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Expression analysis of lipid transfer protein gene in wheat (*Triticum aestivum*) under drought stress

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Abstract

Drought stress causes changes in morphology, physiology and gene expression profile in the plants. One of the ways to respond to this stress is to change the synthesis of specific polypeptides such as LTP (Lipid transfer proteins). For this purpose, *LTP* expression level was investigated under three osmotic potentials of -2, -4 and -6 bar in combination with different time courses of 0, 3, 6, 10, 24, 48 and 72 h after applying stress using RT-qPCR in DN-11 and Marvdasht genotypes. Also, the soluble sugar content in both genotypes was measured by the phenol-sulfuric acid method after each stress level. Furthermore, promoter analysis of *LTP* was studied using bioinformatics tools. The results showed that the highest expression level of *LTP* in both genotypes occurred at -6 bar osmotic potential level and 48 h after stress in DN-11 and 72 h after stress in Marvdasht. There was no significant difference between 48 h and 72 h after stress in the DN-11 genotype and between 72 h and 3 h after stress in Marvdasht genotype at P-value of ≤ 0.01, but there was a significant difference among other time courses at P-value of ≤ 0.01. Besides, the soluble sugar content increased with increasing stress levels in both genotypes, so that its amount was higher than control at -6 bar stress level. The promoter analysis showed that several domains and motifs in the *LTP* promoter region are activated in response to drought stress and increase its expression. Therefore, it can be concluded that *LTP* gene can be used as a drought resistance gene in gene transformation and genetic engineering programs.

***Key words*:** Drought stress, Gene expression analysis, *LTP*, RT-qPCR, *Triticum aestivum*.

INTRODUCTION

Plants respond to environmental changes through changes in metabolic pathways, physiological reactions, and growth and development. One of the ways to respond is to change the synthesis of certain polypeptides including LTPs (Lipid Transfer Proteins), which are synthesized in response to drought, salinity and temperature stresses (Key *et al.*, 1981; Heikkila *et al.*, 1984; Ramagopal, 1987). Plant LTPs are small (6.5-10.5 kD) and secretory molecules, mainly found in the epidermis tissue of the aerial parts of plants (Jung *et al.*, 2005). A study showed that LTP expression in young tissues is higher than old tissues in tobacco so that LTP is over-expressed in areas with the highest growth (Fleming *et al.*, 1992). LTPs have several roles in plants (Wang *et al.*, 2009), which has led many researchers to study them. LTPs are involved in various abiotic stresses, as their expression changes under these stresses (Jang *et al.*, 2002). Drought stress is the most common environmental stress and has several impacts on plant productivity (Wang *et al.*, 2009). Therefore, it is necessary to investigate the expression of the genes involved in drought stress, such as *LTP* genes, for

protecting the plants. Various studies have identified the genetic isoforms of LTP proteins (Boutrot *et al.*, 2008; Wang *et al.*, 2008) and described the structure of these proteins (Pacios *et al.*, 2012). However, functional studies are important for determining the differential expression of these genes using specific expression techniques such as Real-time quantitative-polymerase chain reaction (Moraes *et al.*, 2015).

Wheat is a strategic crop, so that it provides human nutrition needs. Since climate changes has led to the emergence of drought stress, it is necessary to investigate the changes in drought stress related genes such as *LTP* expression under drought stress. In a study, *LTP1*, *LTP2*, *LTP3*, and *LTP1500* were identified in wheat and the high levels of *LTP* expression in the tissue layers between the vascular bundles at the apex of the plant indicate the role of this gene in adapting to the drought stress (Jang *et al.*, 2005). In addition to drought stress, the factors such as salicylic acid, ethephon, hydrogen peroxide and wounding can increase the *LTP3* expression in wheat (Jang *et al.*, 2005).

The drought stress is one of the most important environmental stress and occurs due to low rainfall, salinity, high and low temperature, and high intensity of light (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Therefore, according to climate changes in world, the selection of a genotype or production of a genetically modified crop will be necessary to deal with the damages caused by drought stress in plants. Hence, in this study, *LTP* expression level and soluble sugar content were investigated at different osmotic potentials in two genotypes of resistant and sensitive wheat, including DN-11 and Marvdasht, respectively. In addition, the structural features of *LTP* were investigated using bioinformatics tools.

MATERIALS AND METHODS

Plant materials and stress application

In this study, two genotypes of wheat designated DN-11 (tolerant) and Marvdasht (sensitive) were used. The seeds were obtained from Zarghan Agricultural-Jahad Research Center.

