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Abscisic acid regulated gene expression in bread wheat (*Triticum aestivum* L.)

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Abstract

The studies about the elucidation of molecular components of drought response in major crops have become an urgent priority. Since abscisic acid (ABA) is one of the major plant hormones influencing main stress responses including drought, cold, and heat, there has been overwhelming interest in investigation of ABA-associated genes. Therefore, the identification and characterization of ABA-associated genes from bread wheat is significant for gaining better insights about the multiple stress-response mechanisms. Due to the pivotal role of ABA in stress response mechanism, we tried to determine spatiotemporal expressional characteristics of ABA-related genes in wheat. The comparative differential transcript profiling experiments revealed a total of 60 differentially-regulated cDNA fragments, thirty of which were sequenced to obtain more details about their functional identities. Some of the sequenced fragments showed significant similarity with the genes known to be associated with ABA-induced signaling networks; e.g., Germin-Like Proteins, Tonoplast Intrinsic Proteins, MAP kinases and leucine-rich repeat (LRR) receptor-like proteins. To get more details about ABA-associated genes in wheat, the expressional characteristics of the transcripts from the initial screening was further investigated by using quantitative Real Time PCR. For example, the amount of transcripts *MAPK4* and *TIP1* homologs in leaf tissue of bread wheat is gradually increased and eventually peaked in about 2 hours after ABA treatment, and subsequently declined with the prolonged exposure. On the other hand, induction in the *GLP* homolog expression was much faster than the aforementioned transcripts; reaching to the apex in roughly an hour after the treatment. This study provided insights about the expressional characteristics of some ABA-associated genes from bread wheat. This is the first study investigating the early response genes under ABA-treatment in hexaploid bread wheat.

Keywords*: Triticum aestivum L.;* drought; abiotic stress; mRNA Differential Display; transcript profiling, Abscisic Acid, quantitative Real Time PCR; MAP Kinase; Tonoplast Intrinsic Protein 1; Germin-Like Protein 1
Abbreviations*:* ABA_Abscisic Acid; MAPK_Mitogen Activated Protein Kinase; *TIP1*_Tonoplast Intrinsic Protein-1*; GLP1*_Germin Like Protein-1; α-EXPA2_alpha-Expansin-2; mRNA DD_mRNA Differential Display; qRT-PCR_Quantitative Real Time PCR

Introduction

Triticum aestivum L. (modern bread wheat) is one of the major cereal crops cultivated in the world (Lagudah et al. 2001) accounting for 58.1% of total grain production in 2008. Of all the abiotic stresses that reduce crop productivity, drought is one of the most important environmental constraints for agriculture (Moussa and Abdel-Aziz, 2008; Zaidi et al., 2008). ABA is a universal plant stress hormone regulating drought response, which acts through the regulation of stomatal aperture and by triggering the expression of downstream drought-related genes. ABA is also the major internal signal enabling plants to survive adverse environmental conditions such as salt and cold stress (Marcotte et al., 1992; Koornneef et al., 1998; Taji et al., 2004). Besides stress response mechanism, ABA is also involved in the regulation of plant growth and development (Zhu, 2002; Verslues and Zhu, 2005; Sheard and Zheng, 2009). Because of the major role of this hormone in both abiotic and biotic stress response mechanisms, gaining insights about the ABA-associated pathways in different plant species is important (Pennisi, 2009). Therefore, the identification and characterization of ABA-regulated genes is helpful in terms of improving our knowledge about stress response mechanism in wheat.The treatment of plants with exogenous ABA creates similar symptomal effects to drought stress (Zeevard and Creelman, 1988; Trewavas and Jones, 1991; Davies et al., 1991, 1994). ABA-dependent and -independent pathways of drought response mechanism in many plant species has been extensively studied (Yamaguchi-Shinozaki and Shinozaki, 2005). Moreover, overlap of several stress regulatory pathways such as cold, salinity, and drought has been suggested, therefore supporting the interconnectedness of these responses; i.e., stress signals and ABA-dependent pathways share common elements in the signal transduction (Schroeder and Hagiwara, 1989; Leung and Graudat, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein and Lynch, 2000; Finkelstein et

al., 2002; Rabbani et al., 2003). Thus the identification of the transcripts differentially induced by exogenous ABA-treatment would allow to the identification of the genes that could play role in multiple-stress response pathways.The main purpose of this study was the identification and the characterization of the transcripts induced by exogenous ABA-treatment from modern bread wheat (*Triticum aestivum* L.) through mRNA Differential Display (mRNA DD) profiling approach. In more detail, this study involves two main parts: 1) Screening of the ABA-associated genes in bread wheat by comparative analysis of transcript profiles of the plants treated with 50 µM ABA and untreated control) More detailed characterization of the expressional properties of the transcripts identified from the first screen by quantitative Real Time PCR (qRT-PCR).

