Research Article

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Identification of Drought-Responsive Genes in Turkish Bread Wheat (*T. aestivum* L.) Cultivar Gerek 79 by mRNA Differential Display Analysis

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ABSTRACT

Wheat is one of the most important cereal crops in the world. Many parts of the world depend on wheat as a source of food and animal feed. Drought stress negatively affects its development and greatly reduces its production. Drought response is a complex genetic mechanism involving multiple genes, transcription factors, miRNAs, hormones, proteins, co-factors, ions, and metabolites. Understanding the genetic basis of the drought tolerance mechanisms is very important for genetic improvement of this trait in wheat. Wheat is also an important cereal crop in Turkey. In this study, it is aimed to identify drought stress-regulated genes in bread wheat (Triticum aestivum cv. Gerek 79) and gene expression profiling using mRNA differential display (mRNA DD) was performed for seedling leaves of control and drought-stressed plants (62.4% of RWC). The comparative profiling study showed a total of 20 differentially- expressed cDNA bands and 10 of them were cloned and sequenced. The inserts with poor quality reads were eliminated. The nucleotide sequences of the remaining two cDNAs named G1 and G2 were subjected to similarity analysis. G1 and G2 showed a high degree of homology the mRNA of purple acid phosphatase and glycosyltransferase family 92 protein-like sequences of Triticum aestivum and some other plants, respectively. Purple acid phosphatases have been shown to be involved in plant responses to abiotic and biotic stresses. Similarly, the role of glycosyltransferases in thermotolerance has been reported in rice besides their functions in cellular homeostasis and detoxification pathways in plants. These reports and our findings have laid a foundation for further investigation of G1 and G2 cDNA clones. The investigation of differential expression of these gene fragments corresponding to purple acid phosphatase and glycosyltransferase family 92 protein-like sequences under drought conditions at the RNA level is ongoing. Further characterization of these genes could be important in understanding the functions of these gene/s in drought response.

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Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world. Many parts of the world depend on wheat as a source of food and animal feed. Drought is a major environmental stress that negatively affects its development and greatly reduces its production [1]. The development of wheat varieties with improved drought resistance can help ensure food security and improve the sustainability of agricultural systems. Plants respond to drought stress at the morphological, physiological and molecular levels. The developmental stage, age, plant genotype, severity and duration of

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drought influence their responses [2]. Molecular response to drought stress is very complex [3]. Numerous genes, transcription factors, microRNAs, metabolites, stress hormones, co-factors and drought-related proteins [4] are involved in this response. A large number of genes induced during water deficit function in the stress signaling pathways and the protection of cells from stress by production of functional proteins such as aquaporins and osmoprotectants. Although many genes and signaling pathways involved in drought response have been identified, the complex gene network that mediates plant responses to drought stress remains largely unclear. Therefore, understanding the molecular mechanisms of drought stress tolerance is very important for genetic improvement of this trait. The identification of drought-responsive genes and understanding of their functions in stress adaptation provide insight into the molecular mechanisms underlying the plant's response to drought stress. So far, hundreds of genes involved in the key processes of the plant response to drought have been identified using different genetic and genomic techniques [5]. These genes play crucial roles by strictly controlling the physiological and biochemical responses to stress during a water deficit. Different gene expression technologies such as differential display reverse transcription-polymerase chain reaction (DDRT-PCR), SAGE, SSH, cDNA-AFLP, microarray and RNAseq have been employed to study stress-related genes in many plant species [6-10].

mRNA Differential Display (DD) technique is a powerful technique for the identification and isolation of differentially expressed eukaryotic genes [11]. Several abiotic and biotic stress-related genes have been identified in plants using this genome-wide transcript profiling technique. For example, the stress responsive *psb A* gene was isolated from rice (*Oryza sativa* L.) using DD [12]. In a study performed by [13], a desiccation-responsive small GTP-binding protein (Rab2) was characterized from the desiccation-tolerant grass *Sporobolus stapfianus* using RT-PCR product identified with DD. A cDNA clone, *Bnuc1*, encoding a nuclease gene was isolated from salt-stressed barley leaves by using the DD technique [14]. The different members of a heat shock protein gene family, HSPI 6.9 were identified in wheat using a modified version of DD [15]. The DD technique was successfully used for detecting transcriptome changes in durum wheat upon exposure to Cd and identified *NADH dehydrogenase subunit 1* and *PsaC* genes [16]. Several transcripts differentially induced by exogenous ABA

treatment were identified in bread wheat using DD profiling [17].

