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Research Article
(Araştırma Makalesi)

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Isolation of ascorbate peroxidase (APX) gene in lentil (*Lens culinaris* Medik.) and expression analysis under drought stress conditions

Mercimekte (*Lens culinaris* Medik.) askorbat peroksidaz geninin izolasyonu ve kuraklık stresi koşullarındaki ifadesinin belirlenmesi

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ABSTRACT

Objective: The objective of this study was to isolate partial cDNA that belongs to the ascorbate peroxidase (*APX*) gene of lentil (*Lens culinaris* Medik.) and to express Lc*APX* gene in lentil seedlings under drought stress conditions.

Material and Methods: To identify the relationships between drought stress and LcAPX gene expression, lentil seedlings grown for 2 weeks were subjected to drought stress through not irrigating for 6, 13, and 20 days. Effects of drought stress were determined by measuring the stem relative water content (RWC). Gene expression changes in lentil seedlings were determined with real-time RT-qPCR.

Results: The Lc*APX* gene expression levels of both drought-tolerant Firat-87 and drought-sensitive Ozbek cultivars varied with the severity of drought stress. The gene expression of Lc*APX* reached the highest level in Firat-87 cultivar on the 6th day, whereas a significant increase was observed only on the 20th day of the Ozbek cultivar, and this increase was relatively low as compared to the Fırat-87 cultivar.

Conclusion: From the study conducted, it was concluded that time-dependent changes of the expression of Lc*APX* gene indicates that Lc*APX* gene had a highly specific gene expression profile and complex regulation in lentil drought response.

ÖZ

Amaç: Bu çalışmada, mercimekte (*Lens culinaris* Medik.) askorbat peroksidaz (APX) geninin partial cDNA klonu izole edilmiş ve Lc*APX* geninin kuraklık stresi koşullarında mercimek fidelerinde değişen gen ifadesi seviyesi belirlenmiştir.

Materyal ve Yöntem: Kuraklık stresi ve Lc*APX* gen ifadesi arasındaki ilişkiyi anlamak için, 2 hafta süre ile yetiştirilen mercimek fidelerine 6, 13 ve 20 gün süre ile sulamama şeklinde kuraklık stresi uygulanmıştır. Kuraklık stresinin etkileri, sap nispi nem içeriği (RWC) ölçülerek belirlenmiştir. Mercimek fidelerinde meydana gelen gen ifadesi değişimleri eş zamanlı kantitatif PCR (Real-time qPCR) ile belirlenmiştir.

Araştırma Bulguları: Hem kuraklığa dayanıklı Fırat-87 hem de kuraklığa duyarlı Özbek çeşitlerinin Lc*APX* gen ekspresyon seviyeleri, kuraklık stresinin şiddetine göre değişiklik göstermiştir. LcAPX gen ekspresyonu Fırat-87 çeşidinde 6. günde en yüksek seviyeye ulaşırken, Özbek çeşidinin sadece 20. gününde önemli bir artış gözlenmiş ve bu artış Fırat-87 çeşidine göre nispeten düşük kalmıştır.

Sonuç: Sonuç olarak, Lc*APX* geninin ekspresyonunun gün bazında değişmesi, Lc*APX* geninin mercimeğin kuraklığa tepkisinde oldukça spesifik bir gen ekspresyon profiline ve karmaşık bir regülasyona sahip olduğunu göstermektedir.

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is among the oldest cultivated edible legumes (Bahl et al., 1993). It is a self-pollinating annual cool season plant (Arumuganathan & Earle, 1991; Muehlbauer, 1992) and has a diploid genome (2n=2x=14) with a genome size of 4063 Mbp (Arumuganathan & Earle, 1991). Lentil is an important source of protein (22-35%), fiber, minerals, and carbohydrates for human diet (Pala et al., 2018). It is a cool season crop plant (Tullu et al., 2001) and cultivated over 610 thousand hectares worldwide and annual production is around 6.3 million tons (FAO, 2018). Turkey with an annual production of 353 thousand tons is ranked fourth in the world lentil production after Canada, India and the USA (FAO, 2018). There were significant increases in lentil cultivated lands during the last decade (FAO, 2018), but sufficient yield levels were not achieved because of biotic and abiotic stresses exerted on plants (Rahimi et al., 2016; Sehgal et al., 2017; Singh et al., 2017; Bakır, 2019; Köse et al., 2019).

