The comparison of ABA and humic acid on the expression of some drought related genes in wheat

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Research Article

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Abstract

Drought is a common abiotic stress restricting plant growth and yield. Plants develop multiplicity responses such as abscisic acid (ABA) which is a broad-spectrum phytohormone involved not only in regulating development but also in coordinating various stress signal pathways during abiotic stresses. Numerous genes related to drought are regulated by ABA-dependent and independent pathways. Moreover, the effects of humic acid (HA) which is a biostimulant, on growth, yield and protection of plant resistance in various abiotic stresses are a well-known issue in plants. However, it is not clear the relationship between drought related genes and HA. Here, we aimed to determine the effects of the drought stress generated by -6 bar and -8 bar PEG6000, ABA and HA treatments on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels, expression of drought genes (TaSRG, TaCP, Sal1, Era1 and TdPIP1) on tolerant (Aksel 2000 and Kırkpınar 79) and susceptible (Atlı 2002 and Kırmızı Kılçık) bread wheat (*Triticum aestivum* L.) genotypes. According to the results, the expression of the genes varied depending on genotype and tissue and it was determined whether the genes used ABA-dependent or ABA-independent mechanisms to provide tolerance to stress in our promoter analysis. Sal1 and Era1 genes are thought to be biotechnologically useful in determining tolerant and susceptible genotypes. As a result, the irregularities of the biochemical changes and expression of genes that were caused by drought stress could be improved by ABA, but HA could be used as an alternative to ABA, considering the positive effects on genes.

Introduction

Wheat is an important cereal in terms of both one of the most cultivated and consumed food for a large portion of human populations. While wheat can be grown in a wide range of abiotic stress conditions, these conditions can be a threat that results in a significant reduction in its yield and quality. Among them, drought is one of the main environmental stress that imposes adverse effects on plant growth, morphology and physio-biochemistry (Wang et al. 2018). Also, drought may promote the overproduction of reactive oxygen species (ROS) [superoxide radical ($O_2^{\cdot-}$), H_2O_2 , and hydroxyl radical ($HO^{\cdot}$)], which are extremely reactive molecules that have high affinity to membranes, DNA, or proteins in plant cells (Dietz and Pfannschmidt 2011). Even if, plants are divided into tolerant and susceptible genotypes according to their traits to avoidance and protection from drought, there are various mechanisms for dealing with drought in both genotypes. One of these mechanisms is to be an increase of abscisic acid (ABA) production, which is known as a plant stress hormone, regulate the relationship between plant and water (Taiz and Zeiger 2008). ABA is overproduced during drought and is transported from the roots where it was first synthesized to the stem and leaves, causing stomata to close to prevent water loss (Yordanov et al. 2003; Osakabe et al. 2014) and also induces many genes.

In many studies, it has been observed that external ABA application to stressed plants activates certain genes related to drought and does not affect others (Khan et al. 2012). Therefore, it has been suggested that the response to drought is regulated by two different mechanisms (ABA-independent and ABA-dependent pathways) at the transcriptional level under the influence of transcription factors (Yamaguchi

Shinozaki and Shinozaki 2005; Budak et al. 2013). Genes in the ABA-independent pathways are under the control of the drought responsible element (DRE), C-repeat (CRT) cis-elements and the transcription factors of the DRE-binding protein (DREB) and Zinc finger home domain (ZFHD) (Liu et al. 2018). In the ABA-dependent pathway, many drought-related genes have been found to contain conserved ABA-sensitive elements (ABRE) with ACGT sequences in the promoter regions (Uno et al. 2000). Also, myelocytomatosis (MYC) and myeloblastosis (MYB) transcription factors are among the transcription factors in this pathway that activate the expression of target genes.

