Research article

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Quantitative mRNA Expression Profiles of Germin-Like and Extensin-Like Proteins under Drought Stress in *Triticum aestivum*

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ABSTRACT

Drought stress can severaly damage plant growth and the most important factor in the reduction of wheat yield in cultivated areas. Development of new methodologies to improve wheat productivity and quality under drought conditions have a primary importance. Extensin-like (*TaExtLP*) and Germin-like Protein (*TaGLP*) transcripts were selected from our RNAseq data for their relation with defense mechanism. We aim to show the expression patterns of these genes in drought tolerant (Gerek 79 and Müfitbey) and non-tolerant *T. aestivum* (Atay) cultivars under drought stress conditions using qRT-PCR technique. Extensin is the most abundant proteins present in the cell wall of higher plants and has an important role in plant defense through strengthening the cell wall and preventing tissue damage. GLPs are involve in different biological processes; e.g., disease resistance and superoxide scavenging metabolism. We established different mRNA expression regulation of Extensin like and Germin-like mRNAs in root and leaf tissues of tolerant and non-tolerant *T. aestivum* cultivars under drought stress. We observed GLP transcript was significantly up-regulated (5 fold) in 4 h drought- stressed root tissues of tolerant cultivar Gerek and then decreased in 8 h. On the other hand, there was no dramatic difference in leaf tissue of each cultivar. Extensin-like gene up-regulation was approximately 6 and 3.5 fold in 4 h stressed root tissues of tolerant cultivars. In leaf tissues, different expression pattern was observed in tolerant and non-tolerant cultivars. Drought stress caused to up-regulation (4 fold) in 4 h stressed leaf tissues of tolerant cultivar. On the contrary, down-regulation (4 fold) was identified in non-tolerant stressed leaf tissues. These results suggest that overexpression of Extensin-like gene under drought stress conditions may enhance drought tolerance. The qRT-PCR results from root and leaf tissues from 3 different cultivars were in agreement with our previous RNAseq data. This is the first report shows the expression profiles of these defense proteins under drought stress conditions in *T. aestivum*.

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Introduction

Bread wheat, *Triticum aestivum* L. is one of the main fundamental crops for many countries including Turkey. Drought is the major factor affecting wheat yields throughout the world however; it is more problematic factor for wheat agriculture in arid regions

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including central and eastern Anatolian regions of Turkey. Especially in central Turkey, yield losses could reach up to 80% in some years (http://www.turkstat.gov.tr). Therefore, development of new methodologies to improve wheat productivity and quality under drought conditions has a primary importance. Improvement in wheat breeding for drought tolerance is difficult because of the complexity of quantifying and measuring drought traits.

In higher plants, drought stress causes physiological changes, including loss of turgor, reduced leaf water potential and osmotic adjustment [1, 2]. Turgor pressure is a crucial factor for cell growth regulation. The management of cell enlargement depends on the cell wall extensibility [3, 4]. Water stress causes a low turgor pressure and then leads regression of growth by reducing cell extensibility and cell expansion [5]. From our previous studies, drought-stress related genes were identified in *Triticum aestivum* (bread wheat) cultivars under drought conditions by RNASeq technology (Illumina HiSeq2000) [6]. The selection of most promising drought stress tolerant and sensitive genotypes was performed by slow drought treatment experiments with three biological replications for 12 bread wheat cultivars. Three of them were selected as drought tolerant (Gerek 79 and Müfitbey) and non-tolerant (Atay-85) bread wheat cultivars based on the results of the physiological and biochemical analyses [6]. From RNAseq data, we selected differently expressed Extensin-like protein (*TaExtLP*) and Germin-like (*TaGLP*) proteins important for the defense mechanism in biotic stress [7, 8, 9]. In this report, we aimed to investigate mRNA expression profiles of these genes in the root and leaf tissues of different *T. aestivum* cultivars under drought stress conditions. As a member of the family of Hyp-rich glycoproteins (HRGPs), extensin is the most abundant protein group present in the cell wall of higher plants and regulated developmentally in a tissue-specific [7, 8]. They play an important role in plant defense through strengthening the cell wall, preventing tissue damage, enabling attachment of symbiotic organisms or limiting the pathogen invasion and propagation. Germins and GLPs were firstly reported in wheat as a specific marker for the start of germination [9]. Dwarfism induction, cell morphology changes and disease increase (sheath blight and blast fungal) were observed in GLP1 downregulated in transgenic *O. sativa* [10].