Drought is an important abiotic stress factor influencing about 26% of cultivated lands worldwide (Kalefetoğlu & Ekmekçi, 2005). Drought stress results in stomal closure, thus reduces photosynthesis rates, turgor pressure, cell growth and division, increases oxidative damage, reduce plant height and leaf size, in brief, destructs several plant activities (Kalefetoğlu & Ekmekçi, 2005; Jaleel et al., 2009; Samarah et al., 2009; Qados, 2011; Örs & Ekinci, 2015; Kabay & Şensoy, 2016; Laxa et al., 2019; Marchin et al., 2020). Plants are more sensitive to such negative effects of drought stress in generative stage (Barnabas et al., 2008; Morgil et al., 2019). It was reported that drought stress limits the growth and yield of lentil especially in reproductive period and grain-filling periods (Rahimi et al., 2016; Sehgal et al., 2017) and resulted in yield losses varying between 6 and 54% (Oweis et al., 2004).

Drought stress facilitates the production of reactive oxygen species (ROS) like superoxide (O_2-), hydroxyl (OH−), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) and generates oxidative stress on plants (Harb et al., 2015). Ascorbate peroxidase (APX) enzyme (EC 1.11.1.11, APX) catalyzes H_2O_2, largely produced in plant cells, into H_2O and O_2 and prevent accumulation of H_2O_2 to toxic levels and plays an important role in homeostasis of reactive oxygen species in plants (Mittler & Zilinskas, 1992; Mittler, 2002; Sato et al., 2001; Punchuk et al., 2002; Shigeoka et al., 2002; Güneş et al., 2006; Xu et al., 2011; Gökçay, 2012; Chugh et al., 2013; Harb et al., 2015; Çevik & Ünyayar, 2015; Bartwal & Arora, 2017; Laxa et al., 2019). APX in higher plants exists in four isoforms: cytosolic, stromal, glycosomal, and thylakoid membrane bound (Chen & Asada, 1989; Miyake & Asada, 1992) and these isoforms increase response to different stress conditions. The changes in APX gene expression and APX enzyme activity vary in drought-tolerant and resistant species and such an increase in APX enzyme activity of drought-tolerant genotypes is related to increasing expression levels of different APX isoforms (Secenji et al., 2010). Previous studies showed that the APX gene expression and APX enzyme activity increased with drought stress in many plants such as wheat, sorghum, common meadow, pea, soybean, tomatoand maize etc. (Mittler & Zilinskas, 1994; Morita et al., 1999; D'Arcy-Lameta et al., 2006; Ünyayar & Çekiç, 2006; Jiang et al., 2010; Secenji et al., 2010; Terzi et al., 2010; Xu et al., 2011; Kauser et al., 2012; Chugh et al., 2013; Bartwal & Arora, 2017; Akbudak et al., 2018).

The number of studies about APX enzyme activity of lentil under drought stress is quite limited (Aksoy, 2008; Öktem et al., 2008; Gökçay, 2012; Sing et al., 2017) and there is no study in order to identify relationship between APX gene expression and drought stress in lentil plants in the literature. The objectives of the study were to isolate cDNA clone of ascorbate peroxidase (APX) gene in lentil (*Lens culina*ris Medik.) and to determine changes in Lc*APX* gene expression levels in lentil seedlings under different drought stress conditions.

MATERIALS and METHODS

Plant material

Drought-resistant Firat-87 lentil cultivar (GAP International Agricultural Research and Training Center) (Aslan, 2014; Ceritoğlu, 2019) and drought-sensitive Ozbek cultivar (Field Crops Central Research Institute) (Güneş et al., 2006; Elkoyunu, 2013; Tekin, 2019) were used as the plant material for the experiments. Seeds of these cultivars were sterilized with 10% sodium hypochlorite, imbibed in water for a day and sown into viols and grown under controlled conditions (23°C temperature, 70% relative humidity, 16/8 light/dark photoperiods). Before drought stress treatments, plants were irrigated with ½ Hoagland solution in every 3 days. This study was carried out at Erciyes University, Betül Ziya Genome and Stem Cell Center in 2017.