Fungicide-impregnated seeds were dipped in dishwashing liquid for 5 min and were rinsed 3 times with sterile water. After germination in Petri dishes, they were placed in a mixture of coco-peat and perlite (1:1) containing half-concentration of Hoagland medium (pH=6.5) (Kerepesi and Galiba, 2000) in conditions of 16 h light/ 8 h dark at 25 °C.

In order to apply drought stress, thirteen-day–old seedlings were used for four levels of polyethylene glycol (PEG6000) with osmotic potentials including 0 (as a control), -2, -4 and -6 bar. The leaf samples were harvested at 3, 6, 10, 24, 48 and 72 h after applying the drought stress, and then used for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA extraction was carried out using the RNX-Plus™ kit of Sinagene Co. (S-1020-1) according to the manufacturer's instructions. RNA samples were treated with DNaseI to remove any DNA contamination. The RNA concentration was measured using Nano-drop (Thermo Scientific Co.) and the RNA quality was evaluated by electrophoresis. Afterwards, the synthesis of cDNA was performed using the Frist Strand cDNA Synthesis kit of Thermo Scientific Co. (K1622).

Gene expression quantitation using RT-qPCR

Gene expression profiles of *LTP* in response to drought stress were analyzed using Real-time PCR (Bio-Rad, Hercules, CA) with *LTP* specific primers (Table 1). Specific primers of *LTP*, *18S rRNA*, and *ef1α* were designed by Vector NTI and AlleleID software and then primer blast was conducted using the NCBI database. The synthesized cDNA was diluted five times with sterile water and used as the template for Real-time PCR. The obtained data were analyzed using a thermocycler Line GeneK with the software Line GeneK Fluorescent Quantitative Detection system (BIOER Technology, Hangzhou, China). The mean of Ct values of two housekeeping genes of *18S rRNA* and *ef1α* was used as the internal control. The

Table 1. Primers used in the Real-time PCR.

Gene	NCBI accession number	Sequences (5' to 3')	Annealing temp. (°C)
LTP	DQ286560.1	F: TCCTCACAGCCACAGACG	60
		R: AGCCCACCAGCAGCACTC	60
18S rRNA	AY049040	F: CGCTCCTACCGATTGAATGG	56.7
		R: CCTTGTTACGACTTCTGCTTCC	56.7
ef1α	M90077	F: GCCACACCTCGCACATTG	55.2
		R: GCCAGCATCACCATTCTTG	55.2

relative expression level was calculated using the ΔΔCt method.

Measuring the soluble sugars content

The soluble sugars were extracted and measured at 490 nm according to the phenol-sulfuric acid method (Buysse and Merckx, 1993). The different concentrations of glucose were used to create the standard curve.

Bioinformatics analyzes

Nucleotide sequence of *LTP* was retrieved from the NCBI database (ID: DQ286560.1). The coding region of *LTP* was recognized using ORF Finder tool software. The promoter sequence of *LTP* was recognized using the Phytozome database and analyzed using Plantcare database. Finally, the domains in the coding region of *LTP* were recognized using Pfam, Smart and InterproScan sequence search databases. In addition, the protein sequence of *LTP* was retrieved from NCBI database (ID: ABB90545.1) and then the model was generated using Swiss-model server. The model was compared with the LTP protein of rice (PDB ID:1BV2) and maize (PDB ID:1FK5), and then the identity percentage was calculated using UCFS Chimera 1.14 software.

Statistical analysis

All experiments were carried out in three separate experiments and each with 10 repeats for expression analysis. Analysis of variance of factorial experiment based on completely randomized design was carried out using GLM procedure at a P-value of ≤ 0.01 in the SAS 9.0 software. Treatments were grouped based on multiple Duncan test.

RESULTS

Expression pattern of *LTP* in response to drought stress

The expression profile of *LTP* in leaves in response to stress in two genotypes of wheat was evaluated using Real-time PCR. The results showed that generally, *LTP* expression level in drought-tolerant cultivar was higher than drought-sensitive cultivar (Figure 1).

In DN-11 genotype, the highest and lowest expression levels were obtained in the treatments of 48 h with osmotic potential of -6, and 6 h with osmotic potential of -6 in comparison with control, respectively (Figure 1). Also, in Marvdasht genotype, the highest and lowest expression levels were obtained in the treatments of 72 h with osmotic potential of -6, and 6 h with osmotic potential of -6 in comparison with control, respectively (Figure 1).