We aim to illuminate the mRNA expression profiles of Extensin-like and Germin-like proteins under shock dehydration stress conditions in showing different drought tolerance *T. aestivum* cultivars. QRT-PCR analyses were performed for these defense proteins in the root and leaf tissues under normal and two different dehydration stress conditions.

Materials and Methods

Growth conditions

Three *T. aestivum* cultivars Gerek 79 and Müfitbey (drought-tolerant) and Atay 85 (non-tolerant) were used in this study. Seeds were surface sterilized with 70% alcohol and 30% sodium hypochlorite and pre-germinated in Petri dishes for 10 days at 4°C in the dark. Seedlings were transferred to 10 L plastic pots containing moistened perlite after the germination and grown in a plant growth room under 16/8 h; temperature 22-18°C; relative humidity 60%. Seedlings that at the same developmental stage were moved to hydroponic (continuously aerated ½ Hoagland's solution) culture, renewed every 3 days, and grown under controlled conditions in the plant growth room. Plants were removed from the hydroponic culture at the age of four leaf stage and treated dehydration shock stress for 4 h and 8 h under the same temperature and light conditions. From 3 wheat varieties (2 drought tolerant and 1 non-tolerant) root and leaf tissues were harvested and frozen with corresponding controls and stored -80°C until RNA isolation [11].

RNA extraction and cDNA synthesis

Total RNA extraction was performed by using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) from 3 different *T. aestivum* cultivars according to the manufacturer's instructions. Extracted high-purity total RNAs from 4 h and 8 h drought stressed and corresponding control root and leaf tissues were measured by nanodrop (Thermo). RNA samples were treated with DNase I (10 U) (Roche, GmbH, Germany) for removing residual genomic DNA and purified following to the method described previously [12]. The integrity of RNA samples was controlled by running on 1% formaldehyde agarose gel and first-strand cDNA synthesis was performed to the manufacturer's (Roche High Fidelity cDNA synthesis kit) instructions in a 20 µl final volume containing 5 µg total RNA, 200 U MMLV RTase, 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, and 20 U RNase inhibitor [12].

Quantitative real-time PCR

qRT-PCR reactions contained 75–200 ng of the cDNA as a template, 10 pmole of each primer, 12.5 µl SYBR Green (Roche FastStart Universal SYBR Green Master, Rox) in a total volume of 25 µl. QRT-PCR experiments were carried out in 96 well polypropylene plates and performed in triplicate for each sample with IQ5 System (BioRad, Hercules, USA). The following standard thermal profile was employed: After 95°C for 5 min for polymerase activation, amplification and quantification cycles (45 times) 95°C for 30 s, 55°C for 1 min. Melting-curve analysis was carried out for the specificity of the primer pairs after 45 amplification cycles (55-95°C). Housekeeping gene β-actin was used (AY663392) as an internal control. The following primer pairs (Table 1) were designed to amplify a 115-bp *TaExtLP* fragment and a 102 bp *TaGLP* 9.1 fragment. All the primers used in qRT-PCR experiments were designed by Primer 3 program. Three technical replicates were carried out in order to quantify transcript level accurately. The ΔΔCq values for all the transcripts were averaged across all the treatments and experimental replicates. Student's t-test (GraphPad Prism 6) was applied to check for the statistical significance between drought-treated and –untreated control groups.

Table 1 QRT-PCR Primer list

Primer Name	**Sequence 5'—3'**	**Product Size**
β-Actin F	GACAA TGGAACCGGAATGGTC	110 bp
β-Actin R	GTGTGATGCCAGATTTTCTCCATg	
***TaExtLP* F**	AACCAGGGAAAACACAT CTT	115bp
***TaExtLP* R**	GGCAACAACAACAACAACTA	
***TaGLP* 9.1 F**	CACCAG GGATCACTAGACTA	102bp
***TaGLP* 9.1 R**	TGTCCGGAA ATCATGAAACT	

Results

From our RNAseq data, we found Germin-like protein 9-1 and Extensin-like protein gene expressions were differentially expressed in root and shoot tissues [6]. The abundance of *TaExtLP* and *TaGLP* 9-1 mRNA under 4 h and 8 h shock drought stress treatment was examined in root and leave tissues of drought tolerant and non-tolerant *T. aestivum* genotypes (Fig. 1).

Fig 1 Drought non-tolerant Atay85, Drought tolerant Müfitbey, and Gerek 79 *T. aestivum* cultivars

After total RNA extraction from root and leaf tissues, removal of genomic DNA was carried out. Semi-quantitative RT-PCR method was used to control the synthesized cDNAs (Fig 2).