In recent years, with the increase in drought and cultivation of drought tolerant genotypes, rapid advances in molecular biology, functional genomics and transgenic technologies have facilitated the studies related to drought, and significant progress has been made in defining related genes and gene regions. From these drought related genes which identified in wheat, TaSRG is essentially a transcription factor that increases salt tolerance in plants growing on saline soils (He et al. 2011), however not only in salinity stress but also drought, cold (He et al. 2011), hot and heavy metal stress (Ergun et al. 2014) have been found to change its expression. The TaCP gene is a cysteine protease (CP). The primary role of CPs is to metabolize the degradation of peptides and proteins to ensure protein remobilization in response to stress (Grudkowska and Zagdanska 2004). The Sal1 gene, defined as the negative regulator of the stress signal, is closely related in response to stress in plants. Sal1 encodes a bifunctional enzyme with both 3' (2'), 5'-bisphosphate nucleotidase (3'-phosphoadenosine 5'-phosphate) (PAP) and inositol polyphosphate 1-phosphatase activities (Quintero et al. 1996). The Era1 gene encodes the β subunit of the farnesyltransferase enzyme involved in protein farnesylation, which determines the substrate specificity. Farnesylation is the negative regulator of the ABA signalling pathway in stoma guard cells and protein farnesylation in plants is best described by the response to drought from ABA-dependent pathway (Cutler et al. 1996; Pei et al. 1998). The other gene is TdPIP1 which is an aquaporin (plasma intrinsic proteins; PIPs), known as water channels, are conserved major structural proteins, particularly abundant in plasma and intracellular membranes, and maintain the current water state to prevent rapid fading of plants exposed to severe drought or salt stress (Aharon et al. 2003).

While plants develop quite a number of internal protection mechanisms against environmental stress, externally applied biostimulants like humic substance (HS) contribute to the yield and development of the plants. HSs, known as organic carbon pools, consist of plant or animal residues through biological or chemical transformations in the soil (Canellas et al. 2015). Besides, humic acids (HAs) reduce the evaporation of water from the soil. This feature is of great importance in arid regions (Ay 2014). It has been shown to have an effective role in the protection of plant resistance in various abiotic stresses (drought, salt, heavy metal) (Siddiqui et al. 2011). Although it is known that humic components have transcriptionally interacted both biochemical components and signalling pathways (Garcia et al. 2016), as well as a protective effect on genomic template stability and DNA methylation in plant under stress (Yildirim et al. 2014), the role of HSs in the molecular mechanism, has not been exactly elucidated yet.

In our present study, we were aimed (a); to identify susceptible and tolerant wheat genotypes to drought (b); to determine the effects of external ABA and HA applications on the biochemical changes

[malondialdehyde (MDA) content and H_2O_2] and mRNA transcript levels of drought stress related genes (TaSRG, TaCP, Sal1, Era1 and TdPIP1) in wheat genotypes. Additionally, we identified cis-elements within the promoter region of these genes and we had an idea whether these genes are corroborated with ABA-dependent or independent pathways.

Materials And Methods

Plant material

Drought-tolerant (Aksel 2000; Kırkpınar 79) and drought-susceptible (Atlı 2002; Kırmızı Kılçık) bread wheat (*Triticum aestivum* L.) genotypes were selected in a high dose drought tolerance study and used as experimental materials which were provided by Atatürk University, Faculty of Agriculture.

Growth conditions and PEG6000, ABA and HA treatment procedures

The equal sizes of wheat seeds were sterilized with 2.5% sodium hypochlorite for 10 min, washed thrice with deionized water and then hanged on 70% ethanol for 1 min and washed thrice with deionized water again. 20 of sterile seeds for each treatment were planted in perforated plastic pots which include hydrotones and these pots were placed on trays containing Hoagland solution (Sigma H2395-10L), (Hoagland and Arnon 1938). The air-pump system was used to provide oxygen into the solution. The seeds were germinated under the conditions of $20\pm 1^\circ\text{C}$ (16 h light/8 h dark), $300\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ light intensity and 70% relative humidity for 14 days. Fourteen-day-old seedlings were randomly divided into nine groups: control (CK)- untreated; MD- moderate drought (-6 bar PEG6000); HD- high drought (-8 bar PEG6000); ABA- abscisic acid (100 μM) ; MD+ABA- seedlings treated with -6 bar PEG6000 and ABA (100 μM); HD+ABA- seedlings treated with -8 bar PEG6000 and ABA (100 μM); HA-humic acid (0.001%, w/v); MD+HA- seedlings treated with -6 bar PEG6000 and humic acid (0.001%, w/v); HD+HA- seedlings treated with -8 bar PEG6000 and humic acid (0.001%, w/v). Each treatment included three biological replicates. Osmotic potentials (-6 bar for moderate drought and -8 bar for high drought) were prepared according to Michel and Kaufmann (1973). While PEG6000 solutions were added to trays, ABA and HA solutions were sprayed to leaves in the fifteenth day. To perform the biochemical assays and RNA extraction, young leaves and roots were separately collected 7 days after treatments and kept in liquid nitrogen immediately and stored at -80°C until further use.