Quantifying *LTP* expression level under different osmotic potential levels

In the early hours of -2 bar stress level, the increase in the expression of *LTP* in the DN-11 genotype occurred earlier than the Marvdasht genotype. Although the amount of gene expression in Marvdasht genotype

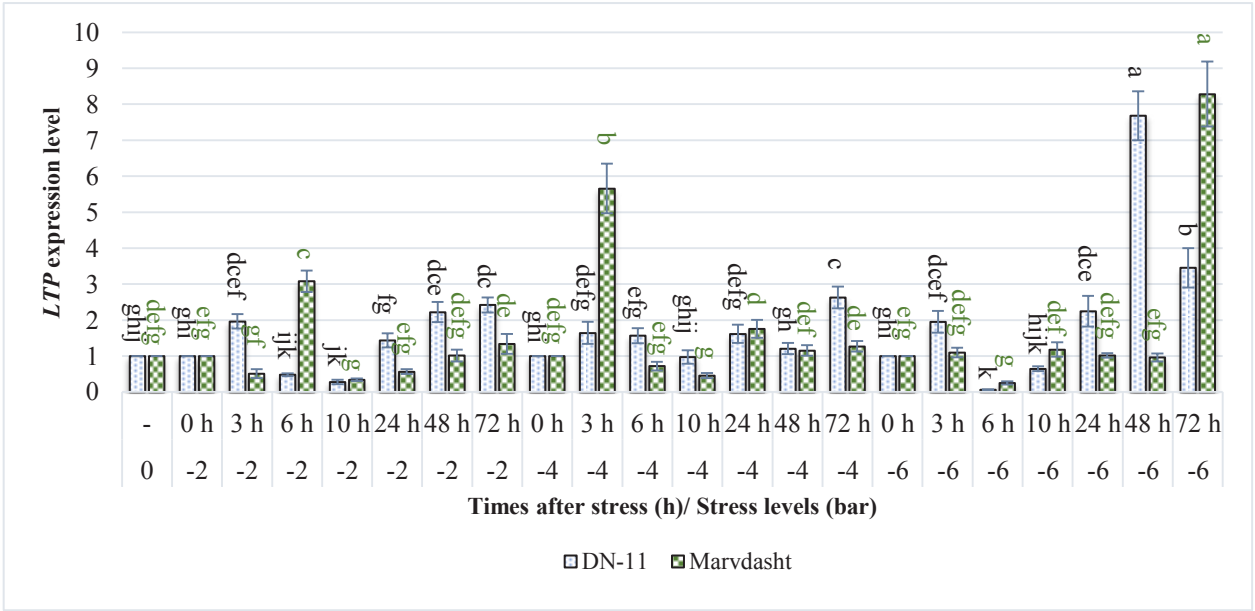

Figure 1. The investigation of *LTP* expression level in DN-11 and Marvdasht genotypes. The mean comparison revealed significant difference at P-value of ≤0.01 between two genotypes. The numbers of 0-72 h and -2- -6 bar indicate the time of stress and stress level, respectively.

Table 2. The variance analysis of expression level in Marvdasht genotype at P-value of ≤0.01.

Source of changes	df	Sum of squares	Mean of square	F-value
Stress level (a)	2	26.24	13.12	18.73**
Time after stress (b)	6	119	19.83	28.31**
a*b	12	351.49	29.29	41.81**
Error	180	126.09	0.7	

**: Significantly at P-value ≤1%.

Table 3. The variance analysis of expression level in DN-11 genotype at P-value of ≤0.01.

Source of changes	df	Sum of squares	Mean of square	F-value
Stress level (a)	2	19.04	9.52	15.88**
Time after stress (b)	6	160.71	26.78	44.68**
a*b	12	143.28	11.94	19.92**
Error	165	98.91	0.6	

**: Significantly at P-value ≤1%.

was higher than the DN-11 genotype 6 h after stress, DN-11 genotype responded earlier to the stress. It can be concluded that DN-11 adapted more quickly to the stress (Figure 1).