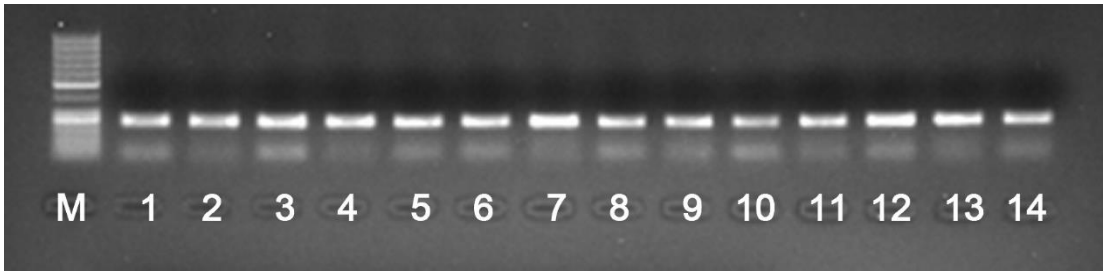

Fig 2 Agarose gel electrophoresis of amplified first-strand cDNAs by semi-qRT-PCR with housekeeping β-actin gene primers (M: GeneRuler 100bp DNA Ladder, MBI Fermentas)

Extensin-like protein (Arabidopsis, cell wall extensin) mRNA Expression: Extensin-like protein is the most abundant protein group present in the cell wall of higher plants

[7, 13]. Extensin expression in response to wounding, pathogen infection and ethylene treatment supports for the role of extensins in plant defense [14, 15, 16, 17, 8]. In our qRT-PCR experiments, it was observed that drought stress caused the up-regulation of this gene in root tissues. Maximum mRNA expression was observed in 4 h drought-stressed root tissues of tolerant and non-tolerant genotypes (Fig 3A, B, and C) .

In leaf tissues, *TaExtLP* mRNA was dramatically increased under 4 h drought-stressed Müfitbey.

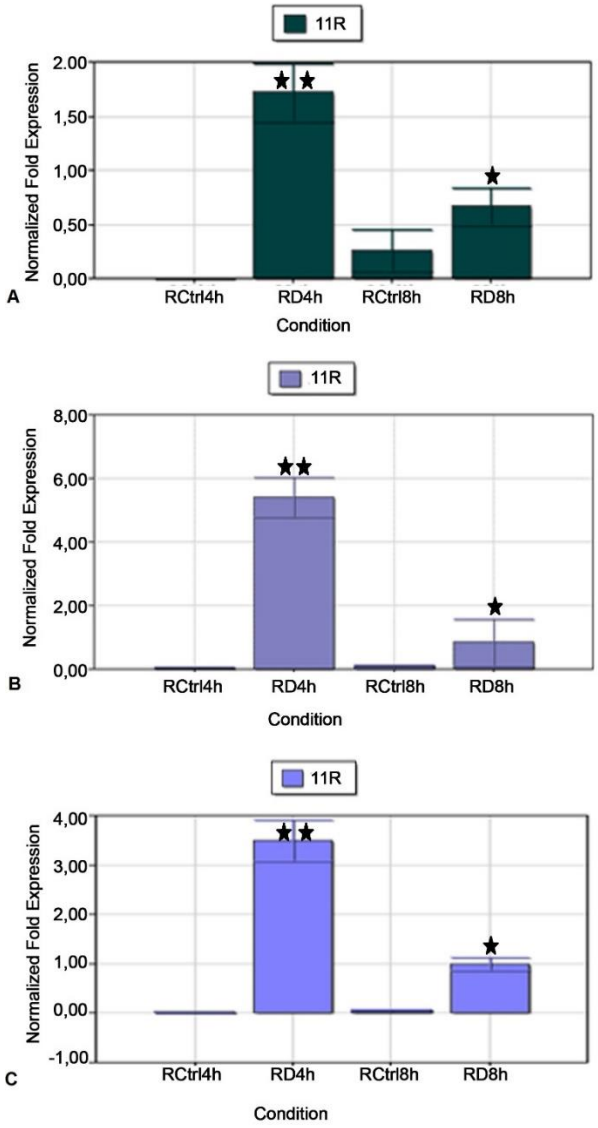

Fig 3 mRNA expression pattern of *TaExtLP* (cell wall) in 4 h and 8 h drought stressed root tissue of drought non-tolerant genotype Atay 85 (A), and Gerek (B), Müfitbey (C) cultivars. **RCtrl:** Root Control 4 h, **RD4h:** Root Drought 4 h. **RCtrl8h:** Root Control 8 h, **RD8h:** Root Drought 8 h. Error bars indicate the standard deviation of qRT-PCR each performed in triplicate. (*): $p \leq 0.05$, (**): $p \leq 0.01$.