Materials and methods

Plant Material

Widely-cultivated drought-sensitive, hexaploid bread wheat, *Triticum aestivum* cv. Atay 85, provided by Anatolian Agricultural Research Institute (Eskisehir, Turkey), is used as plant material. The seedlings were grown under white fluorescent light, 108-135 µmol m^{-2} s^{-1} at 25 ^{o}C with 65% relative humidity on pots containing special soil mixtures. The plants were watered with Hoagland solution for every day during 8h light/16h dark cycles. For the ABA treatments, ABA was added into Hoagland solution with a final concentration of 50 µM and 100 µM, and applied to the pots directly for every other day after the germination. The leaf samples from the ABA-treated and the untreated 4-week-old plants were harvested, flash-frozen in liquid nitrogen, and stored at –80 ^{o}C until being used for RNA isolation. For qRT-PCR analyses, plants were grown for four weeks and leaf tissues were harvested at different time intervals (control, 1, 2, 4, 8, and 24h) after the treatment with 50 µM ABA.

RNA Isolation and cDNA Synthesis for mRNA DD

Total RNA was isolated from whole leaf tissues by using Plant RNeasy extraction kit (Qiagen USA, Valencia, CA, USA) according to the manufacturer's instructions. The RNA samples were treated with 10 U of RNase-free DNaseI (GenHunter, Message Clean Kit) for an hour at 37 ^{o}C to remove genomic DNA; which is followed by phenol chloroform (3:1) extraction and ethanol precipitation. Finally, DNA free total RNA samples were resuspended in 20 µl DEPC-treated ddH_2O and were quantified with spectrophotometer (Nanodrop, ND1000). Three reverse transcription reactions were prepared for each RNA sample. Each reaction contained 0.2 µg DNA-free total RNA, 1X Reverse Transcription Buffer, 20 µM dNTP, and 4 µM of either one of the $HT_{11}M$ anchored primers, where M is G, A or C, in total volume of 20 µl (Table 1a). After incubation at 65 ^{o}C for 5 min, each reaction mixtures was cooled to 37 ^{o}C for 10 min, before the addition of 200 U of MMLV-Reverse Transcriptase (GenHunter, Nashville, TN). Finally, the reverse transcription reactions were conducted at 37 ^{o}C for 50 min, and then the enzyme is denatured at 75 ^{o}C for 5 min without damaging mRNA/cDNA duplex. The samples obtained from this step are directly used for the mRNA DD reactions.

Table 1A. List of anchored primers

Primer	Sequence
H-T_{11}G	5'-AAGCTTTTTTTTTTG-3'
H-T_{11}A	5'-AAGCTTTTTTTTTTA-3'
H-T_{11}C	5'-AAGCTTTTTTTTTTC-3'

Table 1B. List of arbitrary primers of mRNA DD.

H-AP49	5'-AAGCTTTAGTCCA-3'
H-AP50	5'-AAGCTTTGAGACT-3'
H-AP51	5'-AAGCTTCGAAATG-3'
H-AP52	5'-AAGCTTGACCTTT-3'
H-AP53	5'-AAGCTTCCTCTAT-3'
H-AP54	5'-AAGCTTTTGAGGT-3'
H-AP55	5'-AAGCTTACGTTAG-3'
H-AP56	5'-AAGCTTATGAAGG-3'
H-AP17	5'-AAGCTTACCAGGT-3'

mRNA Differential Display

mRNA DD experiments were carried out according to the method described by Liang and Pardee (1992) with minor modifications (Cevher Keskin, 2006) with RNAmap kit (GenHunter, Nashville, TN) as directed by the manufacturer. Each reaction mixture contained 2 µl of the reverse transcription reaction samples from the previous step, 2 µM dNTP, 1X PCR Buffer, 4 µM of one of the $HT_{11}M$ anchored primers (Table 1b), 4 µM of one of the different specific arbitrary 10-mer oligonucleotides (Table 1b), α^{33}-P dATP and 1 U Taq DNA polymerase (Qiagen, Valencia, CA, USA) in a total volume of 20 µl. The thermal cycling parameters were: 94 ^{o}C for 30 s, 40 ^{o}C for 2 min, 72 ^{o}C for 30 s for 40 cycles, followed by a final extension step at 72 ^{o}C for 5 min. For denaturation, after addition of an equal volume of "DNA sequencing stop buffer", the amplicons were incubated at 80 ^{o}C for 5 min. Finally, 2 µl of the denatured products were loaded on 6% denaturing polyacrylamide gel and the electrophoresis was performed at 60 W until xylene-cyanol dye reached to the bottom of the gel. The gels were dried with a gel drier at 80 ^{o}C for an hour and exposed directly to Kodak BioMax MR Film at least 72 hrs at –80 ^{o}C.