Determination of MDA content and H_2O_2

To measure lipid peroxidation, we determined the final product of lipid peroxidation called MDA. The concentrations of MDA was measured according to Madhava and Stresty (2000) with minor modifications. The leaf and root samples (0.5 g) were ground in liquid nitrogen, homogenized in 5 ml of

0.1% trichloroacetic acid (TCA), and centrifuged for 5 min at 12,000 *g*. Then, 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added to 1 ml of the supernatant. The mixture was heated for 30 min in a water bath at 95 °C, immediately centrifuged again for 10 min at 12,000 *g* at 4 °C. The absorbance of the samples was recorded at 532 nm and 600 nm. MDA concentration was calculated using the extinction coefficient ($\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$). H_2O_2 level is determined according to the method of Velikova et al. (2000). In this method, 0.1 g leaf samples from each group were homogenized with 5 ml TCA and centrifuged at 12,000 *g* for 15 min at 4 °C. 0.5 ml of supernatant was mixed with 0.5 ml of buffer (potassium phosphate 10 mM, pH: 7) solution and 1 ml of 1M potassium iodide buffer. Absorbance values at 390 nm were measured by UV-VIS spectrophotometer.

The data obtained from both MDA and H_2O_2 were analyzed by ANOVA using SAS PROC GLM (SAS version 9.4, SAS Institute Inc., Cary, NC) program according to full chance test plan in [9 (application) x 4 (genotype)] factorial order. The differences between the averages of the characters were compared with the Fisher's LSD (Least significant Difference) multiple comparison test at 5% level.

Total RNA extraction, cDNA synthesis and gene expression

The leaf and root samples were harvested from three different plants for each replication of treatments. The strategy of bulk mRNA sampling was applied. Total RNA of 100 mg plant material (leaves and roots) were extracted using RNeasy Plant Mini Kit (Qiagen) according to suppliers' instructions. After checking the integrity of RNA on 1.2% agarose gel electrophoresis, the concentration and purification of each sample were tested using NanoDrop-1000 spectrophotometer ($\text{OD}_{260/230} > 2$). First-strand cDNA synthesis was carried out using 0.5 μg total RNA for the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific). Each quantitative PCR (Q-PCR) reaction (25 μL) contained 2.5 μg of cDNA. Amplification was performed with Maxima SYBR Green/ROX qPCR Kit (Thermo Scientific) following the manufacturer's protocol. The primer sequences for the TaSRG, TaCP, Sal1, Era1 and TdPIP1 genes are provided in Table 1. The Q-PCR reactions were run in a Qiagen Rotor-Gene and the cycling conditions were 10 min at 95°C, 40 cycles of amplification at 95°C for 15 s, at 56°C -65°C for 30 s and extension at 72°C for 30 s. The relative gene expression levels were determined using the $2^{-\Delta\Delta\text{Ct}}$ equation were calculated to get the expression fold change (Livak and Schmittgen 2001). Three biological replicates were examined and b-Actin gene (F: TGCTATCCTTCGTTTGGACCTT; R: AGCGGTTGTTGTGAGGGAGT) was used as the reference gene for the normalization of Q-PCR data. The sample of each biological replicate was analyzed in two technical replicates. The gene expression level was subjected to variance analysis (ANOVA) based on experimental design. Genotype x treatment interactions were analyzed based on simple main effects. Fisher's protected LSD test at 0.05 level was used to determine in differences between treatment for each genotype.