Stress treatments and physiological measurements

Seedlings were grown under controlled conditions (23 °C temperature, 70% relative humidity, 16/8 light/dark photoperiods) for 10 days and then drought stress was exerted on these plants. Drought stress treatments were applied in without irrigating for 6 days (normal drought stress), 13 days (moderate drought stress) and 20 days (severe drought stress). The stem water potential of each stress and control plant was measured with a pressure chamber (Model 600, Wescor, Inc.). A pool was generated for RNA isolation based on the treatment timings with homogeneous stem water potentials of 5 plants between -0.6 and -1.8 MPa for stress-treated plants and between -0.2 and -0.4 MPa for control plants.

Total RNA isolation and cDNA synthesis

Total RNA isolation was performed with the use of TRIzol Reagent (Thermo Fisher Scientific, USA) in accordance with the kit protocol. Purity and concentration of isolated RNAs were determined in Nanodrop ND-1000 spectrophotometer and RNA integrity was checked in 2% formaldehyde agarose gel. The cDNA synthesis was performed with the use of random hexamer, 2 µg total RNA, and Transcriptor First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

Primer design and amplification of APX gene

APX gene amplification was conducted with the use of PCR primer design, Consensus Degenerate Hybrid Oligonucleotide Primer (CODEHOP) (http: //bioinformatics.weizmann.ac.il/blocks/codehop.html) (Rose et. al., 1998) algorithm, *Medicago truncatula* L-ascorbate peroxidase mRNA (XM_003606462.2), *Arabidopsis thaliana* L-ascorbate peroxidase mRNA, (AY081646.1), *Pisum sativum* APXI mRNA for ascorbate peroxidase (X62077.1) sequences. Designed primers were tested in lentil cDNAs and APX_F1R5 primer yielding a clear band was selected for sequence analyses. PCR reactions including 15 µl reaction volume, 200 ng cDNA, 10 pmol primers (dAPX_F1 5'-CCCCACAGTGAAGCCAGACtaymaraargc-3' and dAPX _R5 5'- GCTGCAGCAGGCCGtcyttytcncc -3'), 2.5 mM dNTP, 0.1 unit Taq DNA Polymerase (Thermo Fisher Scientific, USA), 1.5 mM $MgCl_2$, 5x buffer conducted at 94ºC for 5 min, followed by 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min, 35 cycles and 72ºC for 10 min. PCR products were checked in 2% agarose gel, purified with ExoSAP (Thermo Fisher Scientific) PCR purifying system and sequenced in Applied Biosystems Prism 3500 Genetic Analysis System (Applied Biosystems, USA) with the use of BigDye Terminator v3.1 Cycle Sequencing Kit. Resultant sequence results was aligned with the use of BLAST software and primers were designed for lentil APX gene with the use of Primer3 (http: //bioinfo.ut.ee/primer3-0.4.0/) software.

Real-time RT-qPCR analysis

Amplification of APX gene was performed using single-strand cDNA synthesized from the control and drought stress-treated lentil seedlings. The qPCR reactions were conducted with the use of Light Cycler ® SYBR Green 1 Master mixture (Thermo Fisher Scientific, USA) in accordance with the kit procedure. In brief reactions were performed in 20 µl total volume of LightCycler® 480 (Thermo Fisher

Scientific, USA) system including 2 µl cDNA, 10 µl SYBR Green 1 Master mixture, 10 pmol primer (Lc*APX*1_F 5'- TGGAGCCTCTTAAGGAGCAA-3' and Lc*APX*1_R 5'- TCCCTCAAATGGTCAGATCC-3'). Amplification conditions were as follows; following pre-denaturation stage at 95°C for 10 min, 45 cycles at 95°C for 10 s, 50 °C for 10 s, 72°C for 8 s. PCR products were checked with melting curve analysis to verify the specificity of PCR reactions. The analyzes were performed with 3 biological (with 3 technical replicates each) for each sample.

Statistical analysis

Experimental data obtained from the control and drought stress-treated plants were subjected to statistical analyses in accordance with $2^{-\Delta\Delta CT}$ method of Livak & Schmittgen (2001). For normalization of Ct/CP values of gene expression, "housekeeping" gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (GenBank no. X75327.1) (GAPDH_F TGGGCGAAAACTCCACTTTG and GAPDH_R GAATTGCTGCAGCCTTGTGA) control gene were used (Saha & Vandemark, 2013).