In -4 bar stress level, *LTP* expression in DN-11 genotype was induced 2-fold higher at 72 h after stress, compared to the control, while it had a slight increase in expression at other times (Figure 1). However, the highest expression level in *LTP* has been reported upon the longest stress time (72 h) by other studies (Jang *et al.*, 2004; Jang *et al.*, 2005; Choi *et al.*, 2008; Guo *et al.*, 2013). *LTP* expression increased in Marvdasht genotype 3 h after stress induction, but decreased rapidly at other times after stress (Figure 1). Also, the increase in the expression of *LTP* in DN-11 genotype started with a delay compared to Marvdasht genotype. It is likely that DN-11 is more tolerant to drought stress than Marvdasht genotype and or is able to respond more strongly to drought stress by increasing the expression of *LTP* under the influence of other factors such as MYB and MYC transcription factors or ABA hormone (Jang *et al.*, 2004).

Regarding -6 bar stress level, which is a severe stress, the level of *LTP* expression increased in DN-11 genotype 3 h after stress induction, but it decreased again 6 h after stress. The expression level increased to 7-fold in comparison to the control, 48 h after stress induction, which is similar to the results of previous studies (Jang *et al.*, 2004; Jang *et al.*, 2005; Choi *et al.*, 2008; Guo *et al.*, 2013). Although expression level decreased 72 h after stress induction, it was still high, compared to the control (Figure 1). In Marvdasht genotype, the level of *LTP* expression did not change much compared to the control. However, it decreased 6 h after stress induction and increased severely 72 h after stress induction.

Soluble sugar content analysis

The increasing amount of soluble sugar in different levels of stress in two genotypes showed that there was a direct correlation between the level of stress and the amount of soluble sugar (Figure 2). There was a significant difference between the osmotic potential of -6 bar and others in both genotypes. Generally, the soluble sugar level remained high in control, but this level was reduced at the osmotic potential of -2 bar and then increased again to make the plant compatible with stress conditions.

Bioinformatics analysis

The sequence of LTP was investigated on the NCBI database and characterized by the ORF Finder tool, which included a coding region from nucleotide 63 to 410 (348 bp), encoding a protein containing 115 residues (data no shown). Also, the full sequence of *LTP* searched on the Phytozome database, was found to be 1500 bp. According to Jang *et al.* (2004), *TaLTP1* has no intron in comparison to its cDNA and its genomic DNA sequence (Jang, Lee *et al.,* 2004). Promoter sequence analysis of *TaLTP1* 2856 bp in the upstream region of the transcriptional initiation revealed that the *Cis* elements located in the 337 bp segment of the promoter may control the transcription of *TaLTP1* during drought and salinity stresses in wheat. This segment contains five sequences of MYC-like and one sequence of MYB-like. The product of

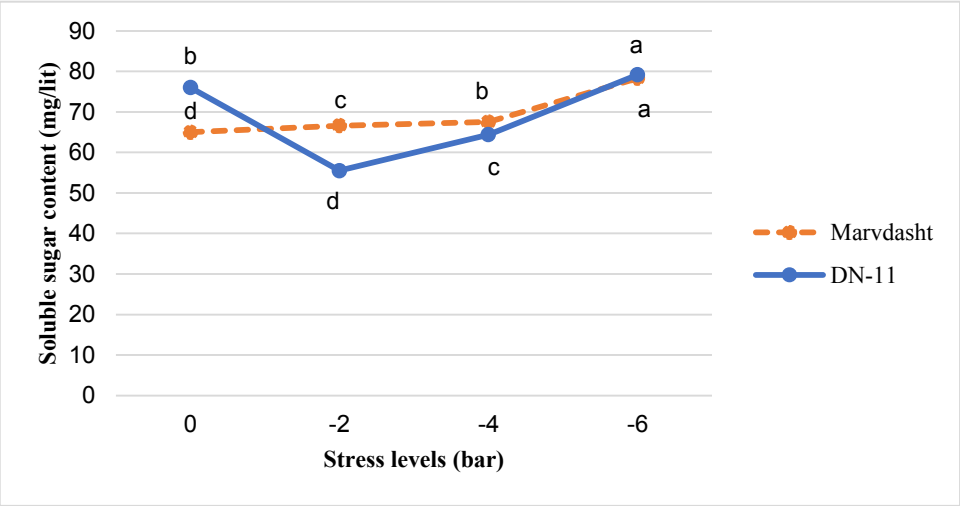

Figure 2. The soluble sugar content under different levels of stress in both genotypes. The soluble sugar content in both genotypes had a significant difference at the P-value of ≤0.01.