Table 1. List of primers used in Q-PCR

Primers	Nucleotide sequences (5' >3')		References
TaCP	F	GAGTGC GACCCAGCAGAAC	Zhang et al. 2010
	R	ACTCCGCCGATGTATGTCTG	
TaSRG	F	ATAGGATGCAGGGCGAAGTG	He et al. 2011
	R	TTGTCCCTGACCTCCATCTTC	
Sal1	F	ACAATTGGTTGTGGTGCTGA	Manmathan et al. 2013
	R	AAAAACGAGGCATTCACTGG	
Era1	F	TGAAGCTCATGGTGGGTACA	Manmathan et al. 2013
	R	AGCCAATCAAGCTAGGCAAA	
TdPIP1	F	GAGCACACCCAACTGTCACACTG	Ayadi et al. 2011
	R	ACAATGCCTGGAACGACCAATGG	

Bioinformatics Analysis of the Promoters

The 1.5 kb 5' upstream promoter regions of each gene was scanned for the presence of cis-acting regulatory elements involved in ABA-dependent or independent pathways based on the PlantCARE program ([http:// bioinformatics.psb.ugent.be/webtools/plantcare/html/](http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)).

Results And Discussion

MDA content

According to the average of the applications, the amount of MDA in the root tissue of genotypes varied between 7.86-9.29 uMol/gr and the average was determined as 8.40 uMol/gr (data not shown). The order of genotypes was Kırmızı Kılçık > Atlı 2002 > Kırkpınar 79 > Aksel 2000 and it was determined that sensitive genotypes (Kırmızı Kılçık and Atlı 2002) had higher MDA amount than tolerant genotypes (Fig. 1).

This result confirms that the MDA test is a reliable test to measure the negative effects of stress on plants. Similar to our study Al Ghamdi (2009) reported that MDA levels were higher in sensitive genotype (Sids-1) than tolerant wheat genotype (Veery) which exposed to water stress. In another study, it was determined that membrane integrity was weakened in sensitive wheat genotypes under drought stress (Simova Stoilova et al., 2010). The lower MDA amount of tolerant genotypes may be due to their higher anti-oxidative ability. Indeed, Dhanda et al. (2004) reported that drought tolerant genotypes had higher antioxidative capabilities.

On the other hand, the average amount of MDA in leaf tissue changed between 8.95-12.02 uMol/gr and 10.42 uMol/gr has been determined as an average (data not shown). The order of genotypes was the same as in root tissue. According to the average of genotypes, MDA amount of applications ranged between 6.94 uMol/gr (HA) -16.48 uMol/gr (HD). Also, we determined that MDA amounts were higher in MD and HD treatments in both tissue types compared to control (Fig.1). On the amount of MDA, the main effects of application and genotype and their interaction effect were very important ($p < 0.01$) on both roots and leaves.

MDA which is the final product of the lipid peroxidation process determines the oxidation in the membrane (Al Ghamdi 2009). Peroxidation of lipids is the most hazardous condition for all organisms (Renu and Devarshi 2007). In this study, when the MDA analysis results of root and leaf were compared in all applications, we observed that the values obtained from the leaves were considerably higher than from the roots (Fig. 1). In conditions where water is insufficient for plant growth, the root:shoot ratio is always higher since the plants do not receive sufficient water. In this case, plants increase the root length in order to get water from anywhere in the soil, so the sensitivity of the roots is lower than the stem and leaves (Wu and Cosgrove 2000). Therefore, it is inevitable that the amount of MDA is lower in this tissue type.

Furthermore, when the effects of ABA and HA on genotypes were examined under stress conditions, it was found that both ABA and HA applications resulted in a decrease in the amount of MDA in both tissue types in all genotypes (Fig. 1). Similar to our findings Kaur et al. (2014) reported that ABA application in stress conditions caused a decrease in MDA amount. The possible role of ABA in decreasing the amount of MDA can be explained by the induction of enzymatic and non-enzymatic antioxidant system to suppress the adverse effect of ROS on the plasma membrane. Considering the effects of HA on MDA amount, HA application caused more decrease in MDA compared to ABA, so it can be said that HA may be more effective than ABA in tolerant to drought stress. This can be attributed to the fact that HA changes the fluidity of the lipid layer of the membranes.

H₂O₂ content

According to the average of applications, the amount of H₂O₂ ranged between 796.7-1040.6 uMol/gr in the roots and 881.6-1139.6 uMol/gr in the leaves (data not shown). The highest amount of H₂O₂ was determined in susceptible genotype Atlı 2002 and the lowest in the tolerant genotype Kırkpınar 79 in both tissue types. The order of genotypes was as follows: Atlı 2002 > Aksel 2000 > Kırmızı Kılçık > Kırkpınar 79, in both tissue types (Fig. 2). MD and HD applications increased H₂O₂ in both tissue types compared to control. The main effects of application and genotypes and their interaction effects were very important on the amount of H₂O₂ in both roots and leaves ($p < 0.01$).

