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Identification of differential expressed transcripts of almond (Prunus dulcis 'Sefied') in response to water-deficit stress by cDNA-AFLP

1   **Identification of differential expressed transcripts of almond (*Prunus dulcis var.***

2   ***Sefied*) in response to water-deficit stress by cDNA-AFLP**

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4

5   **Abstract**

6   Drought is the major abiotic stress with adverse effects on growth and productivity of plants. It induces

7   the expression of various genes that are involved in stress response and tolerance/sensitivity phenotypes.

8   In this study, the expression of several genes were analyzed in response to dehydration of almond (*Prunus*

9   *dulcis var. Sefied*) to shed light on underlying genetic basis of water-deficit tolerance in almond. The

10   advantages of using almond as a model system for studying dehydration tolerance in woody species

11   include its small diploid genome and its adaptation to drought. Differential expression technique cDNA-

12   AFLP (amplified fragment length polymorphism) was used to find transcripts accumulated in young trees

13   subjected to water-deficit treatment. Twenty transcript-derived fragments with differential expression

14   between control and stress conditions generated, amplified, and sequenced. The genes that had high

15   homology with those of known function were selected and further validated by quantitative real-time

16   PCR (qRT-PCR) and their possible function(s) were discussed. These genes include: 2-deoxyglucose-6-

17   phosphate phosphatase (*ABI2*), protein kinase *MK5* (*ACF2*) and Urease, which up-regulated by 1.61, 2.39

18   and 4.87 fold respectively under water-deficit stress condition. In addition, network analysis unraveled a

19   drought response mechanism displaying activation of ABA signaling pathway via phosphorylation by 2-

20   deoxyglucose-6-phosphate phosphatase and protein kinase *MK5*. Protein kinase *MK5* (*AFC2*) was a

21   central element in drought response network, displaying numerous interactions with RNA-splicing

22   proteins, sugar mediated signaling pathway and epigenetics response (histone phosphorylation).

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24   **Keywords:** cDNA-AFLP, Gene Network, *Prunus dulcis var. Sefied*, Transcript-derived fragments

25   (TDFs), Water-deficit stress.

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1 **Introduction**

2   Drought is a major abiotic stress stimulus that limits crop yields in several regions of

3 the world. Plants respond to drought through modification of their morphology and their

4 physiological and metabolic processes. Plant cells have evolved to perceive different signals

5 from their surroundings, to integrate them and to respond by modulating/repressing gene

6 expression. The products of these genes involved not only in stress tolerance but also in the

7 regulation of gene expression and signal transduction (Bartels and Sunkar 2005; Zhu 2001).

8   Several major classes of genes have been noted which alter in response to water-deficit

9 stress, including genes involved in signaling, regulation and encoding proteins that support

10 cellular adaptation to water-deficit stress (Hazen et al. 2003; Hazen et al. 2005; Ito et al. 2006;

11 Yamaguchi-Shinozaki and Shinozaki 2005).

12   Almond is one of the oldest nut tree crops, governing the largest commercial production

13 of nut tree nowadays. Almond species grow in regions of the world characterized as subtropical

14 Mediterranean climate, with mild wet winters and warm and dry summers. Iran, with a total land

15 area of 1,648,195 square kilometers, lies between $25°$ and $39°$ N latitude and $44°$ and $63°$ E

16 longitude with primarily subtropical in the southern half of the country, temperate in the northern

17 half, and mostly desert in the middle provides a suitable climate for almond production (Sorkheh

18 et al. 2009). *Sefied* is an important variety of almond in Iran with rectangular fruit and a light,

19 thin woody shell. It shows early flowering and ripening habits (Mousavi Ghahfarokhi et al.

20 2009).

21   Almond is an ideal model for studying drought stress responses in woody species (Campalans et

22 al. 2001) regarding its simple genomic organization (X = 8, IC = 0.27pg) and its available saturated

23 linkage maps (Viruel et al. 1995; Joobeur et al. 1998). We used the differential expression technique

24 cDNA-AFLP (amplified restriction fragment polymorphism derived technique for RNA fingerprinting)

25 (Bachem et al. 1996; Durrant et al. 2000) to identify transcripts that are accumulated in the young almond

26 tree (*Prunus dulcis var. Sefied*) in response to water-deficit treatment. cDNA-AFLP is a sensitive and