RESULT and DISCUSSION

Physiological changes

A gradual decrease was observed in stem water potential of the cultivars with increasing drought stress. While greater decrease was observed in drought-sensitive Ozbek cultivar on 6th day, decrease in stem water potential of drought-resistant Firat-87 cultivar was greater than Ozbek cultivar on 13th and 20th days. There is a reverse relationship between resistance to drought stress and stem water potential (Joshi & Karan, 2013). Stem water potentials of the control plants varied between 0-0.5 MPa (Figure 1).

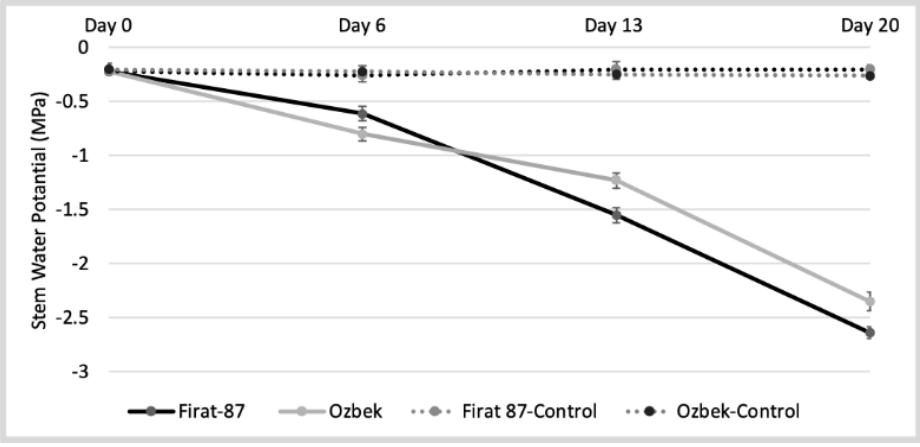

Figure 1. Stem water potential (MPa) measurements under drought stress conditions.

Şekil 1. *Kuraklık stres koşulları altında gövde su potansiyeli (MPa) ölçüm sonuçları.*

Decrease in relative water content is an early indicator of water deficits in plant tissues (Valentovic et al., 2006). Several other researchers reported that the relative water content in plant and stems under drought stress decreased (Jiang et al., 2010; Terzi et al., 2010; Ghaderi et al., 2011; Xu et al., 2011; Cao et al., 2017). Singh et al., (2017) conducted a study with two lentil genotypes, drought-tolerant and sensitive, and reported 28.6% decrease in relative water content of tolerant genotype and 60.1% decrease in relative water content of sensitive genotype under drought stress. Sehgal et al., (2017) investigated the effects of temperature and drought stress on 8 lentil genotypes and reported 32-35% decrease in relative water content of tolerant genotypes and 51-57% decrease in relative water content of sensitive genotypes (Sehgal et al., 2017).

Isolation of partial APX cDNA from lentil

From drought stress-treated lentil (*L. culinaris* Medik.) leaves, 564 bp long cDNA was isolated. Isolated cDNA fragment was named as *Lens culinaris* cultivar Firat87 ascorbate peroxidase I mRNA and submitted to GenBank (KY428918). In nucleotide sequence analyses of the resultant sequence, it was observed that lentil APX gene exhibited a high homology with *Pisum sativum* ApxI *(*X75327.1) (94%) *Medicago sativa* ascorbate peroxidase isoform 2 mRNA and complete cds (93%) sequences.

The samples of the same phylogenetic branches located in the same cell sections of different plant species are more closely related than the samples of different phylogenetic branches of the same species (Dabrowska et al., 2007). Cowpea cytosolic APX cDNA had high homology with pea (92%) and turnip (80.8%); peroxisomal APX cDNA with squash (84.7%) and barley (75.7%); chloroplastic APX cDNA with spinach (77.5%) and pumpkin (79.3%) (D'Arcy-Lameta et al., 2006). High homology of spinach was reported with pea (74%) and *A. thaliana* (72%) (Webb & Allen 1995) and between pea and *Arabidopsis thaliana* (79%) (Mittler & Zilinskas, 1992).

