA3* (GenBank: DQ196430.1), which was characterized and submitted to GenBank by Barrera-Figuero et al. (2007), is a group three late embryogenesis abundant protein coding gene found in *Phaseolus vulgaris*.

Free radicals as a cause of oxidative stress can interact with the whole structure of the cell but the lipids are the most sensitive structures to this interaction (Cheeseman, 1993). Malondialdehyde is one of the most reactive final products of lipid peroxidation and is caused by the effects of free radicals on the tissues (Rao et al., 2005). Determination of plasma MDA levels is one of the sensitive indicators of lipid peroxidation and oxidative stress (Knight et al., 1988).

In the current study, increased MDA levels were measured compared to the untreated controls in all bean varieties subjected to 3 h and 27 h of NaCl and PEG stress conditions except for Göynük-98 and Yunus-90. This result indicates the effects of oxidative stress on lipid peroxidation in all bean varieties except Göynük-98 and Yunus-90 under NaCl and PEG stresses. According to the MDA analysis, Göynük-98 and Yunus-90 are the most tolerant while Karacaşehir-90 is the most sensitive bean variety. The rest of the bean varieties are determined to be intermediate forms.

Previous studies proved that MDA levels can be used for assessment of the tolerance capabilities of plant species against oxidative stress (Terzi et al., 2010). Results of MDA analysis obtained in the current study appear to be consistent with those of previous studies. The study by Terzi et al. (2010) suggested that Yunus-90 was the most drought tolerant bean variety among Göynük-98, Karacaşehir-90, Şehirali-90, Eskişehir-855, and Yunus-90 according to some biochemical parameters including MDA analysis. In the same study, Karacaşehir-90 was also the most sensitive bean variety, which appears to be consistent with the current study.

qRT-PCR using SYBR Green I was performed following the MDA analysis to evaluate mRNA levels of the *PvLEA3* gene in root and leaf tissues of different bean varieties. All the data, which were normalized against the most stable housekeeping gene (*ACT*), revealed the alterations in mRNA levels of the *PvLEA3* gene in all bean varieties under all stress conditions.

When all bean varieties were compared, Yunus-90 and Göynük-98 were found to be the varieties with the highest mRNA levels of the *PvLEA3* gene under all stress conditions. On the other hand, when these two varieties were compared, root and leaf tissues of Göynük-98 revealed higher mRNA levels than did Yunus-90.

Stress exposure times of 3 and 27 h were selected to maintain a 24-h interval between the two time points to avoid the changes in MDA and *PvLEA3* mRNA levels being under the control of the circadian clock. With regard to evaluating the stress exposure times, it was seen that *PvLEA3* was expressed more at 3 h of both stress treatments than at 27 h. Even lower mRNA levels than the untreated control in all bean varieties except for Göynük-98 and Yunus-90 were determined at 27 h of both salt and drought stress treatments. All these results showed that the *PvLEA3* gene has an effective short-term response to both salt and drought stress in bean varieties.

The current study provides the first evidence for the importance of the *PvLEA3* gene in response to NaCl and PEG stresses and the usefulness of this gene to evaluate tolerance capacities of different bean varieties.

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