H₂O₂, known as a ROS, was first thought to be the major redox metabolite in redox detection, signalling and regulation. However, in subsequent investigations, it has been found that H₂O₂ acts as a signalling molecule to initiate cellular events such as cell shape changes and initiation of proliferation by taking in cells and tissues and thus serves as a basic regulatory function beyond the role of damage signalling in metabolism (Sies 2017). Therefore, it can be said that the reason for the increase in the amount of H₂O₂ as stress dose increases is to strengthen tolerance to stress. Variation in the amount of H₂O₂ of the genotypes according to the applications caused a significant effect of application x genotype interaction in both tissue types (Fig. 2).

When the effects of ABA and HA were examined, it was found that ABA caused a near but insignificant increase and HA application caused a higher increase compared to control on both tissues. Also, both of them caused an increase in drought stress conditions. In different studies consistent with our data, they explained the reason for the increase in H₂O₂ amount with ABA application that H₂O₂ acted as a downstream signal during ABA signalling (Zhang et al. 2007). Also, Kaur et al. (2014) suggested that the increase in H₂O₂ content in the application of drought stress with ABA was due to the lack of ABA-dependent mechanism in some genotypes. Besides that, Garcia et al. (2012) determined that the application of HA increases H₂O₂ production in the roots and leaves of rice. It is thought that H₂O₂ acts as a signal molecule to activate the stress response pathway and increase tolerance to stress.

Q-PCR analyses

According to the results of TaSRG gene mRNA expression analysis, there was an increase in expression rate in both MD and HD applications. Compared to leaves, tolerant genotypes have higher expression rates than sensitive genotypes in root tissue (Fig. 3). Similar to these findings, Rana et al. (2015) stated that TaSRG expression ratio of the salt tolerant genotype was higher than the sensitive genotype, also the expression rate of this gene was less in the leaf compared to root in salt stress. In Arabidopsis, when the TaSRG gene was upregulated, it was determined that Na²⁺ ratio was low and K⁺/Na²⁺ ratio was quite high. Although TaSRG gene is known to be a salt response gene, upregulation in drought stress in this study indicates that both types of stress use similar stress pathways. In our study, it was determined that the application of ABA alone caused upregulation of TaSRG gene in both tissue types at very high values. Similarly, He et al. (2011) stated that the ABA applied to the salt-tolerant mutant RH8706-49 wheat genotype caused upregulation of the TaSRG gene. In addition to this, when ABA applied with MD and HD, TaSRG gene rather upregulated compared to stress applications alone (Fig. 3).

It is known that externally applied ABA acts as a signal molecule to increase the level of internal ABA during stress and it provides a stronger acclimation against stress (Nemoto and Sasakuma 2002). In our promotor analyses, we determined ABA related ABRE, ABRE3a and ABRE4 elements and drought related DREcore ve DRE1 elements (Table S1). Although the mechanism which TaSRG uses ABA-dependent or ABA-independent pathways is not yet known, it can be said that the increase of expression of this gene

may be due to the presence of ABRE elements. Indeed, Nemoto and Sasakuma (2002) found that the expression of the WESR3 gene was increased by ABA application and that only the ABRE element was present in the promoter region of this gene. Apart from this, HA application significantly increased TaSRG gene expression in both tissues (Fig 3). Although there is very little literature on the effect of HA on genes, it can be explained by the fact that HA increases TaSRG gene expression by acting as a signal molecule and showing hormone-like activity. TaCP gene was upregulated in all genotypes in drought stress applications in both tissues (Fig 4). Simova Stoilova et al. (2010) supports our study that the proteolytic activity of TaCP gene encoding cysteine proteases increased in wheat under water stress.