On the other hand, Ext-like protein mRNA transcript was not much changed compared to the control tissue in 4 h drought stressed leaf tissues in non-tolerant genotype Atay. After 8 h of drought, mRNA expression level was not changed in leaf tissues (Fig 3).

Conversely, the different expression pattern was observed in leaf tissue of tolerant and non-tolerant genotypes. In non-tolerant genotype Atay 85, maximum mRNA was observed in 4 h drought stress treated leaf tissue and there was no significant difference in 8 h drought stress (Fig 4A). Whereas in the same tissue, down-regulation of this gene was shown in tolerant genotype Müfitbey (Fig 4B) and Gerek (data not shown).

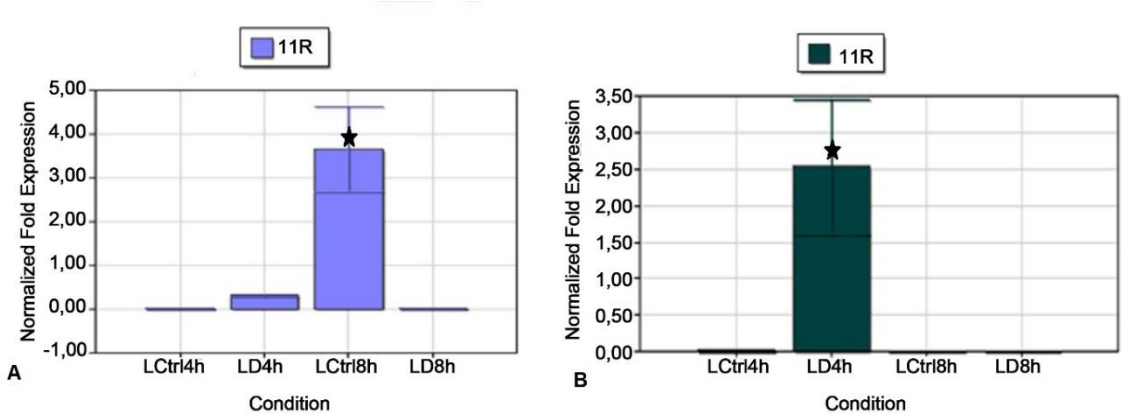

Fig 4 mRNA expression pattern of *TaExtLP* (cell wall) in 4 h and 8 h drought stressed leaf tissue of drought non-tolerant genotype Atay 85 (A), and non-tolerant Müfitbey (B) cultivars. **LCtrl4h:** Leaf Control 4 h, **LD4h:** Leaf Drought 4h, **LC8h:** Leaf Control 8h, **LD8h:** Leaf Drought 8h. Error bars indicate the standard deviation of qRT-PCR each performed in triplicate. (*): $p \leq 0.05$.

Germin-like proteins (GLPs) have been shown to implicate as plant cell defenders in many species to different conditions and diseases [19]. In root tissue, *GLP9-1* mRNA expression was induced by 4 h and 8 h drought stress (Fig 5A, B, and C). In the leaf tissue of non-tolerant genotype, there was no dramatic difference between control and drought stress. On the contrary, up-regulation was obtained in 4 h and 8 h drought stressed leaf tissues of tolerant genotype Müfitbey (Fig 6 B).

