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Quantitative expression analysis of *TaMPK4* and *TaTIP1* genes in drought tolerant and non-tolerant wheat (*Triticum aestivum* L.) cultivars

Birsen Cevher-Keskin*, Yasemin Yıldızhan, Oktay Kulen, Selma Onarıcı

TUBITAK, The Scientific and Technical Research Council of Turkey, Marmara Research Center, Genetic Engineering and Biotechnology Institute, Plant Molecular Biology Laboratory, P.O Box: 21, 41470 Gebze, Kocaeli Turkey

***Corresponding author:bcevherkeskin@gmail.com; birsen.keskin@tubitak.gov.tr**

Abstract

The regulation of plant responsive genes to drought stress comprises very complex mechanisms. Plant signal transduction cascades are stimulated by the sensing of water stress signals, and then expression of different genes and signaling molecules. Expression patterns of these genes are different; some of them respond to drought very rapidly, while others are induced slowly after the accumulation of ABA. The mitogen-activated protein kinase (MPK) cascade elements and tonoplast intrinsic proteins (TIPs) take place in abiotic signaling pathway and water movement regulation, respectively. We aim to show the expression patterns of *MPK4* and *TIP1* mRNA in drought tolerant and non-tolerant *T. aestivum* cultivars treated with two different shock dehydration stresses using qRT-PCR technique. The patterns of *MPK4* and *TIP1* mRNA accumulation was different in non-tolerant wheat cultivar, up-regulated in 4h and 8h drought-stressed root and leaf tissues. The reason for early response to drought stress in the cultivar Atay might be related to drought sensitivity. Drought tolerant cultivars showed *MPK4* up-regulation in 8h stressed roots implying that, increased expression of *MPK4* might play an important role in drought tolerance of *T.aestivum* by regulating the stress signaling. There was no significant difference *in TIP1* mRNA expression level between drought stressed and control root tissues in both tolerant cultivars. Although down-regulation was observed in TIP1 transcript level under 4h drought stress, an induction was found under 8h drought stress in two drought tolerant cultivar leaves. Similar results were obtained from RNAseq data performed with the same cultivars and stress applications. These results suggest that ABA-dependent *MPK4* and *TIP* genes are also involved in ABA-independent pathway and there might be some relationships between *TIP1* and *MPK4* gene expresion in wheat under drought stress. Since the functions of *TIP1* and *MPK* genes have not been completely identified yet, detailed protein expression analyses will allow us to get a better idea about the possible role of these genes in drought-response mechanism in plants.

Keywords: *Triticum aestivum*, *MPK4*, *TIP1*, qRT-PCR, drought stress, ABA-dependent and ABA-independent signaling pathways.
Abbreviations: ABA_Abscisic Acid; MPK4_Mitogen Activated Protein Kinase; qRT-PCR_quantitative Reverse Transcription Polymerase Chain Reaction; TIP1_Tonoplast Intrinsic Protein; RNA-seq_RNA sequencing.

Introduction

Bread wheat (*Triticum aestivum* L.) is the most important cereal crop in terms of the size of growth area and volume of the trade (FAO statistics database: http://apps.fao.org). This crop has been the basic staple food for major civilizations in Europe, West Asia and North Africa and continues to be a major food source for humanity (Curtis, 2002). Drought stress is prevalent in many regions of the world, especially in the semi-arid areas and in other parts of the world where the majority of poor people live. If we take into account the growing human population, irrigated agriculture should be expanded 30% by the year 2025. However, expansion is expected to be limited to 5-10% due to expected slowdowns in dam buildings and irrigation investments, as well as falling ground water tables (Cosgrove, 2003). The drought problem in the world is likely to only get worse, as the destabilizing effects of global warming may increase the variability of both rainfall and temperature (Curry et al., 1995). Because of the diversity and instability of drought events, as well as the difference of the drought-tolerance mechanisms developed by plant, breeding of tolerant crop cultivars to drought stress is a demanding research area. Severe drought conditions could result in yield losses in strictly affected areas ranging from 10-15% compare to irrigated lands. Thus, it is important to find and develop new strategies to improve wheat cultivars more productive with less amount of water. Wheat breading progress for drought tolerance is laborious due to the complexity of measuring and quantifying drought traits and other parameters associated with the trait itself. In the adaptation of plants to abiotic stresses, Abscisic acid (ABA) is an important phytohormone which regulates many important events. It is a major internal signal enabling plants to survive adverse environmental conditions such as salt, cold, and drought stresses (Marcotte et al., 1992; Koornneef et al., 1998; Taji et al., 2004). Both ABA-dependent and -independent pathways of drought response mechanisms have been widely studied in many plant species, but gene regulation of these pathways have not been completely understood (Yamaguchi-Shinozaki and Shinozaki, 2005). Despite the differences in transcriptional activation, molecular mechanisms show that there is a cross talk between the ABA-dependent and -independent pathways in controlling gene expression under abiotic stresses (Xiong et