Cysteine protease enzyme activity increases in response to oxidative stress and plays a role in the protection of cell metabolism by degrading denatured proteins under stress conditions or increasing protein turnover (Grudkowska and Zagdańska 2004; Kidrič et al. 2014). Therefore, an increase in proteinase activity during abiotic stress can be considered beneficial to plants. In our study, the TaCP gene expression rates determined in MD and HD applications in tolerant genotypes were found to be very low compared to sensitive ones in both tissues (Fig. 4). Similarly, Hieng et al. (2004), demonstrated that proteinase enzyme activity was more in drought-susceptible *Phaseolus vulgaris* L. genotype. This can be explained that some of the proteins required for growth and development under normal conditions are broken down into amino acids for use as building blocks for the synthesis of proteins required under stress conditions. In ABA treatments, there was a discrepancy among the tolerant and susceptible genotypes in both tissues (Fig. 4). Fukayama et al. (2010) reported that ABA increased protease enzyme activity in rye leaves. Poret et al. (2017) stated that ABA had an important role in increasing protein degradation and cysteine proteinase activity in *Brassica napus* during senescence, whereas ABA applied externally increased the level of intrinsic ABA. In drought stress conditions, ABA treatments had a variance between genotypes and tissues. In our cis-element analysis, the presence of ABRE element and gibberellin response element (P-BOX) in the promoter region of TaCP gene showed that expression of this gene may be due to ABA and gibberellin (Table S2). Also, we found that HA application caused to upregulation of TaCP gene in low levels especially in roots of tolerant genotypes (Fig. 4). Humic substances having low molecular weight are known to interact with proteins located in plasma membranes and contribute to protein synthesis in plant tissues due to their conformational flexibility and hydrophilic properties (Nardi et al. 1996, 2012; Vaccaro et al. 2015). So it can be thinkable that HA may contribute to TaCP gene expression in this way.

In our study, it was determined that Sal1 gene upregulated in susceptible genotypes and downregulated in tolerant genotypes in drought applications in roots, while it upregulated that only in susceptible Kirmızı Kılçık genotype in leaves (Fig. 5).

Downregulation of Sal1 in tolerant genotypes shows that genotypes are preserved in stress tolerance mechanism. Sal1 is one of the genes that act as a negative regulator in the formation of stress resistance in the abiotic stress mechanism. In addition to drought, Sal1 gene had the tolerance to stress by negatively regulating the transcriptional and metabolic pathways in salt stress (Xiong et al. 2001), cadmium stress (Xi et al. 2016) and cold stress (Quintero et al. 1996). Also, we determined that ABA

application alone caused the Sal1 gene to be downregulated a negligible value in the root and leaf tissues of Aksel 2000, Kirkpınar 79 and Atlı 2002 genotypes, but is upregulated in Kırmızı Kılçık genotype (Fig. 5). Xiong et al. (2004), determined that Sal1-deficient mutants showed a high degree of sensitivity to ABA. The Sal1 gene, also called the Fry1 protein, is a negative regulator of the ABA signal. Chen et al. (2011) found that ABA caused to increase of Fry1 gene expression in Arabidopsis and that they used the same metabolic pathway with ABH1 (ABA hypersensitivity), which is one of the ABA regulators of Fry1 gene. It is known that the Sal1 gene mostly acts as a negative regulator of the ABA-independent pathway and in part the ABA-dependent pathway. The absence of an ABA-related cis-element of the Sal1 gene in our promoter region analyses indicated that this gene uses the ABA independent pathway in the stress mechanism (Table S3). However, it was determined that HA application caused to downregulation of Sal1 gene in both tissue types in all genotypes (Fig. 5). This may be related to the glutathione mechanism because of humic components increase the content of organic metabolites such as alanine, proline and glutathione under abiotic stress conditions.

The Era1 gene analyses showed that it downregulated in drought stress applications in tolerant genotypes and upregulated in susceptible genotypes (Fig. 6).