27 efficient technology for the discovery and identification of genes (Dinari et al. 2013, Qian et al. 2014). It

28 is a highly reproducible differential display method based on restriction enzyme digests and selective

29 PCR amplification under high stringency condition. Compared to the other differential display techniques

(Liang and Pardee 1992), cDNA-AFLP technique greatly reduces the number of false positives by ligating adapter molecules to the digested double-stranded cDNA, thereby allowing high stringency PCR conditions (Campalans et al. 2001). In addition, due to its high detective sensitivity of analysis, some rare transcripts could also be detected by this method (Cao et al. 2013). The cDNA-AFLP technique has been used to recognize a wide range of candidate genes involved in flower pigmentation (Habu et al. 1997), nematode development (Jones and Harrower 1998), fruit ripening processes (Jones et al. 2000) and a novel recessive genic male sterility mutant in sesame (Wu et al. 2014). This method has been widely used to pinpoint genes with altered expression under different environmental conditions, due to its efficiency, technical simplicity and lack of requirement of genomic sequence information of the species of interest (Si et al. 2009; Vuylsteke et al. 2006). We find differential expression of transcripts that were up regulated in response to water deficit stress in young trees of *Prunus dulcis var. Sefied*. Twenty transcript-derived fragments (TDFs) with differential expression between control and stress conditions were selected, analyzed and sequenced. Three genes, 2-deoxyglucose-6-phosphate phosphatase (*ABI2*), protein kinase *MK5* and Urease were further validated by quantitative real-time PCR (qRT-PCR). Network construction and *in silico* promoter analysis were employed to unravel underlying regulatory mechanism of the observed gene expression pattern. These results may lay the basis of the almond variety breeding in the future.

Materials and Methods

Plant material, water-deficit stress treatment and measurement of relative water content (RWC)

Prunus dulcis var. Sefied saplings were prepared from Agriculture organization (Jihad-e-Agriculture), Chaharmahal and Bakhtiari, Iran and planted in 10 kg pots during first week of March 2011. The pots were transferred to a greenhouse under 16h/8h light/dark regime, at temperatures ranging from 25-30 °C, and ambient relative humidity 50%, and then moved to field condition during last week of May. Ten well-grown saplings of almond *P. dulcis var. Sefied* were subjected to a water deficit stress treatment that involved withholding water for ten days during the fourth week of June in field conditions when the midday temperature was around 30 °C. Leaves (one fully expanded leaf from top) were harvested from each replication at midday initially from well-watered control replications and after the stress treatment ten days later to measure relative water content (RWC; average ten pots). Young leaves were frozen immediately in liquid nitrogen for RNA extraction and stored at -80 °C. RWC was calculated as:

1   RWC = [(FW-DW)/(TW-DW)×100]

2   FW: Fresh Weight [The leaves were weighed immediately after collection.]

3   TW: Turgid Weight [The leaves were rehydrated by floating for 4h on distilled water and then were

4   weighed.]

5   DW: Dry Weight [The leaves were weighed after oven-drying at 85 $^\circ$C for 24h to obtain the dry weight.]

6   (Smart and Bingham 1974).

7   **Total RNA isolation and cDNA synthesis**

8   Total RNA was extracted from almond *Prunus dulcis var. Sefied*'s control and stressed leaves

9   following the protocol of Chang et al. (1993). The extracted RNA was purified with RNase Free DNaseI

10   (Fermentas Inc.) and dissolved in DEPC-treated water. The concentration and purity of the RNA was

11   determined spectrophotometrically. Spectrophotometric analysis revealed high RNA quality with an

12   A260/A280 ratio of 1.8–2.0. The quality of RNA was tested using 1.5% formaldehyde gel

13   electrophoresis.

14   One µg of RNA from each sample was used for cDNA synthesis. Single stranded

15   cDNA synthesis was carried out with RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas,

16   Inc.) according to the manufacturer's instruction. Second strand cDNA synthesis was carried out

17   as follow: *Escherichia coli* DNA polymerase I (30 units/µl) (Fermentas, St. Leon-Rot, Germany)

18   and *E. coli* RNaseH (1 units/µl) (Fermentas, St. Leon-Rot, Germany) were added to the reaction

19   of single strand cDNA. The reaction was transfered to 15 ℃ for two hours. Then 12.5 units/µl of