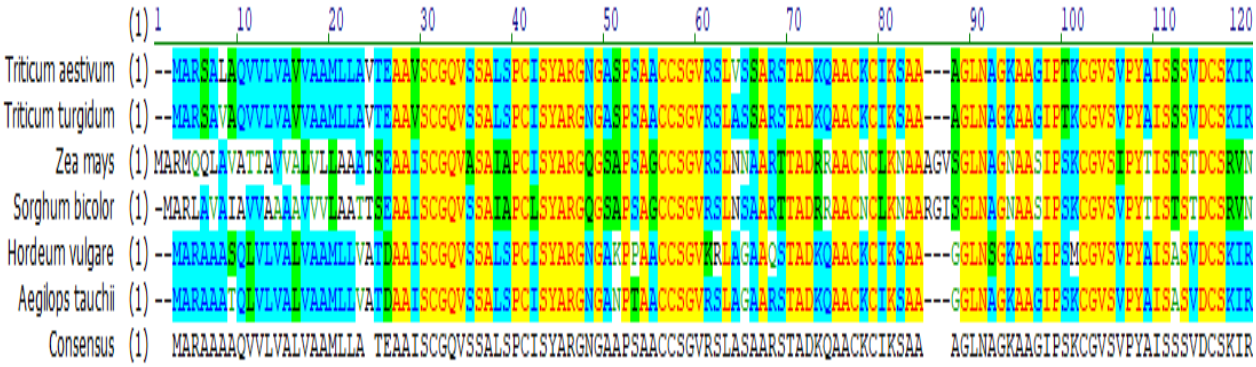

Figure 3. The multiple alignment of related sequences from different cereals to identify conserved regions in the LTP proteins.

TaLTP1 is linked to the *Cis* elements of MYC and MYB in the promoter region of the target genes and through activation of the downstream genes it may contribute to the creation of tolerance to drought stress (Jang *et al.*, 2004).

Promoter motifs studied on the Plantcare database in rice plants had various elements including elements responding to light (ACE, GT1-motif, 3-AF1 binding site, and G-Box) and hormone (ABRE, TGA-element, and TCA-element) (Zhang *et al.*, 2020). Transcription of *TaLTP1* increased under drought stress through treatment with different concentrations of NaCl, abscisic acid, salicylic acid, and ethephon hormones, H_2O_2 and or wounding (Sairam and Saxena, 2000; Lascano *et al.*, 2001; Jang *et al.*, 2004). The presence of the motifs mentioned in the *LTP* promoter suggests that this gene responds to a variety of stresses, especially abiotic stresses, through complex mechanisms and may play a vital role in stress tolerance.

On the other hand, the presence of conserved regions in LTP proteins was identified by multiple alignment among cereals using the Vector NTI software version 10 (Figure 3). Also, the protein model had an identity percentage of 72.22% and 62.22% in comparison with LTP protein from maize and rice, respectively (Figure 4). This showed that LTP protein of wheat has many structural similarities with LTP protein of rice and maize.

DISCUSSION

By applying different levels of drought stress, *LTP* in both Marvdasht and DN-11 genotypes showed different expressions due to morphological, physiological and metabolic differences, which in turn could affect the effective rate and compatibility with the drought stress conditions in both genotypes. In general, it seems that at some levels of stress and in the early hours after stress

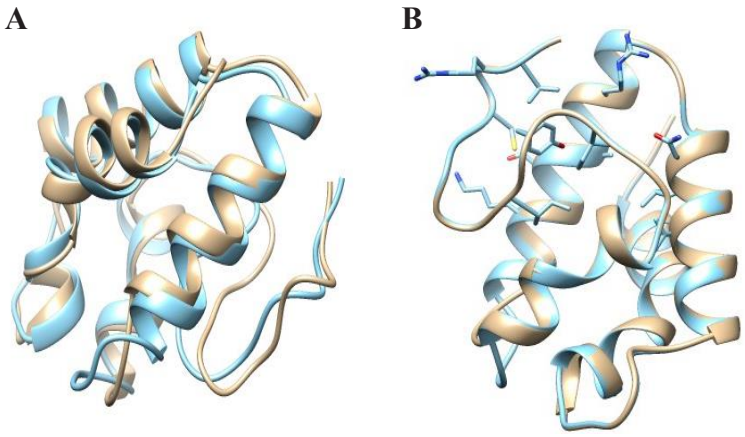

Figure 4. The alignment of protein model of LTP in wheat with the LTP protein of **A:** rice and **B:** maize.

induction, an intense increase in the expression of *LTP* was observed in both genotypes, meaning that the plant reacts quickly with stress, and to tolerate the stress, it starts a cascade of events including increase in the expression of *LTP*. On the other hand, *LTP* expression declined at 6 h after stress in the DN-11 genotype and 10 h after stress induction in both genotypes. In the interpretation of this issue, it is supposed that the proteins involved in increasing the *LTP* expression have been produced in the first hours.