Reamplification of cDNAs

The differentially expressed cDNA fragments were excised from the dried gels and eluted by boiling in ddH_2O for 10 min. After ethanol precipitation, the cDNA fragments were reamplified in a 40 µl reaction volume by using the same primers and the same reaction conditions as in the mRNA DD experiments with 10 times more dNTP. The amplicons were checked by running on 2% (w/v) agarose gels.

Cloning and Sequencing of cDNAs

The reamplified fragments ranging in size between 350-550 bp were cloned into PCR-TRAP vector (GenHunter, Nashville, TN). The inserts were amplified with universal primers to verify the fragment sizes, and the clones with inserts of 350-550 kb in size were considered for further analysis. A total of 30 positive clones with the desired insert sizes were sent for sequ-

Table 2. Up and down regulated genes with significant homology to the annotated sequences in GenBank by Blastn analysis in *T. aestivum* cv.Atay ESTs transcriptionally regulated during ABA treatment

Clone	GenBank Accn	Annotation	Organism	Expression*	E-value
BCK1	FG203253	No match		+	
BCK5	FG203273	dbj|AB016801.1| *MAPK-4*	*Zea mays*	+	1.00E-17
BCK6	FG203274	No match		+	
BCK8	FG203275	gb|GH730788.1| Unknown	*Triticum aestivum*	-	1.00E-78
BCK9	FG203276	AY589584 α -ekspansin *EXPA2*	*Triticum aestivum*	+	6.00E-28
BCK10	FG203254	emb|Y15962.1|Germin-Like Protein-1 (*GLP1*)	*Hordeum vulgare L.*	-	2.00E-24
BCK11	FG203255	No match		+	
BCK12	FG203256	dbj|AP008209.1|Unknown	*Oryza sativa*	+	1.00E-12
BCK16	FG203260	X87686 Leucine Rich Like Protein	*Aegilops tauschii*	+	1.00E-87
BCK17	FG203261	No match		-	
BCK19	FG203263	AL816094 Unknown	*Triticum aestivum*	+	2.00E-85
BCK21	FG203265	No match		+	
BCK22	FG203266	gb|AF497474.1| leucine-rich-like protein gene	*Aegilops tauschii*	-	2.00E-87
BCK23	FG203267	HD95L20 cytosolic acetyl-CoA carboxylase (*Acc-2*) and putative amino acid permeases genes	*Aegilops tauschii*	+	5.00E-105
BCK24	FG203268	U86762.1 γ-Tonoplast Intrinsic Protein (*TIP1*)	*Hordeum vulgare*	+	3.00E-32
BCK25	FG203269	No match		+	
BCK27	FG203271	AK252878 Unknown	*Hordeum vulgare*	-	3.00E-44

* Down-regulated (-) or up-regulated (+) gene expression as determined by mRNA DD

encing (www.iontek.com.tr). Seventeen sequences were obtained in sufficient quality to be considered for further studies. The sequences were compared by using BLAST algorithm with different online publicly available wheat nucleotide and protein sequence databases from the sites such as NCBI (www.ncbi.nlm.nih), TIGR (www.tigr.org), and Gramene (www.gramene.org) to elucidate the functional identities.

cDNA Synthesis for qRT-PCR

The relative transcript levels of three transcripts identified as a result of mRNA DD analyses, *MAPK4, GLP1*, and *TIP1* genes in leaf tissues were investigated with qRT-PCR. The leaf samples were harvested from 4-week-old plants at 1, 2, 4, 8, and 24h after the treatment with 50 µM ABA. Total RNA was isolated from whole leaf tissues by using Plant RNeasy extraction kit (Qiagen USA, Valencia, CA, USA). 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 was quantified with spectrophotometer (Nanodrop, USA). After checking the integrity on formaldehyde agarose gel electrophoresis, the RNA samples with sufficient integrity and quality was used as template for cDNA synthesis with MMLV reverse transcriptase (Roche Applied Science GmbH, Germany). The efficiency of cDNA synthesis reactions were assessed through the use of wheat β-actin primers for end-point RT-PCR (More details about the primer sequences could be seen at Table 3).The primers for qRT-PCR were designed from the sequenced cDNA fragments by using Fast PCR program (Kalendar et al., 2009; www.biocenter.helsinki.fi/bi/Programs/) (Table 2). Each RT-PCR reaction was set up in total volume of 25 µl, containing 12.5 µl SYBR Green PCR SuperMix (BioRad Laboratories, Hercules, USA Laboratories, Hercules, USA), 10 pmole of each primer and 75–200 ng of the cDNA. qRT-PCR reactions were carried out with an IQ5 System (BioRad Laboratories, Hercules, USA Laboratories, Hercules, USA) with cycling parameters: 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. Each cDNA sample was analyzed in three different reactions with three technical replicates and negative controls. In order to determine, if there are significant differences in expression at the different time points, three biological replications have been performed for each transcript. The relative abundance levels of *MAPK4*, *GLP1*, and *TIP1* transcripts for different reactions were normalized with respect to the loading standard, β-actin. The relative fold expression differences were calculated by using the comparative CT method (Schmittgen et al., 2000). The ΔCT values for all of the transcripts were averaged across all treatments and experimental replicates. Finally, Student's t-test was applied to check for the statistical significance between ABA-treated and –untreated groups.