al., 1999). In the present study, we aimed to search mRNA expression level of *MPK4* and *TIP1*, important for intracellular signal transduction and water movement in drought stress respectively. From our previous studies, we found different mRNA expression patterns of these genes by exogenous ABA application. It was reported that MAPKs were involved in biotic and abiotic signaling, and developmental and hormonal signaling (Colcombet and Hirt, 2008). It was shown that brassinosteroid regulates stomatal development by activating the MAPK kinase kinase (MAPKKK) in Arabidopsis (Kim et al., 2012). It has been reported that the last MAPKs in the signaling cascade has an inhibitory role on the initiation of stomatal development, thus limiting the number of stomatal opening and preventing water loss (Bergmann et al., 2004, Zhang et al., 2006). ABA-induced MAPK activation was reported in Arabidopsis (Pagnussat et al., 2004, Zhang et al., 2007). In plants, PIPs (Plasma Membrane Intrinsic Proteins) and TIPs (Tonoplast Intrinsic Proteins) are belong to aquaporins and regulate water movement across membranes (Jung et al., 1994; Walz et al., 1997). Althought there are many studies about TIP genes and abiotic stress tolerance, the relationship among TIP, water status and plant tolerance under different stress conditions remains unclear. Many studies have demonstrated that the expression levels of TIP genes are regulated by different kinds of stress conditions (Ludevid et al., 1992; Boursiac et al., 2005; Sakurai et al., 2005; Li et al., 2009), but the effect of TIPs on plant tolerance to abiotic stresses remain limited (Sade et al., 2009). The expression level of *TIP1*-like gene and *MPK4* was significantly increased with the application of exogenous ABA in our previous study. Thereby our findings suggests a possible involvement of these transcripts in the ABA-dependent stress response pathways in hexaploid bread wheat (Keskin et al., 2010). The objective of this study was to analyze the expression level of *TIP1* and *MPK4* in ABA-independent pathway. We hypothesize that there may be some relationship or crosstalk for these genes in ABA-dependent and -independent pathways. Quantitative real-time PCR analyses were performed in the root and leaf tissues for the *TIP1* and *MPK4* genes to observe differential induction under shock dehydration stress in drought-tolerant and non-tolerant *T. aestivum* cultivars.

Results

Drought tolerant Triticum aestivum cultivar Müfitbey

We examined *MPK4 and TIP1* mRNA abundance under 4h drought application in drought-stressed and control root tissues. *MPK4* expression was up-regulated with 4h stress treatment compare to control in root tissues (Fig. 1A), on the other hand, there was no significant difference in *TIP1* expression level. Similar results were observed after 8h of drought stress for *MPK4* and *TIP1* mRNA expression patterns (Fig. 1B). In leaf tissues, *TIP1* was strongly decreased under 4h drought stress treatment; *MPK4* mRNA expression was not significantly changed compare to the control tissue (Fig. 2A). Conversely, after 8h of drought application, mRNA expression level of *MPK4* and *TIP1* was induced in wheat leaves (Fig. 2B). Increased *MPK4* expression was shown in 4h drought stressed roots. The effects of drought stress were first determined in root tissues than leaf tissues that could be explained that roots were suffered to drought earlier than leaves. When plants were suffered to drought, plant leaves could be triggered by signal transduction and then stomata in leaf tissues might be closed to reduce the negative effects of the stress that could be another explanation why plant root tissues increased *MPK4* expression earlier.

Drought non-tolerant Triticum aestivum cultivar Atay 85

When the same experiments were repeated with non-tolerant cultivar Atay 85, *MPK4* and *TIP1* expression were up-regulated under 4h and 8h stress treatments compare to control root tissues (Fig. 3A and B). Similarly, the expression of *MPK4* and *TIP1* transcrips were significantly increased in response to drought stress after 4h and 8h in leaf tissues (Fig. 4A and B).

Drought tolerant Triticum aestivum cultivar Gerek 79

In drought tolerant Gerek 79, *MPK4* mRNA expression was declined in root tissues after 4h and 8h drought stresses. On the other hand, there was no significant difference in the expression pattern of *TIP1* in both drought conditions in root tissues (Fig. 5A and B). *MPK4* was approximately 8-fold down-regulated in 4h drought stressed leaves, while *TIP1* was 3-fold down-regulated in the same leaves compare to control tissues (Fig. 6A). Conversely, *MPK4* and *TIP1* were shown to be dramatically up-regulated after 8h of drought stressed leaves (Fig. 6B). *MPK4* and *TIP1* expression were not significantly increased in leaf tissues of Gerek 79 after 4h drought treatment. *MPK4* and *TIP1* expression rates in drought tolerant leaf tissues are even lower than control leaf tissues. However, expression of *MPK4* and *TIP1* in leaf tissues was significantly increased after 8h drought application. It was shown that response to drought was earlier in leaf tissues compared to root tissues in the drought tolerant wheat cultivar.