The expression analyses of *LcAPX* gene

The changes in lentil APX gene expressions under drought stress conditions were analyzed with real-time quantitative PCR (RT-qPCR) method. The Lc*APX* gene expression in lentil seedlings exhibited differences under drought stress. Drought-resistant Firat-87 cultivar had greater gene expression than drought-sensitive Ozbek cultivar under all drought conditions. Gene expression in Firat-87 cultivar reached the highest level on 6th day of drought stress, decreased on 13th day and increased again on 20th day. In Ozbek cultivar, increase in gene expression was parallel to increase in drought stress and reached the highest level on 20th day (Figure 2).

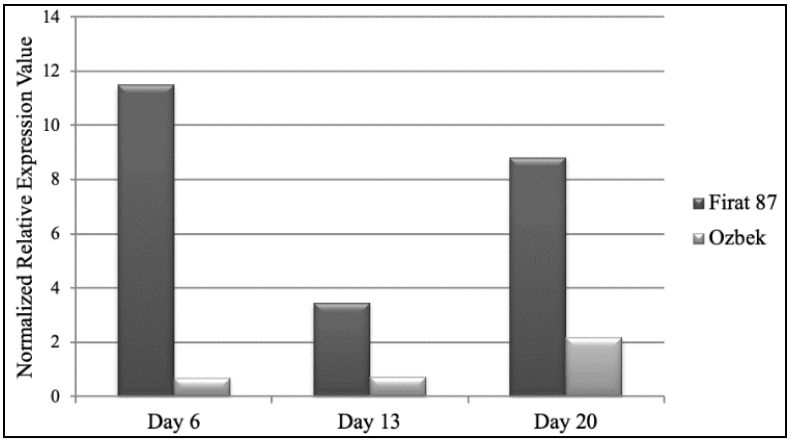

Figure 2. APX gene expression levels in seedlings of different lentil cultivars under drought stress conditions.

Şekil 2. *Kuraklık stresi koşullarında farklı mercimek çeşitlerine ait fidelerde APX geninin ekspresyon düzeyleri.*

D'Arcy-Lameta et al., (2006) applied drought stress to drought-resistant and sensitive cowpea (*Vigna unguiculata*) cultivars in the form of not-irrigated for certain periods of time, isolated APX cDNA and investigated the changes in gene expression with RT-PCR. Similar with the present findings, researchers reported an increase in APX gene expression of drought-sensitive cultivar with drought stress, but greater increase in gene expression of resistant cultivar during the early periods of the drought and throughout the drought. Jiang et al., (2010) applied drought stress to drought-tolerant and sensitive genotypes of prairie junegrass (*Koeleria macrantha*) plants grown for 45 days through not irrigating for 7 days and reported increases in cytAPX gene expression of sensitive and resistant genotypes and indicated that there were no distinct differences in accumulated transcript quantity of the genotypes. It

was reported in the same study that as compared to the control plants, drought stress did not yield significant differences in APX enzyme activity. Similar with the present study, Xu et al., (2011) applied drought stress to Kentucky bluegrass plants grown for 2 weeks through not irrigating for 22 days and reported increased APX gene expression of both tolerant and sensitive cultivars with greater increase in tolerant genotype. Harb et al., (2015) conducted a long-term drought stress experiments in barley and reported that APX enzyme activity and gene expression of sensitive genotypes increased in the early stages of drought stress, but changes were not observed in enzyme activity and gene expression of tolerant genotype in early stages, enzyme activity and gene expression of both genotypes decreased on the 9th day, but significantly decreased in resistant genotype, gene expression of sensitive genotype did not change on the 16th day, but increased in resistant genotype and enzyme activity of resistant genotype did not change. Similarly, in this study, gene expression level of resistant genotype decreased on 13th day of drought and increased again on 20th day. Increasing APX gene expressions were reported in different plants (Populus, paddy, tobacco) in response to drought and role of APX gene in drought tolerance was indicated in previous studies (Li et al., 2009; Zhang et al., 2013; Cao et al., 2017).

As a conclusion, it could be stated that stem water potential decreased and APX gene expression increased in lentil plants with drought stress treatments. Such an increase varied with the plant response to drought and drought durations and increase in gene expression was greater in drought-tolerant cultivar. The findings in this study revealed that increase in APX gene expression of lentil plants under drought stress was related to plant response to drought stress, but such a contribution was different in sensitive and resistant genotypes. Slight increase in gene expression of sensitive lentil cultivar, but not conversion of such an increase into a significant increase indicated that different mechanisms involved suppressed the increase in gene expression.

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