Table 3. List of Real Time PCR primers

ID No	Source	Gene Symbol	Primer seq (5'-3')	Product Size
AY663392	NCBI**	β-Actin	F-GACAATGGAACCGGAATGGTC R-GTGTGATGCCAGATTTTCTCCAT	352bp
TA63689_4565	TIGR*	*MAPK4*	F-CGTACCTAGAGCGGCTTCACGA R-GGTTTGAAGAAGCAGCAACAA	298bp
U86762.1	NCBI**	γ-TIP (*TIP1*)	F-GGAGATCGTGATGACCTTCG R-CTGCTCAGTAGTCGGTGGTG	320bp
Y15962.1	NCBI**	*GLP1*	F-ACACCGTGTACACCAAGACG R-CGGACTTGAGCTTCTTCACC	250 bp

*www.tigr.org; **www.ncbi.nlm.nih

Results

Exogenous ABA-Treatment of Triticum aestivum Atay 85 Plants and Detection of ABA-Associated Genes in Leaves

Two different concentrations of ABA, 50 µM and 100 µM, were exogenously applied to bread wheat. The amount of ABA was based on the previously reported studies (Mohapatra et al. 1988). The plant growth is severely hindered at both concentrations of ABA, 50 µM and 100 µM, (Fig 1), showing very similar physiological symptoms as in drought-treatments. A commonly grown hexaploid bread wheat cultivar, Atay 85, was chosen as the experimental material, which is reported as drought sensitive (Taş and Taş, 2007). Since the plants treated with 100 µM ABA were more severely stunted, we used leaf tissues from the plants treated with 50 µM ABA to identify and characterize e up- or down-regulated or transcripts. A total of 27 different combinations of arbitrary and anchor primers were used for mRNA DD experiments (Table1a, b). At the end of mRNA DD experiments, , we obtained about 180 differentially induced bands and 111 of which was up-regulated, while 69 of the bands were either absent or suppressed in the experimental samples (Fig 2, Fig 3a, b, c, d). Among the differentially-expressed bands, 40 cDNA bands were selected primarily on the basis of their lengths (250-550 bp), only 30 of which were cloned and seventeen of the cloned fragments were sequenced. Six sequences out of 17 did not show any significant match (E-value $\leq 10^{-7}$) to any sequence in the screened databases; two of them were similar to unknown proteins, and the remaining shared important sequential identity with known genes (Table 2). Among those that showed high sequential identity with known genes such as expansin, germin-like proteins, protein kinases, NBS-LRR containing receptor-like proteins, glutamyl tRNA reductase, and tonoplast intrinsic protein were selected as the main interest for further research. Even though most of these genes could be associated with ABA-response mechanism, only three of them, MAP kinase, tonoplast intrinsic proteins and germin-like protein, were chosen to analyze in terms of their temporal expressional characteristics in leaf tissues on the basis of previous studies in other plant species. The genes selected for more detailed expressional analysis are likely to be related to drought stress response and ABA-induced pathways (Casson and Gray, 2008; Ruiz-Lozano et al., 2009). Hence, RT-PCR primers were designed from the sequences, retrieved from NCBI and TIGR, that showed high level of sequential identity *MAPK4:* E-value: $1e^{-17}$(TA63689_4565), *γ-TIP* E-value: $3e^{-32}$(U86762.1) *GLP-1* E-value: $2e^{-24}$ (Y15962.1) with the sequences identified from the initial mRNA DD experiments (Table 2). The expression levels of each gene at