Discussion

Drought response signaling is a well studied but the underlying molecular mechanisms have not yet been illuminated in detail. In plants, multiple MAPKs take place in signaling pathways. One of the important pathways MAPK cascade, consist of MAPKs, MAPKKs and MAPKKKs, and transduce signals by sequential phosphorylation (MAPKKK /MAPKK/MAPK) (Doczi et al., 2012). The last component of this cascade MAPK can phosphorylate specific effector proteins. Phosphorylation of this protein activates the cellular responses (Ichimura et al., 2002). Seven sub-groups (A, B, C, D, E, F, and G) were found according to phylogenetic analyses of MAPKs from wheat, rice and Arabidopsis (Lian et al., 2012). Twenty MAPKs were found in Arabidopsis, 17 in rice, and 21 in poplar (Ichimura et al., 2002, Nicole et al., 2006). Fifteen putative members of the wheat MAPK gene (*TaMPK*) family were identified by an *in silico* search of wheat expressed sequence tags (EST) databases based on the presence of amino acid sequence of Arabidopsis and rice MAPKs (Lian et al., 2012). In Arabidopsis, 10 MAPKKs and 60–80 MAPKKKs were annotated in the genome (Doczi et al., 2012 Samajova et al., 2013). In wheat, MAPK genes are stringently controlled in different organs and different growth stages. Two *TaMPK*s (WCK-1, FLRS) have been characterized and diffrentially regulated by biotic stress in wheat (Lian et al.,2012 Takezawa, 1999, Rudd et al., 2008). It was reported that *MPK3* and *MPK6* are activated by

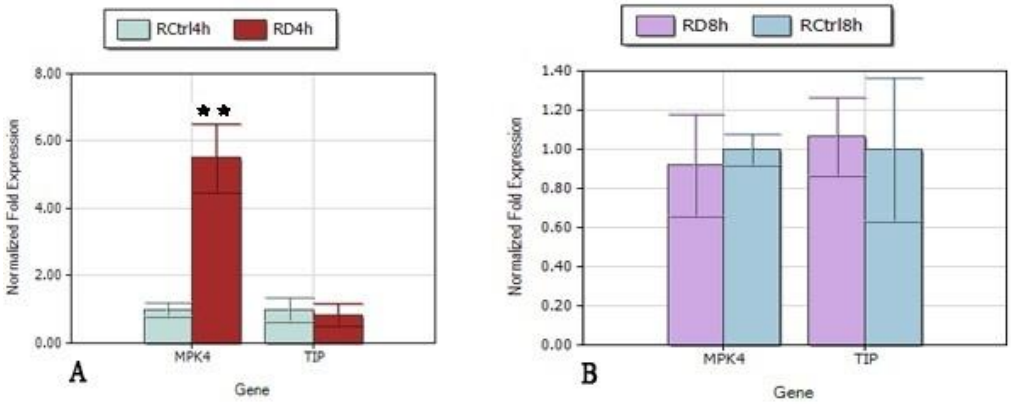

Fig 1. QRT-PCR analysis of *MPK4* and *TIP1* in control and drought stress *T. aestivum* L. cv. Müfitbey root samples. RCtrl4h: Root Control 4h, RD4h: Root Drought 4h. RCtrl8h: Root Control 8h, RD8h: Root Drought 8h. The gene expression was normalized using β-actin as a housekeeping gene. (A) *MPK4* expression up-regulated in 4h stress treatments relative to control in root tissue. On the other hand, there is no significant difference in *TIP1* expression (B) In 8h stress treated root, there is no significant difference in *MPK4* and *TIP1* expression pattern. Error bars are the standard deviation of PCRs each performed in triplicate. Three biological replicates were carried out for each sample. (*): $p \leq 0.05$, (**): $p \leq 0.01$.