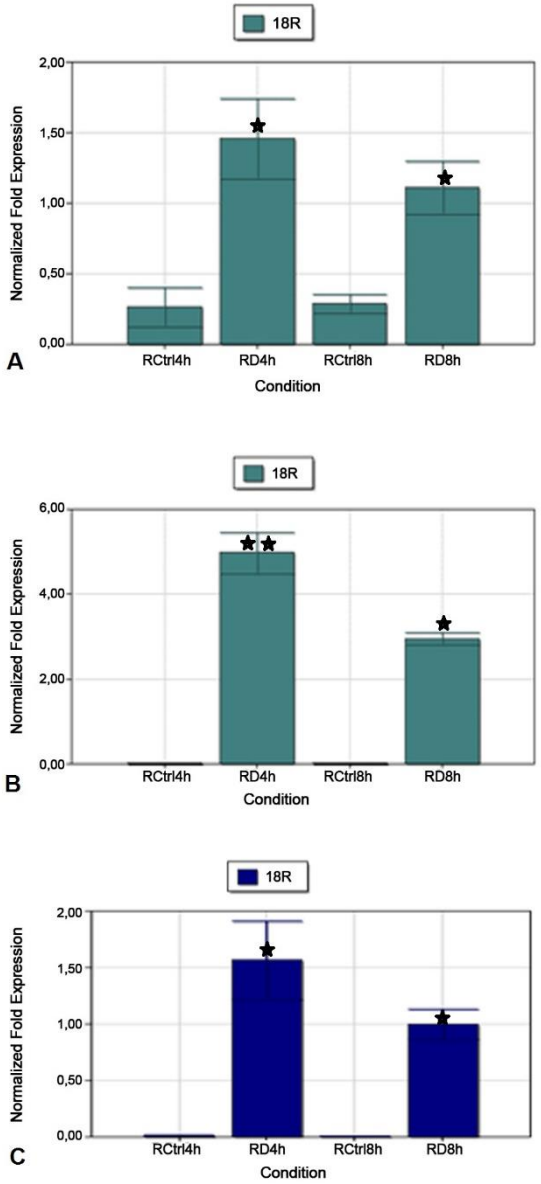

Fig 5 mRNA expression profile of *GLP9-1* in 4 h and 8 h drought stressed root tissue of drought non-tolerant genotype Atay 85 (A), and Gerek (B), Müfitbey (C) cultivars. **RCtrl:** Root Control 4h, **RD4h:** Root Drought 4h. **RCtrl8h:** Root Control 8h, **RD8h:** Root Drought 8h. Error bars indicate the standard deviation of qRT-PCR each performed in triplicate. (*): $p \leq 0.05$, (**): $p \leq 0.01$.

In root tissue, *GLP9-1* mRNA expression was induced by 4 h and 8 h drought stress (Fig 5A, B, and C). In the leaf tissue of non-tolerant genotype, there was no significant difference between control and drought stress. On the contrary, up-regulation was observed in 4 h and 8 h drought stressed leaf tissues of tolerant genotypes Müfitbey (Fig 6 B) and Gerek (data not shown).

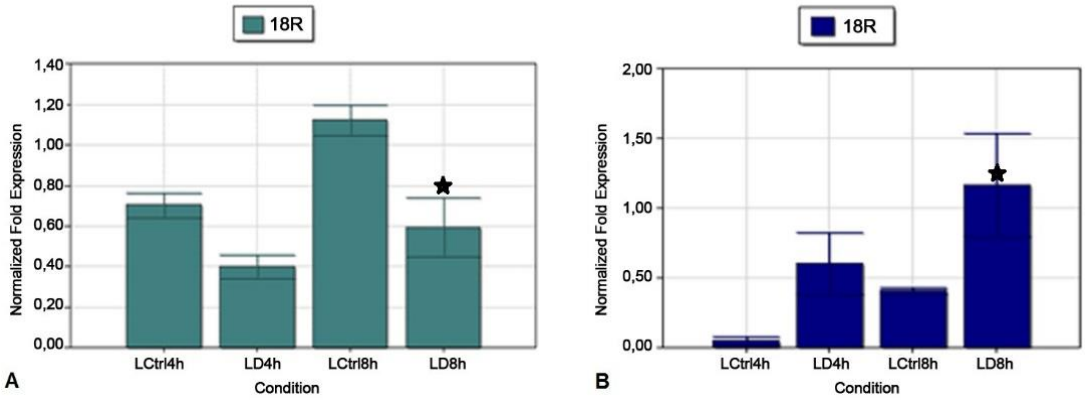

Fig 6 mRNA expression profile of *GLP9-1* in 4 h and 8 h drought stressed leaf tissue of drought non-tolerant genotype Atay 85 (A), and non-tolerant Müfitbey **(B)** cultivars. **LCtrl4h:** Leaf Control 4 h, **LD4h:** Leaf Drought 4 h, **LC8h:** Leaf Control 8h, **LD8h:** Leaf Drought 8h. Error bars indicate the standard deviation of qRT-PCR each performed in triplicate. (*): p ≤0.05.