It seems that at early times after stress application, plants responded to the stress and may the compatible mechanisms like *LTP* induction was activated but after the continuation of stress time and intensity, the induction of *LTP* was detected again. This expression induction was more pronounced in Marvdasht genotype than in DN-11 genotype.

. To prove it, Guo *et al.* (2013) showed that *myb96* plants were more sensitive to drought stress. Wang *et al.* (2009) showed that *ThLTP* expression level in *Tamarix hispida* increased under abiotic stresses such as drought stress, which is consistent with the results of this study. Also, Guo *et al.* (2013) investigated *LTP3* expression level in Arabidopsis and showed that expression pattern increased under drought stress, similar to the *LTP* expression pattern at -6 bar stress level in DN-11 genotype. In rice plant, the overexpression of *Oryza sativa* Drought-Induced LTP (*OsDIL*) increased drought tolerance (Hu *et al.*, 2020).

Many studies have shown that expression levels of some *LTPs* increase under drought stress (Vignols *et al.*, 1997; Liu and Lin, 2003; Wu *et al.*, 2004; Gonorazky *et al.*, 2005; Volpicella *et al.*, 2015; Li *et al.*, 2019). The lipid transfer proteins are involved in the secretion and transfer of extracellular lipophilic components like phospholipids and galactolipids and also the transfer of cutin monomers needed for the synthesis of wax on the plant surface, therefore inducing the expression of these genes in conditions of drought stress seems logical (Sterk *et al.*, 1991; Thoma *et al.*, 1994; Kader, 1996). On the other hand, soluble sugar content is the best marker for improving drought tolerance (Mohammadkhani and Heidari, 2008; Chen *et al.*, 2017; Sallam *et al.*, 2019). Soluble sugars are involved in various metabolic events and act as molecule signals regulating different genes, especially those involved in photosynthesis, sucrose metabolism and osmolyte synthesis (Rosa *et al.*, 2009). In addition, Karpets *et al.* (2020) showed that the accumulation of soluble sugars increases under drought stress in *Triticum aestivum* and *T. dicoccum*.

In general, it seems that inducing the expression of *LTP* in the early hours of stress actives the key related stress tolerant mechanisms, therefore the plant has suppressed the *LTP* expression at the next times after stress application. With prolonged stress induction time, the plant again increases the expression of *LTP*, which probably interferes with MYB and MYC transcription factors and ABA hormone. On the other hand, TaMPS, a MYB transcription factor of wheat, could activate*TaEXPA2* expression by binding to its promoter (Yang *et al.*, 2020). Overexpression of TdLTP4 (from *Triticum turgidum*) and TaMPS (from *Triticum aestivum*) in *Arabidopsis* has increased its tolerance under drought stress (Safi *et al.*, 2015; Yang *et al.*, 2020). Therefore, in response to drought stress in longer periods, a significant difference in *LTP* expression was found, which helped to the continuation of plant resistance reactions and possibly the emergence of some new resistance reactions in the plant. In a study it was shown that *TaLTPs* expression

may help plants better tolerate various abiotic stresses such as drought, because these genes were highly expressed during drought stress (Hairat *et al.*, 2018). In addition, Jacq *et al.* (2017) have mentioned structural role of an LTP from *Arabidopsis*, which helps to tolerate the plant against stress. Not only wheat and Arabidopsis, but also it has been shown to increase *Nicotiana tabacum* and potato tolerance to drought stress through the expression of *NtLT4* and *StnsLTP1*, respectively (Gangadhar *et al.*, 2016; Xu *et al.*, 2018). Finally, Francoz *et al.* (2016) have proven that there are abundant AtLTP2 transcripts in the epidermal cells of embryonic aerial organs at late stages of seed development. It may indicate a relationship between abiotic stresses and seed development. According to our results, isolation of *LTP* gene from wheat and transferring it to drought sensitive plants can prevent some of the damages caused by stress. In addition, since DN-11 genotype showed a higher *LTP* expression in response to drought stress than Marvdasht genotype, this genotype may be a better candidate for attempts to increase drought tolerance in plants.

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