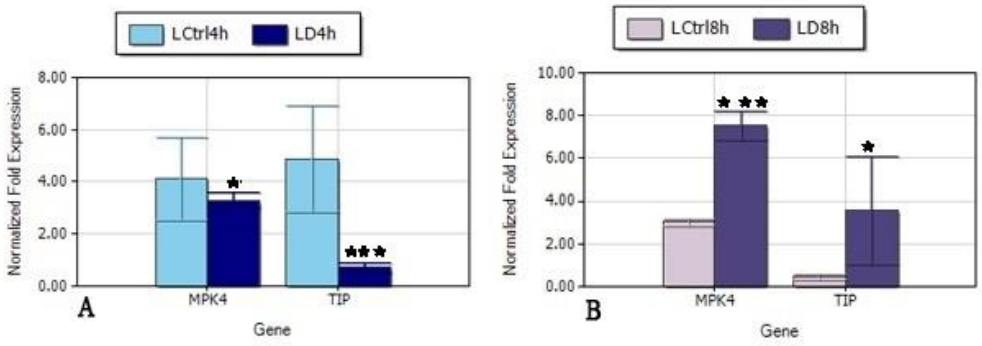

Fig 2. QRT-PCR analysis of *MPK4* and *TIP1* in control and stress *T. aestivum* L. cv. Müfitbey samples. LCtrl4h: Leaf Control 4h, LD4h: Leaf Drought 4h, LC8h: Leaf Control 8h, LD8h: Leaf Drought 8h. The gene expression was normalized using β-actin as a housekeeping gene. (A) *MPK4* and *TIP1* expression down-regulated in 4h stress treatments relative to control in leaf tissue. (B) In 8h stress treated leaf, *MPK4* and *TIP1* expression up-regulated relative to corresponding control tissue. Error bars are the standard deviation of PCRs each performed in triplicate. Three biological replicates were carried out for each sample. (*): $p \leq 0.05$, (**): $p \leq 0.01$, (***): $p \leq 0.001$.

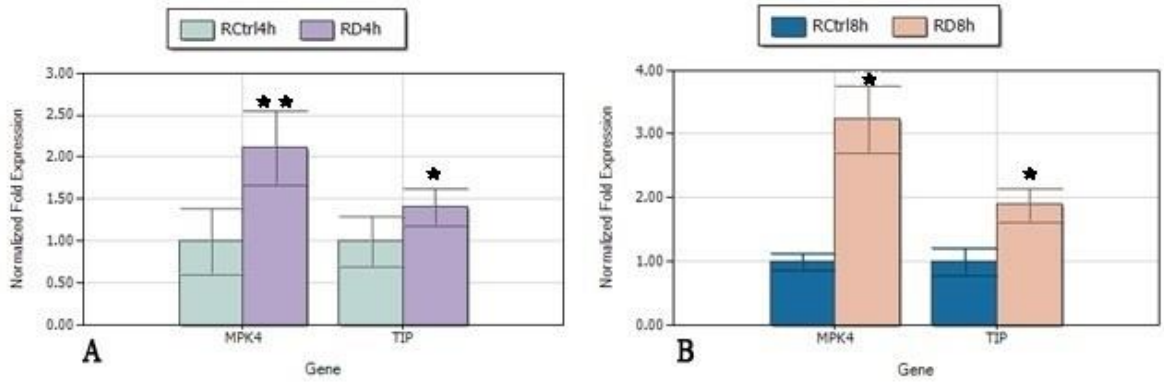

Fig 3. QRT-PCR analysis of *MPK4* and *TIP1* in control and drought stress *T. aestivum* L. cv. Atay 85 root samples. RCtrl4h: Root Control 4h, RD4h: Root Drought 4h. RCtrl8h: Root Control 8h, RD8h: Root Drought 8h. The gene expression was normalized using β-actin as a housekeeping gene. (A) *MPK4* and *TIP1* expression up-regulated in 4h stress treatments relative to control in root tissue. (B) In 8h stress treated root, *MPK4* and *TIP1* expression up-regulated relative to corresponding control leaf tissue. Error bars are the standard deviation of PCRs each performed in triplicate. Three biological replicates were carried out for each sample. (*): $p \leq 0.05$, (**): $p \leq 0.01$.

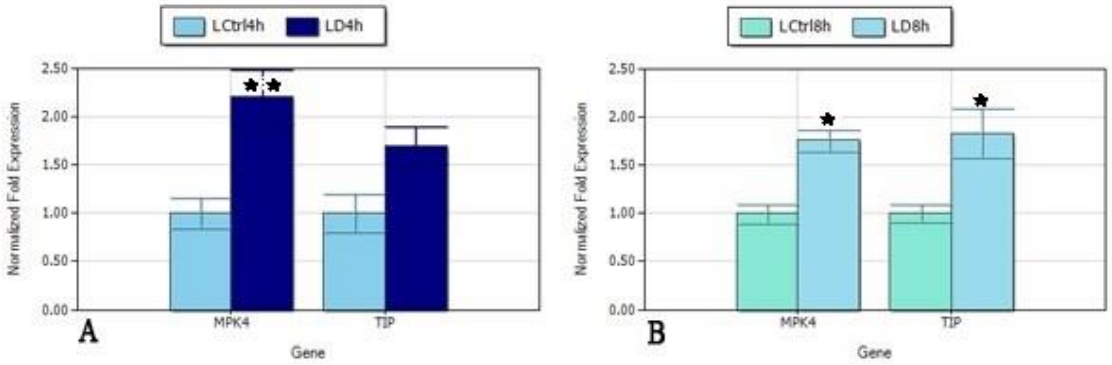

Fig 4. QRT-PCR analysis of *MPK4* and *TIP1* in control and drought stress *T. aestivum* L. cv. **Atay 85** leaf samples. **LCtrl4h:** Leaf Control 4h, LD4h: Leaf Drought 4h. LCtrl8h: Leaf Control 8h, LD8h: Leaf Drought 8h. The gene expression was normalized using β-actin as a housekeeping gene. (A) *MPK4* and *TIP1* expression up-regulated in 4h stress treatments relative to control in leaf tissue. (B) In 8h stress treated leaf, *MPK4* and *TIP1* expression up-regulated relative to corresponding control leaf tissue. Error bars are the standard deviation of PCRs each performed in triplicate. Three biological replicates were carried out for each sample. (*): $p \leq 0.05$, (**): $p \leq 0.01$.