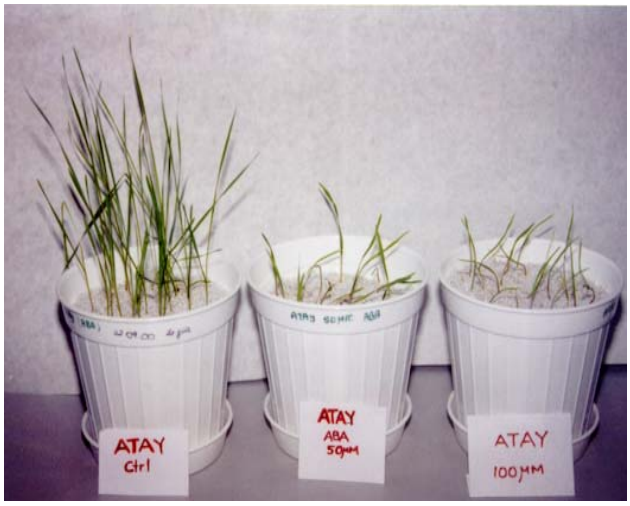

Fig 1. Wheat, *Triticum aestivum* cv. Atay 85, plants were grown under white fluorescent light 108-135 µmol m-2 s-1 at 25 °C with 65% relative humidity. ABA was added into Hoagland solution at final concentration of 50 and 100 µM and applied to the pots directly for every other day. The leaves from ABA-treated and -untreated (Ctrl) 4-week-old plants were harvested and used for RNA isolation.

1st,, 2nd, 4th , 8th and 24th hours after the exogenous application of 50 µM of ABA is checked with qRT-PCR (Fig. 3a, 3b, and 3c).

Expression Pattern of Mitogen Activated Protein Kinase-4 (MAPK-4) in ABA-treated wheat

The significant differences between untreated control and ABA-treated ones at the time points 1, 2, 4, 8 and 24 hours were observed ($p \leq 0.01$). The results denoted that MAPK4 transcript was significantly up- regulated in response to ABA after 2h and then decreased gradually during 4th h and 8th h of the ABA treatment (Fig 4a).

Expression Pattern of γ-Tonoplast Intrinsic Protein-1 (TIP1) in ABA- treated wheat

Maximum *TIP1* expression level was observed two hours after the ABA application and then decreased (Fig 4b). There was

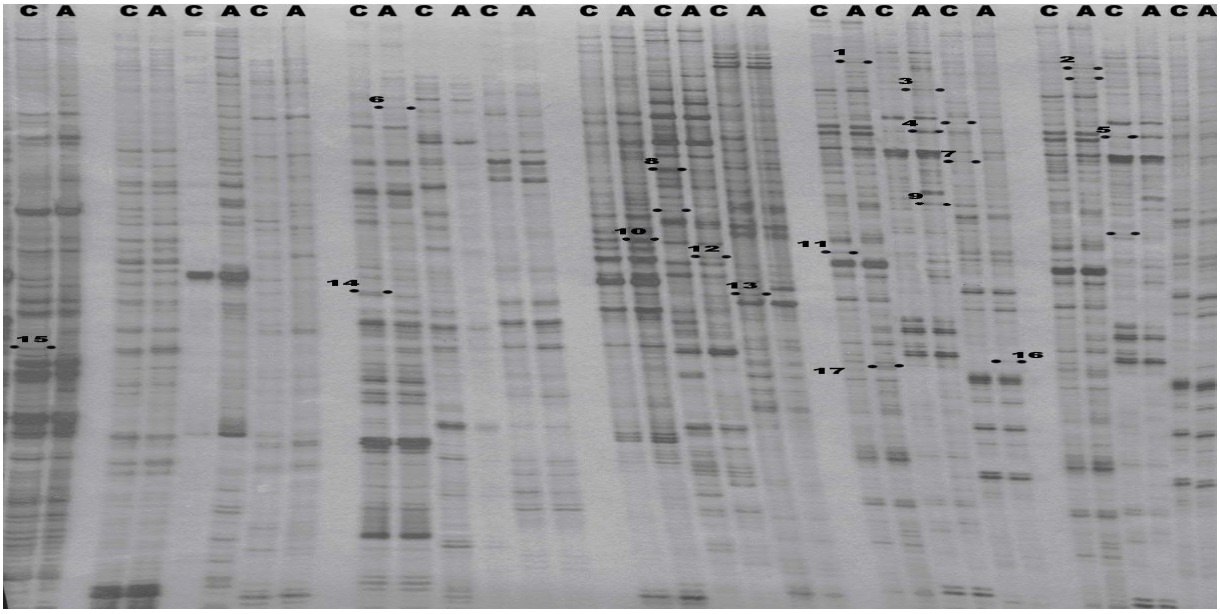

Fig 2. A picture from mRNA DD analyses Total RNAs were isolated from leaves 4-week-old plants. mRNA DD method was performed with RNA map kit (GenHunter, Nashville,TN) with the combination of a anchor pirmerT_{11}MN and other arbitrary 10-mer primer. The amplification products were incubated at 80 ^{o}C for 5 min and loaded on 6% denaturing polyacrilyamide gel. The lanes from the control samples are labeled with "C" and the lanes from the ABA treated plants are labeled with "A". The dots on the picture indicate differentially expressed cDNA fragments. The numbers; 1, 2, 3, 4, 6, 9, 10, 12, 16, 17, depict up-regulated; and 5, 7, 8, 11, 13, 14, 15, indicate down-regulated cDNA fragments

significant difference at the expression levels of this gene between control and experimental control groups 2nd, 4th, 8th and 24th hours of the application ($p \leq 0.01$, Student's t test) ($p \leq 0.01$).