The present study aimed to investigate the genetic response to drought tolerance in bread wheat. The differentially expressed genes upon drought stress were profiled on a whole-genome scale using mRNA differential display technique in bread wheat (*Triticum aestivum* cv. Gerek 79). Here we report some expressed sequence tags (ESTs) that were found to be activated in response to drought stress in the bread wheat cultivar Gerek 79.

Materials and Methods

Plant materials and stress treatment

Gerek 79 (moderately drought resistant) wheat variety was obtained from the Republic of Türkiye Ministry of Agriculture and Forestry Transitional Zone Agricultural Research Institute, Eskişehir and used as plant material. Seeds were washed under running tap water and initially surface sterilized with 70% (v/v) ethanol for 1-2 min followed by treatment with 10% commercial bleach (sodium hypochlorate) containing a few drops of Tween-20 for 15 min. After that, the seeds were thoroughly washed with sterilized water at least six times. The sterilized seeds were then placed between wet sterile filter papers at room temperature for 3 days under dark conditions for germination. Seedlings were transferred to plastic pots with a top diameter of 18 cm and height of 20 cm containing soil/sand mixture (flower soil:garden soil:sand; 1.5:1.5:1). They were grown under white fluorescent light, 108- 135 μmol m^{-2} s^{-1} at 25ºC with 65% relative humidity by watering daily with tap water for 30 days until the pre-flowering stage. When they reached the pre-flowering stage, they were divided into two groups; one treated with drought-stress and one without drought stress as a control. Drought stress was given by completely withholding irrigation until they reached the desired relative water content (RWC) value. The RWC was measured in the daytime using the flag leaf and calculated as follows;

RWC (%) = [{(FW – DW)/ (TW – DW)}] ×100, where FW is the fresh weight of the leaves, TW is the weight at full turgor and DW is the dry weight of the leaves [18].

Plant leaf samples (control and drought-stressed) were harvested and immediately plunged in liquid nitrogen and stored at -80˚C for RNA isolation. Three replicates were performed for control and drought-stressed plants.

Total RNA isolation and DNase I digestion

Total RNA extraction from the collected leaf samples was performed using the hot phenol method [19]. Genomic DNA contamination was removed by digesting total RNA samples with 10 units of DNase I enzyme using the MessageClean Kit (GenHunter Corporation, Nashville, TN, USA) following the manufacturer's instructions. Digested samples were extracted with phenol:chloroform (3:1) and ethanol precipitated. RNA pellets were dissolved in DEPC- treated ddH_2O. The quantity of RNA samples was measured using a NanoDrop1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Extracted RNAs were stored at -80°C until use.

mRNA differential display analysis

Single-stranded cDNA synthesis was performed using RNAImage Kit (GenHunter Corporation, Nashville, TN, USA) according to the manufacturer's instructions. For each RNA sample, three reverse transcription reactions were performed with three different H-T11M primers (M= G, C or A). The template RNA (0.2 µg/reaction) was added to tubes containing 4 µl of 5X reverse transcription buffer, 1.6 µl of 25 µM dNTPs, 2 µl of2 µM H-T11M primer, and 9.4 µl of ddH_2O. Reactions were incubated at 65^{o}C for 5 min and 37^{o}C for 10 min. After that, 1 µl of MMLV reverse transcriptase (100 u/µl) was added to each tube and incubated at 37^{o}C for 20 min and 75^{o}C for 5 min. The reactions were stored on ice or -20^{o}C until use.