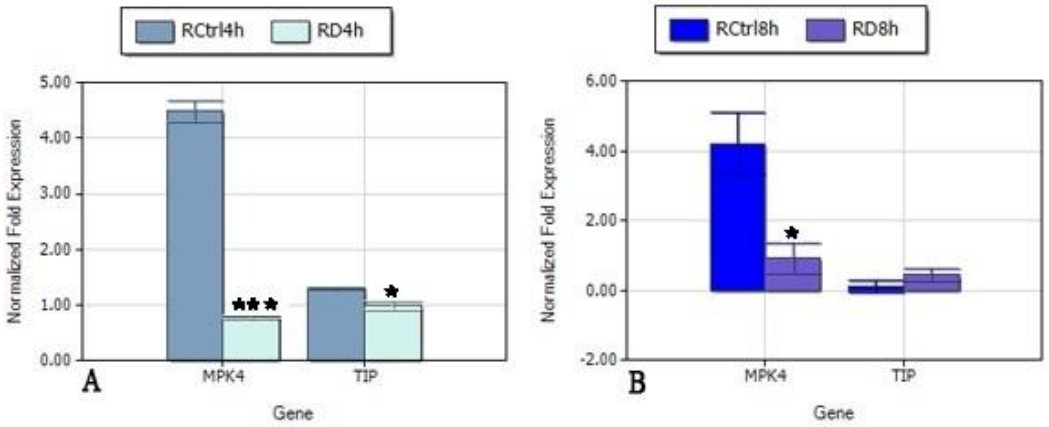

Fig 5. mRNA expression pattern of *MPK4* and *TIP1* in 4h and 8h drought stressed root tissue of *T. aestivum* L. cv. Gerek 79. RCtrl4h: Root Control 4h, RD4h: Root Drought 4h. RCtrl8h: Root Control 8h, RD8h: Root Drought 8h. (A) *MPK4* expression up-regulated in 4 h stress treatments relative to control in root tissue. (B) In 8h stress treated leaf, *MPK4* down-regulated relative to corresponding control root tissue. The gene expression was normalized using β-actin as a housekeeping gene. Error bars are the standard deviation of PCRs each performed in triplicate. Three biological replicates were carried out for each sample**.** (*): $p \leq 0.05$, (**): $p \leq 0.01$, (***): $p \leq 0.001$.

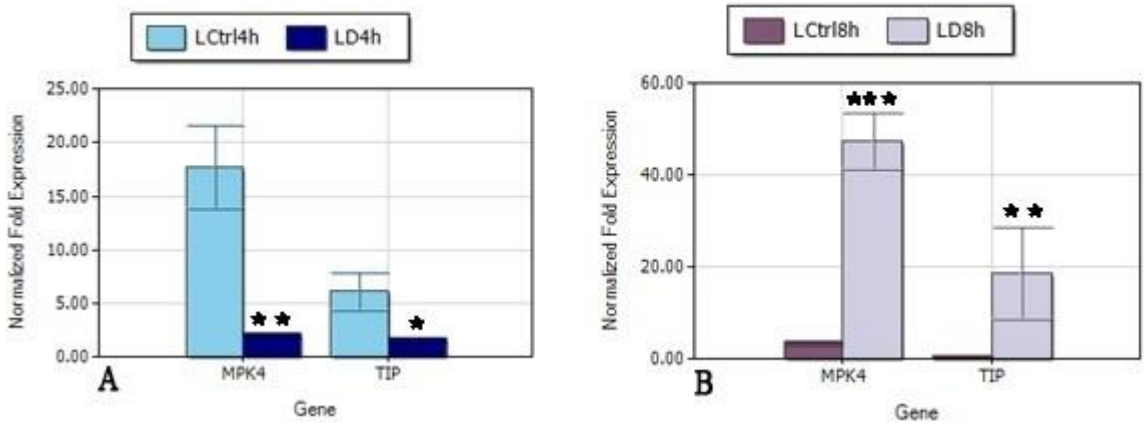

Fig 6. mRNA expression pattern of *MPK4* and *TIP1* in 4h stressed root and leaf tissue of *T. aestivum* L. cv. Gerek 79. LCtrl4h: Leaf Control 4h, LD4h: Leaf Drought 4h. LCtrl8h: Leaf Control 8h, LD8h: Leaf Drought 8h. The gene expression was normalized using β-actin as a housekeeping gene.(A) *MPK4* expression up-regulated in 4h stress treatments relative to control in root tissue. (B) In 8h stress treated leaf, *MPK4* down-regulated relative to corresponding control root tissue. Error bars are the standard deviation of PCRs each performed in triplicate. Three biological replicates were carried out for each sample. (*): $p \leq 0.05$, (**): $p \leq 0.01$, (***): $p \leq 0.001$.