It was observed that water loss in Arabidopsis by loss of function of Era1 and there were some changes in growth and development events (delay of senescence, change of apical and flora meristem, decrease in seed yield etc.,) (Donetta et al. 2000). The Era1 gene is known as a gene that acts as a negative regulator of ABA, which reduces plant susceptibility to ABA. Essentially, protein farnesylation is most widely accepted in responses to ABA in plants, since plants which have a mutation in protein farnesylation have been found to have a better response to ABA (Goritschnig et al. 2008). Also, the usefulness of Era1 as a biotechnological target for drought tolerance was investigated in species such as canola (Wang et al. 2005), wheat (Manmathan et al. 2013) and soybean (Ogata et al. 2017). In our study, it was determined that ABA caused to downregulation of Era1 gene at very high rates in tolerated genotypes in both tissue types. As a matter of fact, the presence of ABRE element in the promoter region of the Era1 gene in the cis-element analysis is an indication that the Era1 gene is involved in the ABA-dependent mechanism (Table S4). It is known that the functional loss of the Era1 gene increases the sensitivity of the guard cells involved in the closure of stomata to ABA. Indeed, Allen et al. (2002) showed that Arabidopsis Era1 mutants had less fading under drought stress and externally treated ABA resulted in a tighter stomatal closure. In our study, it was determined that HA caused to downregulation of Era1 gene in tolerant genotypes and upregulation in sensitive genotypes in both tissue types (Fig. 6). So, it can be said that HA application causes downregulation of Era1 gene in tolerant genotypes in this way it prevents water loss due to transpiration by closing the stomata. Huang et al. (2013) found that a stronger stoma closure occurred with drought stress, which is mediated by HA's activity on stomata during stress.

According to TdPIP1 gene expression analysis results, it was determined that TdPIP1 gene upregulated in all genotypes in leaf tissue, whereas it upregulated in tolerant genotypes and downregulated in sensitive genotypes in drought stress applications (Fig. 7).

Similar to our study Lian et al. (2006) reported that PIP1;5, PIP2;2, PIP2;3 and PIP2;6 genes downregulated while PIP1;4 and PIP2;5 genes upregulated in Arabidopsis in drought stress. So it can be said that up/downregulation status of aquaporin genes against drought stress varies according to plant type, tissue type and genotypes. Aquaporins are integral membrane proteins that transport water and small molecules such as CO₂ and glycerol from membranes and PIPs protect the cell membranes by preventing ROT accumulation in the stress mechanism (Tian et al. 2016). Similar to our study, it has been determined that abiotic stress factors such as drought, salt, cold stress cause upregulation of aquaporin genes and tolerance to stress are provided in previous studies (Gao et al. 2010; Sade et al. 2010). In our study, it was determined that ABA treatments were caused to upregulation of TdPIP1 gene in all genotypes in leaf tissue and it upregulated in tolerance genotypes and downregulated in susceptible genotypes in root tissue (Fig. 7). It has been proven that externally applied ABA enhances water conductivity in the roots and changes the expression of aquaporins in plants such as sunflower, corn, Arabidopsis, tobacco (Siefritz et al. 2001). The up/downregulation of the aquaporin genes varied in these ABA treated plants. So it can be said that genes are associated with both ABA-dependent and ABA-independent signalling pathways. In addition to ABA, hormones such as gibberellin, salicylic acid, ethylene, methyl jasmonate, etc., have a role in stress tolerance mechanisms. Hu et al. (2012) found that ABA inhibited the expression of TaAQP8 gene in tobacco, whereas gibberellin and ethylene induced the gene expression. Therefore, aquaporin genes are dependent on not only ABA but also other hormones. In fact, in our TdPIP1 promoter analysis, both ABRE and TGACG/CGTCA-motif (MeJa-dependent cis-element) and P-Box (gibberellin-dependent cis-element) cis-elements confirm this opinion (Table S5). Also, in our study HA caused to upregulation of TdPIP1 gene in tolerant genotypes and downregulated in susceptible genotypes (Fig. 7). It is thought that the application of HA causes to decrease in the amount of ROS and increase in the antioxidant enzyme activity, thus contribute to the stress tolerance mechanism.

Conclusion

In conclusion, stress effects can be improved with ABA and HA applications, especially in tolerant genotypes, and HA has a healing effect that competes with ABA by regulating the signal in various structural and functional mechanisms in the plant. Considering all the analyzes in our study, it was determined that the most tolerant genotype was Aksel 2000 and the most sensitive genotype was Kırmızı Kılçık in both root and leaf tissues. Thus, wheat genotypes that can be used in future stress tolerance studies were determined. In addition, it was showed that application of HA from the leaves against drought stress in natural conditions would be effective in stress tolerant wheat cultivation.

Supplementary Material

Table S1. The cis-acting elements present in the promoter region of the TaSRG

Table S2. The cis-acting elements present in the promoter region of the TaCP

Table S3. The cis-acting elements present in the promoter region of the Sal1

Table S4. The cis-acting elements present in the promoter region of the Era1

Table S5. The cis-acting elements present in the promoter region of the TdPIP1

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