Differential display (DD) PCR amplification was performed on single-stranded cDNA samples using H-T11M primer (M= G, C or A) in combination with the arbitrary H-AP1-8 and H-AP17-24 primers from RNAimage Kits (GenHunter Corporation, Nashville, TN, USA) following the manufacturer's instructions. Briefly, one-tenth of single-stranded cDNAs were subjected to PCR in a 20 µl reaction mixture composed of 10 µl ddH_2O, 2 µl of 10X PCR Buffer (100 mM Tris.HCl, pH 8.4; 500 mM KCl; 15 mM $MgCl_2$ and 0.01% (w/v) gelatine), 1.6 µl of dNTPs (25 µM), 2 µl of H-AP primer (2 µM), 2 µl of H-T11M primer (2 µM, same one used in the RT-PCR), 0.2 µl of α-(^{33}P)dATP (2000 Ci/mmole) and 0.2 µl of Taq DNA polymerase (5 u/µl) under the following cycle conditions: 40 cycles of 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for amplification, followed by 72°C for 5 min for final extension. The reactions were stopped by adding 3.5 µl of stop solution (95% formamide; 10 mM EDTA, pH 8.0; 0.09% (w/v) xylene cyanol FF; 0.09% (w/v) bromophenol blue). Prior to loading,

PCR products were denatured at 80°C for 2 min and separated on 6% denaturing DNA sequencing gels. After electrophoresis, DNA gels were dried and exposed to X-ray film at -80°C for 2-3 days. The radioactively-labeled cDNA bands were visualized on X-ray films after exposure. Changes in cDNA bands (differential expression) between control and drought-stressed samples were detected for each set of primers. The autoradiograph was properly aligned with the gel and the differentially expressed bands were excised from the gel. Elution of cDNA fragments from gel slices was carried out with 100 µl ddH_2O by incubating at 100°C for 15 min. The gel debris was removed by centrifugation. The cDNA fragments were ethanol precipitated and then resuspended in 10 µl ddH_2O. 4 µl of cDNA fragments were reamplified by PCR with the same primer combinations, in the absence of radiolabeled nucleotides, under the same conditions used for the initial reaction. The reamplified products were electrophoresed on a 1.5% (w/v) agarose gel and visualized under a UV transilluminator.

Cloning, sequencing and homology analysis of differentially expressed cDNAs

The reamplified bands were directly subcloned into the PCR-TRAP vector using PCR-TRAP Cloning System (GenHunter Corporation, Nashville, TN, USA) as instructed. Colony-PCR was performed with Lgh and Rgh primers to verify the presence of inserts. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel. Plasmid DNAs were isolated from overnight bacterial cultures of positive clones using alkaline lysis method [20]. Sequencing of cloned inserts was performed on a CEQ8800 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA, USA) using vector-specific primers. Alignment of insert sequences was performed by BioEdit software [21]. The homology search was performed using the Basic Local Alignment Search Tool (BLAST) [22] provided by the National Centre of Biotechnology Information (NCBI) at National Institutes of Health (NIH) (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

In this study, it is aimed at identifying drought stress-related genes in wheat by using mRNA DD. The seedlings were grown as mentioned above until the pre-flowering stage. When they reached the pre-flowering stage they were divided into two groups; one group served as a control and the other group was subjected to drought-stress by withholding irrigation until plants reached 62.4% of RWC (Figure 1).