Cd in Arabidopsis (Liu et al., 2010). *AtMPK3* and *AtMPK6* genes were strongly activated by abiotic stress (Rentel et al., 2004) and *AtMPK4* was also demonstrated in response to osmotic and cold stresses (Droillard et al., 2000). It was also reported that, overexpression of *OsMPK5* leads to enhanced tolerance to drought, salt and cold stresses in rice (Xiong and Yang, 2003). It was shown that, the last MAPKs in the signaling cascade has an inhibitory role on the initiation of stomatal development, thus limiting the number of stomatal opening and preventing water loss (Zhou et al., 2012; Bergmann et al., 2004, Zhang et al., 2006). Under drought stress condition, silencing of *SlMPK4*, MPK gene homolog of Arabidopsis *AtMPK4*, reduces drought stress tolerance in tomato (Gong et al., 2010) . It was also shown that *SlMPK4* gene decreases disease resistance against *B. cinerea* (Virk et al., 2013). mRNA expression level of *ZmMPK4* was up-regulated by cold, salt and H_2O_2 but down-regulated by ABA (Kong et al., 2011). The *TaMPK4* gene identified from our previous studies was significantly up-regulated (approximately 4 fold) in response to 50 µM ABA treatment after 2h and then gradually decreased after 4h and 8h of ABA treatments (Keskin et al., 2010). In this study, *TaMPK4* expression level was examined in 3 different *T. aestivum* cultivars (2 drought tolerant and 1 non-tolerant) under 4h and 8h drought stress in roots and shoots. In drought tolerant cultivar Müfitbey, the highest *TaMPK4* expression (approximately 5-fold) was observed in 4h stressed roots, and then expression level was returned to control levels after 8h stress induction. In leaf tissues, *TaMPK4* was 2-fold up-regulated in 8h drought stressed roots compared to controls. There was no significant difference between 4h stressed and control leaf tissues of tolerant cultivar. Different *TaMPK4* expression pattern was observed in the other in drought tolerant cultivar Gerek 79. In 4h and 8h of drought stressed roots, *TaMPK4* was down-regulated when compared to control root tissues. In leaf tissue, *TaMPK4* expression was down-regulated after 4h, but up-regulated after 8h stress treatments. In non-tolerant *T.aestivum* cultivar Atay 85, up-regulation was observed in root and leaf tissues in both drought conditions. Furthermore our RNA seq data shows and confirms that *MPK4* expression was up-regulated in drought treated leaf tissue of non-tolerant cultivar Atay 85. Tonoplast Intrinsic Proteins (TIPs) are related to the genes encoding water channel proteins (aquaporins). The water permeability of the tonoplast is known to be much higher than that of the plasma membrane and the vacuole osmotic buffering capacity of the cytoplasm is performed by this protein (Morillon and Lassalles, 1999). All the results demonstrated that aquaporins were involved in plant response to water stress, and different aquaporins exhibited different regulation patterns under stress conditions (Sarda et al., 1997; Mariaux et al., 1998). Because TIPs are responsible for water exchange between cytosolic and vacuolar compartments, regulation of cell turgor is the most important role of these proteins in plants (Forrest and Bhave, 2007). Tyerman et al (2002) reported that the expression of TIPs is organ specific and influenced by hormones and abiotic stresses in plants ABA induces the TIP expression in rice and rapeseed and decreases the TIP expression in *Craterostigma plantagineum* (Liu et al., 1994, Mariaux et al., 1998, Gao et al., 1999). In Arabidopsis, gibberellin (GA3) also regulates TIP expression (Phillips and Huttly, 1994). Under water deficit stress, TIPs expression is decreased in *Craterostigma plantagineum*, *Helianthus annuus*, *Mesembryanthemum crystallinum*, *Nicotiana glauca*, and Arabidopsis). On the other hand, water deficit was reported to increase the expresison of TIPs in rice, maize and cauliflower (Liu et al., 1994, Sarda et al., 1997, Barrieu et al., 1998, Lopez et al., 2004). In Arabidopsis, the TIP subfamily is divided into five subgroups, *TIP1, TIP2, TIP3, TIP4* and *TIP5*, based on their sequence homology (Johanson et al., 2001). In response to abiotic stress (drought, salinity, cold etc.) different aquaporin gene expression shows that TIPs are important proteins in response of plants to different conditions that affect water usage. The direct associations between aquaporin expression level and several stress conditions including water limitation have been reported (Li et al., 2009, Ruiz-Lozano et al., 2009). Furthermore, water stress was shown to induce the transcription level of *TIP1* in wild emmer wheat (Ergen et al., 2009). *TIP1* (*TaAQP6*) participates in internal water redistribution of wheat seedlings during osmotic stress by regulating outflow of water from water-rich organs to the parts with poor water status under water deficit condition. (Zhang et al., 2008). It was demonstrated that under salt stress the expression of TIP genes was altered in Arabidopsis. Boursiac et al. (2005) showed that constitutive expression of *GsTIP2;*1 increased water loss through leaves but did not affect water absorption through roots in Arabidopsis. Constitutive expression of *GsTIP2*;1 in *A. thaliana* increased dehydration speed and decreased seedling tolerance to salt and dehydration stress. On the other hand, overexpression of plasma membrane aquaporin *BnPIP1* in *Nicotiana tabacum* resulted in an enhanced tolerance to water stress at the whole plant level (Yu et al., 2005). From our previous studies TIP1 like mRNA was significantly induced at the second hour of the 50 µM ABA treatment, subsequently the level of the transcript dropped slowly the reduction rate in the transcript amount was slower than *MPK4* (Keskin et al., 2010). It was shown that *TIP1* expression differentially regulated in leaf and root tissues under both drought conditions in *T. aestivum* L. cv Müfitbey (Fig. 1 and 2). In leaf tissue *TIP1* was down-regulated in 4h of drought and then up-regulated after 8h stress reaching a peak of expression 20-fold higher than the control tissue level in tolerant *T.aestivum* Gerek 79 (Fig. 6A and B) and the other cultivar Mufitbey (Fig. 2B). On the contrary, there was no significant difference between both stressed and control root tissues of tolerant cultivars Mufitbey (Fig. 1A and B) and Gerek (Fig. 5A and B). It could be explained by earlier response might be developed in root tissues of resistant cultivars. The changes of *TIP1* expression level in roots and leaves under dehydration condition were opposite, implying different regulation occurred in root (Fig. 1A and 5A) and leaf tissue (Fig. 2B and 6B) because of the systemic effect. ABA may act as a signal in leaves inducing the accumulation of TIP1 mRNA On the other hand, in non-tolerant cultivar Atay 85, *TIP1* up-regulation was observed in 4h and 8h stressed leaf tissues (Fig. 4A and B). *TIP1* mRNA expression was dramatically up-regulated in roots after 8h of drought-stress (Fig. 3B). Analysis of variance showed significant differences between *MPK4* mRNA level in each tolerant wheat cultivar under 4h drought stresses, while no significant differences were obtained for *TIP1*. Because of the non-tolerant characteristics of Atay. TIP1 might be expressed later than the other two tolerant cultivars. Tolerant cultivar compete with drought stress by stimulating the water storage into vacuoles and also reducing water transport to peripheral tissues. Montalvo-Hernández (2008) reported that drought tolerant pea cultivar showed restricted water transportation that it was not occured in susceptible cultivar. They suggested that restricted water transportation with drought treatment renders increased drought tolerance in pea. Illumina HiSeq 2000 RNA sequencing technology was used to characterize cDNA libraries from drought treated and control Atay, Mufitbey and Gerek 79 cultivars. Different