Expression Pattern of Germin Like Protein-1 (GLP1) in ABA-treated wheat

mRNA levels of *GLP1* in ABA-treated plants rapidly increased in 1h and maximal expression level was reached in 8h (Fig 4c). The significant differences between un-treated control and ABA-treated plants were observed at 4th hr ($p \leq 0.05$) and 8th hr ($p \leq 0.01$). The expression level of this gene dropped significantly after 8th hour of ABA-treatment, and at the end of 24th hour, the transcript level of this gene was not significantly different than the control plants. The temporal expression pattern of *GLP1* gene was different than the other two genes, *TIP1* and *MAPK4*. It was induced by ABA much later, 8 hour of the treatment, and induction time was much shorter.

Discussion

The unraveling of ABA-associated genes starting from the perception stage to the downstream elements has been one of the most intensively studied subjects in plant signaling, because of their involvement in myriad biological processes (Pennisi 2009; Bray 2002). Therefore, the identification and characterization of the genes induced upon exogenous application of ABA in wheat would allow us to develop wheat crops that could resist better to water shortage. Our observations about the effect of the ABA to plant growth were similar to the prior studies on wheat (Ober and Setter, 1990; Ahmadi and Baker, 1999). We chose to use 50 µM ABA, in our treatments, which was the preferred concentration in prior similar studies in other plant species. The majority of the genes identified as a result of the differential display studies were from functional categories; cell wall restructuring or biogenesis associated genes such as expansins, oxidative stress associated genes such as GLPs, transpiration minimizing genes such as ACCs (Acetyl-CoA Carboxylase), osmotic stress induced genes such as TIP, ABA and other stress signaling pathway genes such as MAP kinases, and unknown proteins. ABA-dependent-pathway in drought stress response mechanism in many plants works through the limiting water loss by constricting of stomatal apertures. In other words, drought induces the genes that regulate stomatal distribution on leaf surface and the genes encoding for the characters influencing transpiration rate from leaf surface. For example, the relative abundance of ACC, a key enzyme in lipid metabolism, was significantly increased in drought-tolerant peanut genotype (Kottapalli et al., 2009). Likewise, epicuticular wax deposition on leaves of different plant species was significantly increased in drought tolerant genotypes with abiotic stresses (Mills et al. 2001; Shepherd and Griffiths, 2006). Thus, one of the transcripts, ACC-related (*Acc-2*) gene (Table 2), identified in this study, could be directly associated with drought response in wheat. However, a closer look into the spatiotemporal expressional characteristics of this gene would be required to reach more definite conclusion about the functional identity of this gene. One of the isolated transcript shared high level of sequential similarity with *GLP* proteins (Table 2). GLP's are implicated in the multiple biological processes; e.g., superoxide scavenging metabolism and disease resistance against fungal pathogens (Hurkman and Tanaka, 1996; Zimmermann et al., 2006; Godfrey et al., 2007).