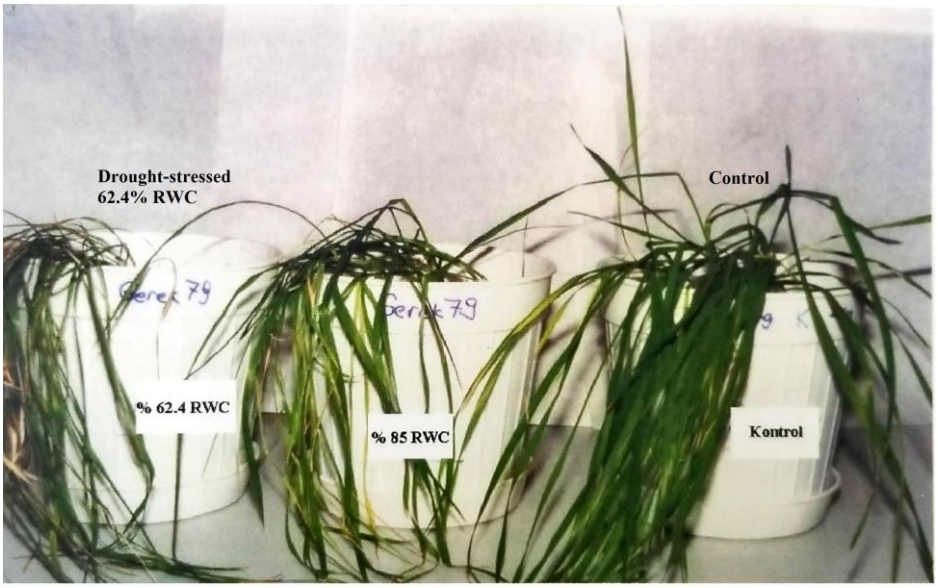

Fig 1 Control and drought-stressed (62.4% RWC) Gerek 79 plants

mRNA DD analysis was performed to analyze changes in gene expression between control and drought-stressed leaf samples and identify differentially expressed transcripts under drought conditions. In total, 48 different combinations of anchored and arbitrary primers were used in the mRNA DD analysis (Table 1).

A total of 20 bands were identified to be differentially expressed (unique and/or upregulated in stress condition) between control and stressed samples and excised from the gels. All of them were reamplified. The randomly selected 10 bands were cloned into the PCR-TRAP vector and sequenced. Eight of the cloned inserts were not sequenced well and subjected to similarity analysis. The nucleotide sequences of the remaining two differentially expressed cDNAs named G1 and G2 were subjected to similarity analysis. The reamplified G1 and G2 fragments with sizes of 324 and 268 bp, respectively separated on 1.5% agarose gel are shown in Figure 2. The DD-RT- PCR gel and DDRT-PCR autoradiographies showing G1 and G2 fragments are given in Figure 3 and Figure 4, respectively. In BLAST analysis, G1 and G2 showed a high degree of homology to the mRNA sequence of purple acid phosphatase and glycosyltransferase family 92 protein-like sequence of *Triticum aestivum* and some other plants, respectively.

Purple acid phosphatases (PAPs) belong to the metallophosphatase superfamily of proteins. They hydrolyze phosphate esters and anhydrides under optimal acidic

conditions [24]. Besides plants, PAPs have also been found in fungi, bacteria, unicellular eukaryotes and mammals [25].

Table 1 Primers used in mRNA differential display

Anchored Primers	
Primer	**Sequence (5′–3′)**
H-T11A	AAGCTTTTTTTTTTTA
H-T11G	AAGCTTTTTTTTTTG
H-T11C	AAGCTTTTTTTTTTC
Arbitrary Primers	
Primer	**Sequence (5′–3′)**
H-AP1	AAGCTTGATTGCC
H-AP2	AAGCTTCGACTGT
H-AP3	AAGCTTTGGTCAG
H-AP4	AAGCTTCTCAACG
H-AP5	AAGCTTAGTAGGC
H-AP6	AAGCTTGCACCAT
H-AP7	AAGCTTAACGAGG
H-AP8	AAGCTTTTACCGC
H-AP17	AAGCTTACCAGGT
H-AP18	AAGCTTAGAGGCA
H-AP19	AAGCTTATCGCTC
H-AP20	AAGCTTGTTGTGC
H-AP21	AAGCTTTCTCTGG
H-AP22	AAGCTTTTGATCC
H-AP23	AAGCTTGGCTATG
H-AP24	AAGCTTCACTAGC

PAPs exist as a multigene family in many plants such as Arabidopsis (29 *AtPAPs*), rice (26 *OsPAPs*) and tomato (25 *SlPAP*s) [26-28]. PAPs are mainly involved in Pi homeostasis but they also have roles in root growth modulation, carbon metabolism, symbiotic association, reproductive development and response to environmental stresses [29-35].

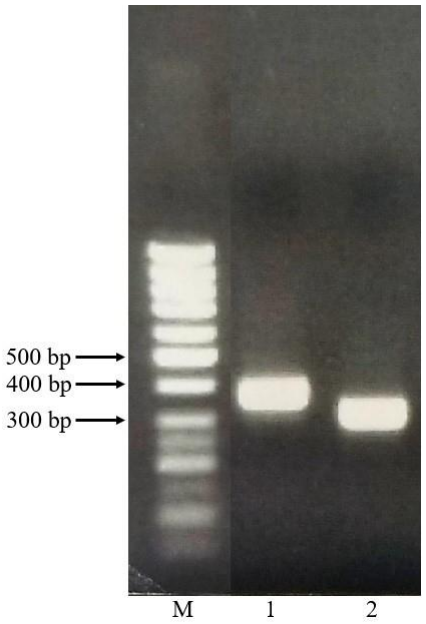

Fig 2 Agarose gel electrophoresis of reamplified G1 and G2 fragments M: GeneRuler 50 bp DNA Ladder (Thermo Fisher Scientific). Lane 1 and Lane 2 represent reamplified G1 and G2 fragments, respectively