TIP1 regulation was observed between tolerant and non-tolerant cultivars in response to shock dehydration drought stress. RNA-Seq data of 4h-drought treated leaf tissues of these cultivars was compared and increased *TIP1* expression was observed in non-tolerant cultivar Atay (Cevher-Keskin unpublished data). In 4h and 8h drought stressed root tissues, *TaTIP1* expression was increased in two drought-tolerant cultivars. Under both drought conditions it was shown that *TaMPK4* and *TaTIP1* were up-regulated in root tissue of non-tolerant cultivar Atay 85. It is remarkable that different expression patterns of these genes in tolerant and non-tolerant wheat cultivars under drought stress were observed. *MPK4* and *TIP1* expression in root and leaf tissues were significantly increased with both 4h and 8h drought treatment in cultivar Atay. The reason for the early response to drought stress might be drought sensivity of the cultivar Atay. RNAseq and qRT-PCR analysis indicates that ABA-dependent *MPK4* and *TIP* genes are also involved in ABA-independent pathway and there are some relationships between *TIP1* and *MPK4* gene expresion in wheat under drought stress.

Materials and Methods

Plant materials

For initial screening, twelve bread wheat cultivars originating from Turkey were used for the selection of the most promising drought stress tolerant and non-tolerant cultivars (Table 1). The seeds were surface sterilized (5 min 10% EtOH and 5 min 5% hypochlorite) and pre-germinated in Petri dishes for 10 d at 4^0C in the dark. Six pots were prepared for one cultivar, three pots were for control and other three were for the application of drought stress. Seedlings of similar germination stage were transferred to six pots (10 seeds per pot) containing a turf: soil: sand mixture (3:3:1). Plants were then grown under a natural photoperiod (16/8 h; temperature 22-18°C.)