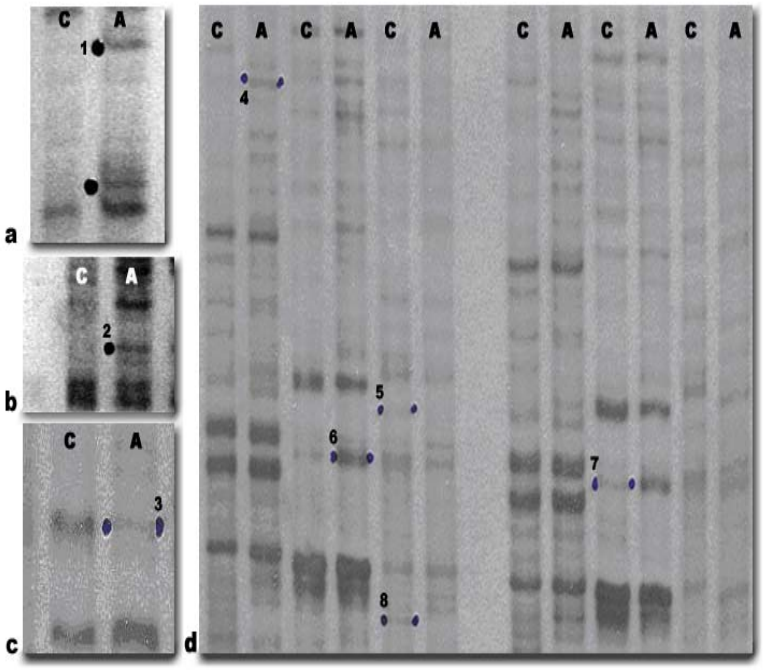

Fig 3. A gel picture from mRNA DD experiments.The lanes with label "C" are from control and with label "A" from ABA treated plants. The spots indicate differentially expressed cDNA fragments. (**a, b)** The bands labeled with 1 and 2 are up regulated cDNA fragments (*MAPK4* and *TIP1*-like cDNA fragments respectively); (**c**) The band labeled with 3 is down-regulated cDNA fragment (*GLP1*). (**d)** The bands labeled with 6 and 7 are up-regulated cDNA fragments, 5 and 8 indicate -down- regulated cDNA fragments

Furthermore, dismutase activity of GLPs from different plant species have been reported (Berna and Bernier, 1997; Tabuchi et al., 2003; Christensen et al., 2004). However, there are discrepancies between different studies about the effect of ABA hormone to the spatiotemporal expressional characteristics of GLPs in different plants. For instance, Tabuchi et al., (2003) found that the expression of GLP is suppressed in the leaves of a halophyte plant, *Atriplex lentiformis*, by ABA treatment. The apparent controversy between different studies could be explained by the fact that there are multiple copies of GLPs in plant genomes and differential spatiotemporal expressional characteristics of paralogs have been observed (Kim and Triplet, 2004; Federico et al., 2006; Dunwell et al., 2008). In other words, different copies of this gene could have diversified with regards to their expressional pattern and functional characteristics. We observed that the transcript level of *GLP1* gene in the leaves of four-week ABA-treated bread wheat plants was down-regulated. On the other hand, the transcript level of GLP gene was significantly induced relative to the control plants at the first hour of ABA treatment and the maximum level is reached at the eighth hour of the treatment (Fig 4c). In wild emmer wheat, Ergen et al., (2009) have shown that the expression level of GLP precursors decreased after 8 hour long drought stress. This apparent contradiction about the expression level of GLP could be explained by the presence of multiple copies of GLPs in wheat genome; i.e., the different copies of this gene could have non-overlapping functions with regards ABA and drought stress induced pathways. Furthermore, the association of ABA hormone level and the enhancement in SOD activity has been demonstrated in several plant species including maize and wheat (Hu et al., 2006; Xian-Wei Fan et al., 2009). Likewise, interconnectedness of ABA and H_2O_2-induced pathways has been also reported (Berna and Bernier, 1999; Wang and Song, 2008; Zhang et al., 2009). In this study, the initial hike in the level of GLP transcript till the 8^{th} hour of the ABA treatment could be explained by the fact that the high amount of exogenous ABA, 50 µM, could have resulted in the formation of superoxides, which, in turn, influence the SOD transcript level. In conclusion, GLP-like transcript identified in this study could have SOD activity. Another gene determined at the transcript profiling step was related to the genes encoding water channel proteins, i.e., aquaporins such as PIPs (Plasma Intrinsic Protein) and TIPs (Tonoplast Intrinsic Proteins). The direct associations between exogenous ABA hormone treatment and aquaporin expression level under several stress conditions including water limitation and ABA have been reported (Jang et al., 2004; Alexandersson et al., 2005; Li et al., 2008; Li et al., 2009; Ruiz-Lozano et al., 2009). Moreover, water stress was denoted to stimulate the transcription level of TIP1 in wild emmer wheat (Ergen et al., 2009). However, multiple copies of aquaporin genes are found in many plant genomes; for instance, rice genome contains 33 copies of aquaporins (Li et al., 2008), which hints a possible functional diversification of these genes. On the other hand, the transcript level of TIP-like gene described in this study increased significantly with the application of exogenous ABA (Fig 1) in comparison to control, thereby suggesting a possible involvement of this transcript in the ABA-dependent stress response pathways in hexaploid bread wheat. Unlike other two transcripts, *MAPK4* and *GLP1*, *TIP*-like protein induced sharply at the second hour of the ABA treatment, subsequently the level of the transcript dropped slowly, the reduction rate in