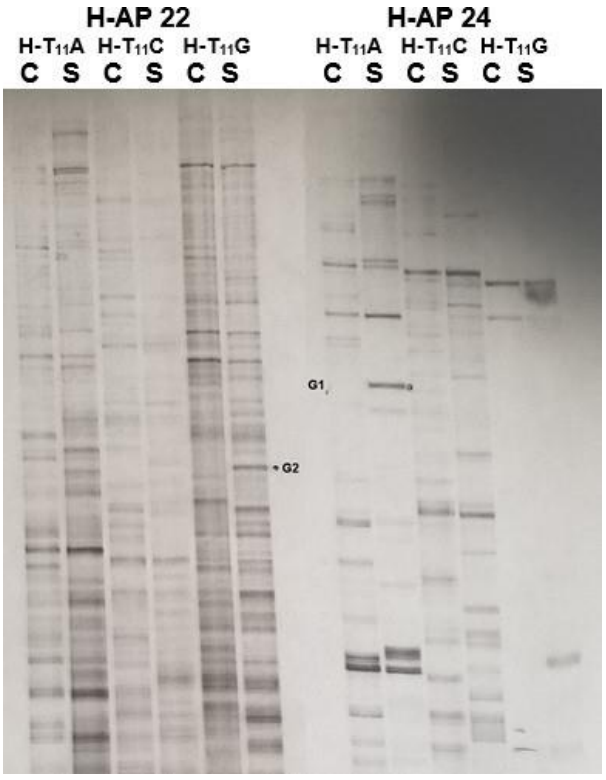

Fig 3 DD-RT-PCR gel showing G1 and G2 fragments C and S indicate cDNA band patterns from untreated and drought-stressed (62.4% RWC) plantsamples, respectively

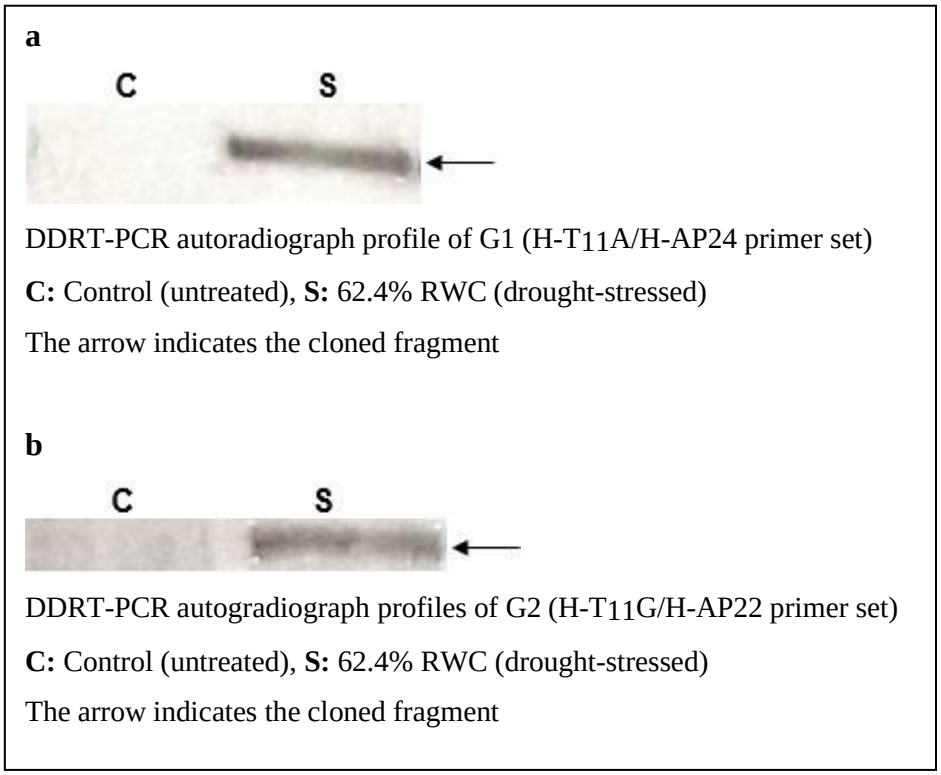

Fig 4 DDRT-PCR autogradiograph profiles of **(a)** G1 (H-T11A/H-AP24 primer set) and **(b)** G2 (H- T11G/H-AP22 primer set)