Discussion

Extensin-like and Germin-like-genes are expressed in an organ-specific fashion and stress-related proteins in plants. Extensins are implicated in different biological processes such as embryonic development [20], root hair growth [21, 22], cell wall assembly and structure [23, 24], and biotic and abiotic stress responses [18, 25, 26]. Increased extensin accumulation and extensin cross-linking has been suggested to help in wound recovery and in the formation of a physical barrier against pathogens, thus avoiding the entry of pathogens into the vascular system [27]. In higher plants, the immune response can differ between leaf and root tissues [28, 29]. RNAseq data from *A. thaliana* showed that a significant number of genes (2424 genes) were differentially expressed between shoots and roots under normal conditions. Root-overexpression of at least nine encoded extensin proteins suggested specific roles of these glycoproteins in root tissues [30]. Glycine max extensin gene, *SbHRGP*3, expression has been shown in hypocotyl and the roots of seedlings [13]. In tomato, *LeExt*1, an Extensin-like protein expression was observed with tip growth, which proposes a role of the *LeExt*1 protein in root hair expansion. Comparative transcript analysis of *LeExt*1/GUS chimeric gene in four different transgenic plant species has proven its role in the regulation of apical/basal polarity in root tissues of transgenic tomato [31]. As a cell wall protein, extensins are also released into the root mucilage. Recently, Castilleux et al [32] reported a model about the influence of cell wall extensin on root secretions.

Up-regulation of cell wall modification related proteins lead to changes in the cell wall composition [33]. Different Expansin (*Exp*) and Extensin (*Xth*) mRNA gene expression profiles were reported at low temperatures in Arabidopsis and rice [34]. Extensin (*Xth*) down-regulation was observed to cold stress in Arabidopsis [35]. Freezing tolerance was improved by the overexpression of *AtXTH*21in transgenic Arabidopsis plants [35].
The Extensin-like gene identified from our RNAseq data was significantly up-regulated (about 4 fold) in response to 4 h and 8 h dehydration stress [6]. In this study, Extensin-like gene up-regulation was approximately 6 and 3.5 fold in 4 h stressed root tissues Gerek and Müfitbey cultivars. In leaf tissues in tolerant and non-tolerant cultivars, the different expression pattern was observed. Drought stress caused to up-regulation (4 fold) in 4 h stressed leaf tissues of Müfitbey. On the contrary, down-regulation (4 fold) was identified in Atay 4 h stressed leaf tissues. These results suggest that Extensin-like gene may have a role in drought tolerance.

Germin-like protein 9-1 (Oryza sativa subsp. japonica – apoplast, manganese ion binding, nutrient reservoir activity): Germins and GLPs were firstly reported in wheat as a specific marker for the start of germination [36]. Different enzymatic activities of six germin subfamilies (GER1-6) were identified with e.g. OXO activity in GER1 and SOD activity in GER2 [10]. The GER1 subfamily has also been reported to be involved in early plant development and germination [37, 38]. Dwarfism induction, cell morphology changes and disease increase (sheath blight and blast fungal) were observed in GLP1 down-regulated in transgenic rice plants [19]. GLPs have been studied in different plant species and implicated as plant cell defenders to biotic and abiotic stress conditions. GLPs have been reported to be resistant to proteases, extreme pH, heat, and sodium dodecyl sulphate [39].
GLP expression in *H. vulgarae* and a QTL on chromosome 8 of *O. sativa* have shown their involvement in disease resistance and complex trait of GLPs in cereal genomes [40, 41]. In our previous studies, we reported differential expression of GLP-like mRNA in ABA- treated wheat by DD mRNA experiments. In ABA-dependent pathway, GLP1 mRNA expression in ABA-treated plants rapidly increased in 1 h and maximal expression level was obtained in 8 h [40]. In the present study, we observed GLP transcript was significantly up-regulated (5 fold) in 4 h drought- stressed root tissues of tolerant cultivar

Gerek and then decreased in 8 h. On the other hand, there was no dramatic difference in leaf tissue of tolerant and non-tolerant cultivars. The qRTPCR results from root and leaf tissues from 3 different cultivars were in agreement with our RNAseq data.

Conclusion

The identification and elucidation of functional characteristics of the genes that play a role in the complex drought-response in wheat will be helpful for making the popular wheat varieties more productive with less amount of water. It is very significant to learn more about stress related genes for the elucidation of stress mechanism by transgenic plants. Although there are many studies about the Ext and GLP genes, the functions of these genes are still elusive. Downregulation or overexpression of Ext and GLP through gene editing methodology may shed more light on the functions of these genes in the future.

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

Abbreviations
qRT-PCR: quantitative real time PCR, *TaGLP*: Germin-Like Protein of *T. aestivum, TaExtLP*: Extensin-like Protein of *T. aestivum*, RNAseq: RNA sequencing

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