Growth conditions

Drought stress treatment (Slow drought stress) was started 3 weeks after transferring the seedlings to the pots and carried out by withholding Hoagland from stress treatment pots. The regular watering regime was performed for the control plants. At the end of 10 d of drought treatment, leaf tissues (third youngest leaf) were collected for relative water content (RWC) measurements. RWC measurements were performed as described by (Barrs and Weatherley, 1962). Harvested tissues were directly frozen in liquid nitrogen and stored at -80°C. For each pot, three different measurements were taken in the afternoon (Babar et al., 2006). Based on the physiological data [SPAD measurements, RWC, soil water content (SWC)], drought sensitive and tolerant wheat genotypes were identified. Three biological replications were performed for 12 cultivars and 3 of them selected as drought - tolerant and non-tolerant.

Shock dehydration stress treatment

The seeds were (Drought-tolerant cultivars-Müfitbey and Gerek 79 and non-tolerant cultivar-Atay 85) surface sterilized in 70% alcohol for 5 minutes and 30% sodium hypochlorite for 10 minutes and then rinsed six times with sterile distilled water for 2 minutes and pre-germinated in Petri dishes for 10 days at 4°C in the dark. After the germination, seedlings were transferred to 10 L plastic pots containing moistened perlite for growth. Seedlings of a similar developmental stage were transferred to continuously aerated ½ Hoagland's solution and renewed every 3 days and grown under controlled conditions (16 h photoperiod, temperature 22/18°C and relative humidity 60%). At the age of four leaf stage, plants were removed from the hydroponic culture, and left dehydration shock stress for 4h and 8h under the same lighting conditions. Root and leaf tissues from each treatment were collected with corresponding controls and frozen in liquid nitrogen.

RNA extraction and cDNA synthesis

Total RNA was extracted from 4 h and 8 h stressed root and leaf tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The RNase free DNaseI (Roche) was used to remove any DNA remains that could otherwise interfere with RT-PCR applications. The concentration of the extracted total RNAs from root and leaf tissues were calculated through the A260/A280 ratios, using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific). High-purity total RNAs (1.95-2.1) were obtained from root and leaf tissues. To eliminate residual genomic DNA, each RNA sample was treated with 10 U of RNAse-free DNaseI (Roche Applied Science GmbH, Germany) for 20 min at 37 ^{0}C and purified according to the method described previously (Keskin et al., 2012). cDNA for qRT-PCR was synthesized using MMLV reverse transcriptase (Roche High Fidelity cDNA synthesis kit) according to the manufacturer's instructions. Five µg of DNase-treated total RNA was used for first strand cDNA synthesis, using 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, 20 U RNase inhibitor, and 200 U MMLV reverse transcriptase in a 20 µl final volume (Keskin et al., 2012).

Quantitative real-time PCR analysis

QRT-PCR was performed in 96 well polypropylene plates sealed with transparent adhesive covers (BioRad Microseal B seal). Each RT-PCR reaction was set up in 25 µl total volumes, containing 12.5 µl SYBR Green PCR SuperMix (Roche SYBR GreenI), 10 pmole of primers and 75–200 ng of the cDNA samples. The RT-PCR reactions were carried out in triplicate for each sample with IQ5 System (BioRad Laboratories, Hercules, USA Laboratories, Hercules, USA). β-Actin was used as an internal reference (GenBank accession AY663392, F-GACAATGGAACCGGAATGGTC R-GTGTGATGCCAGATTTTCTCCATg). Primer sequences are as follows: *MPK4* (GenBank accession TA63689_4565) (F-CGTACCTAGAGCGGCTTCACGA, R-GGTTTGAAG-AAGCAGCAACAA) TIP1 (GenBank accession U86762.1) F-GGAGATCGTGATGACCTTCG, R-CTGCTCAGTAGT-CGGTGGTG). Three biological replicates were carried out for each sample. Three technical replications were performed for each experiment in order to accurately quantify transcript level.

Statistical analysis

After the quantification cycle (Cq) values are measured, "$2^{-\Delta\Delta Cq}$ (Livak) Method" was used to determine the expression level of the target gene (*MPK4* and *TIP1*) in the test sample relative to the calibrator sample. The amplification efficiencies of both target and reference genes were 100% (Guénin et al., 2009) The ΔΔCq values for all of the transcripts were averaged across all treatments and experimental replicates. Finally, GraphPad Prism 6

(Student's t-test) was applied to check for the statistical significance between drought-treated and –untreated control groups.

Conclusion

In conclusion, our present study demonstrated different expression pattern in response to drought in wheat, indicating novel information about these genes in ABA independent pathway. "All these studies show that, manipulation of TIPs' expression levels and protein functioning might be useful for drought stress tolerance. It is very important and necessary to learn more about stress related genes for the illuminate of drought stress mechanism through by transgenics plants.

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