Although they are ubiquitously present in plants, a comprehensive dissection of their roles is still elusive. It was reported that the overexpressing AtPAP15 enhances salt and osmotic stress tolerance and reduces the phytate content in the leaves of Arabidopsis [34]. Recently, functional characterization of the AtPAP17 and AtPAP26 genes has shown their roles in salt tolerance [36]. It was shown that PgPAP18 gene, a heat-inducible novel purple acid phosphatase 18-like gene isolated from pearl millet, plays a defensive role against abiotic stresses such as heat and salt [37]. In another study, GmPAP3 gene from soybean has been shown to be induced by NaCl stress and oxidative stress [38]. As far as we know, the induction of the PAP gene upon dehydration in bread wheat has not been reported before. The reports showing plants PAPs' roles under environmental stress conditions and our findings suggest that the gene fragment corresponding to PAP could play an important role in wheat subjected to drought stress.

The glycosyltransferases (GTs; EC 2.4.x.y) catalyze the transfer of sugar moieties to acceptor molecules such as sugars, nucleic acids and lipids. Plants have an important

and functionally diverse family of GTs. Their major roles are the biosynthesis of cell wall polysaccharides and glycoproteins. They are also important for cellular homeostasis and detoxification of mycotoxins and xenobiotics [40].

The role of glycosyltransferase in thermotolerance in some cereals has been shown before. 1,704 candidate genes (CGs) identified by meta-QTL analysis in wheat were subjected to in silico expression analysis. The analysis results identified 182 differentially expressed genes, which included 36 CGs with known functions previously reported to be important for thermotolerance such as UDP-glucosyltransferase, zinc finger transcriptional factor and K homology domain etc. [41]. Dong et al. (2020) characterized GSA1, which encodes a UDP-glucosyltransferase and redirects the metabolic flux between branches of the phenylpropanoid pathway under abiotic stress in rice. This results in the accumulation of flavonoid glycosides, which protect rice against abiotic stress [42]. To our knowledge, there is no study showing the direct involvement of *Triticum aestivum* glycosyltransferase family 92 protein in drought stress response. Our findings suggest that the gene fragments corresponding to purple acid phosphatase and glycosyltransferase family 92 protein-like sequences could play an important role in plants subjected to drought stress. These results have laid the foundation for further investigation of these clones. The investigation of the differential expression of these putative genes under drought conditions at the RNA level is ongoing. Further characterization of these genes could be important in understanding the functions of these gene/s in drought response. They may be used as candidate genes in molecular breeding approaches to improve drought tolerance of wheat.

Conclusion

Understanding the genetic basis of drought tolerance in wheat is very important for genetic improvement through breeding. mRNA differential display was used to identify candidate drought responsive genes in bread wheat (*Triticum aestivum* cv. Gerek 79). It was found that two clones named G1 and G2 showed a high level of sequence homology to the purple acid phosphatase and glycosyltransferase family 92 protein-like sequences of *Triticum aestivum* and some other plants, respectively. These clones are likely to be related to drought stress responses. Further characterization of these genes could be important in understanding their function in drought response.

Abbreviations
BLAST: Basic local alignment search tool; DDRT-PCR: Differential display reverse transcriptase polymerase chain reaction; DW: Dry weight; EST: Expressed sequence tag; FW: Fresh weight; NIH: National Institutes of Health; RWC: Relative water content; TW: Turgor weight.

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Data availability statement
The author confirms that the data supporting the findings of this study are included within the article.

Compliance with ethical standards
Conflict of interest
The authors declare no conflict of interest.

Ethical standards
The study is proper with ethical standards.

Authors' contributions
In this study, the laboratory experiments were conducted by Diğdem Aktopraklıgil Aksu. Prof. Dr. Abdulrezzak Memon received funding from TUBITAK and supervised her work.

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