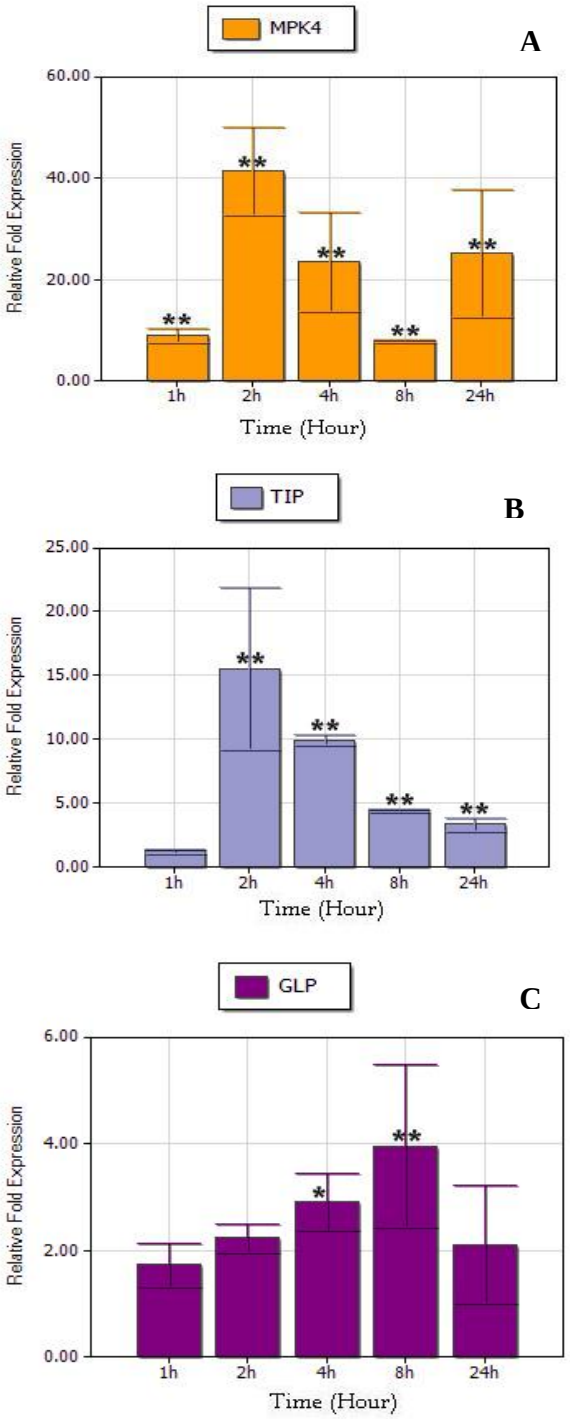

Fig 4. A, B, C: The effect of ABA treatment on the expression level of MAPK4, TIP1 and GLP1 mRNA levels in *T.aestivum* cv. Atay 85 leaves (h). MAPK4 (A), TIP1 (B),and GLP1 (C) β-actin was used as an internal control. The level of significance for the expression differences between control and experimental groups is determined with t-test. (*) depicts the level of significance p ≤0.05 and (**) depicts the level of significance p ≤0.01.

the transcript amount was slower than MAP kinase but faster than GLP-like gene (Fig 4c). This result could be due to hierarchical order of the cascade of ABA-induced genes. In plants, multiple MAPKs take place in signaling pathways. The control of stomata development and distribution pattern is the most critical part of the response mechanism to multiple environmental stresses including drought. The role of MAP kinases in stomatal development and distribution on leaf surfaces has been one of the most widely studied subjects (Bergman et al., 2004; Jonak et al., 1996; Jeong et al., 2006; Hwa and Yang, 2008; Jiang et al., 2008). For example, after the perception of ABA by the receptor molecule, the signal is transducted through *MAPK2* and *MAPK3* and a p38 like MAP kinase (Tena et al., 2001; Ichimura et al., 2002), which, in turn, modulates ROS dependent guard cell response resulting in stomatal redistribution and the constriction of apertures. In another study, it is reported that the last MAPKs in the cascade of signaling pathway 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). However, there are still many unknowns that need to be illuminated about the role of ABA on stomatal development and redistribution mechanism (Casson and Gray, 2008). The MAPK-like gene identified in this study was induced much faster in response to ABA treatment; i.e., only after an hour following ABA treatment, the transcript level in the leaf tissue was significantly different in the treated plants (Fig 4a). This result could be evidence for the possible role of this *MAPK4*-like gene in the ABA-induced pathways. Furthermore, MAPK cascade elements *MAPK1* and, *MAPK7,* are up-regulated in root or leaf tissues after 4 h dehydration (Ergen et al., 2009). In our study, *MAPK4* transcript was significantly up- regulated (approximately 4 fold) in response to ABA treatment after 2h and then gradually decreased during 4th h and 8th h. In conclusion, ABA is one of the most crucial hormones acting in many stress response related mechanisms including salinity, cold, disease resistance and drought (Sheard and Zheng, 2009). In other words, providing insights about the functional characteristics of ABA-associated genes would have an utmost importance in regards to the improvement of crop yield under adverse environmental conditions.

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