20   T4 DNA Polymmerase enzyme (Fermentas, St. Leon-Rot, Germany) was added to mixed

21   reaction and incubated at 15 $^\circ$C for 5 min. Finally the reaction was terminated by adding 5 µl of

22   0.5 M EDTA (pH=8.0) (Fermentas, St. Leon-Rot, Germany). cDNA was purified by

23   phenol/chloroform extraction, ethanol-precipitated and dissolved in a final volume of 25 μl of

24   DEPC-treated water.

25   **cDNA-AFLP analysis**

26   cDNA-AFLP analysis assays were performed as described by Zabeau (1993) and Vos

27   et al. (1995) with minor modifications. Digestion of cDNA with *Pst*I and *Mse*I enzymes, adaptor

28   ligation, pre-amplification reaction, denaturing polyacrylamide gel electrophoresis and silver

29   staining of the gels were carried out as described by Sorkheh et al. (2007). For pre-amplification

1 reactions, selective *Mse*I+1 and *Pst*1+1 primers were applied. The following +3 primer combinations

2 were used for final selective amplifications: *Pst*I-GTT/*Mse*I-CTC; *Pst*I-GTG/*Mse*I-CGA; *Pst*I-ATT/*Mse*I-

3 CGT; *Pst*I-ATT/*Mse*I-CAC; *Pst*I-ACT/*Mse*I-CAG; *Pst*I-ATT/*Mse*I-CAG; *Pst*I-ATT/*Mse*I-CGA; *Pst*I-

4 ACT/*Mse*I-CGA; *Pst*I-GCG/*Mse*I-CTT; *Pst*I-AAT/*Mse*I-GGT.

5 **Isolation, re-amplification, cloning and sequencing of TDFs**

6   Differentially expressed TDFs were excised carefully from the acrylamide gels after drying and

7 purified using QIAquick® Gel Extraction kit (Qiagen, Germany) according to the manufacturer's

8 instruction. 2 µl of eluted TDFs was used as template for re-amplification in a total volume of 20 µl PCR

9 reaction mix (Fermentas, St. Leon-Rot, Germany) using the same set of corresponding selective primers.

10 The PCR product purified using GeneJET^TM PCR purification kit (Fermentas, St. Leon-Rot, Germany)

11 according to the manufacturer's instruction.

12   Cloning and sequencing of TDFs were carried out with minor modification according

13 Alimohammadi et al. (2013). Sequence homology searches were performed in the GenBank database

14 (Benson et al. 2007), accessible through the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/)

15 after deletion of vector sequence. BLASTn and BLASTx search algorithms were used (Altschul et al.

16 1997). Three TDFs (#14, #16 and #17) with a homology of over 70% and *E-value* of less than 10^{-3}

17 (Dubos and Plomion, 2003) were selected for validation by qRT-PCR (Cao et al. 2013; Qian et al. 2014).

18 **qRT-PCR analysis**

19   First strand cDNA was synthesized using a QuantiTect® Reverse Transcription Kit (Qiagen,

20 Hilden, Germany) for three TDFs (#14, #16 and #17) using the same RNA samples which were analysed

21 by cDNA-AFLP. The primers for qRT-PCR were designed with Vector NTI software version 10 (see

22 Table 1). qRT-PCR was performed with Power SYBR Green dye (Takara, Japan) through Real-Time

23 PCR System (Rotor-Gene Q, Qiagen, Germany). One of the housekeeping genes, *Actin*, was applied as a

24 control to check the uniformity of expression (Jain et al. 2006; Livak & Schmittgen 2001). qRT-PCR was

25 done in duplicates for each sample. The number of transcripts of each gene was normalized to the internal

26 *Actin* was analysed using Gene Expression Relative Quantitation Software (Bio-Rad, München,

27 Germany). The whole experiment was repeated twice independently.

28 **Network construction and analysis**

1   Gene/protein interaction network was constructed by making a database of interaction

2   relationships between the 3 significantly up-regulated genes (2-deoxyglucose-6-phosphate phosphatase

3   (*ABI2*), protein kinase *MK5* (*ACF2*), and Urease) in response to water-deficit and other genes based on

4   text (literature) mining approach using Pathway Studio 9 and MedScan softwares and peach genome as

5   reference (Ariadne Genomics, ELSEVIER), according to the procedure previously described

6   (Hosseinpour et al. 2012). Text mining is a novel approach that brings the relevant information together,

7   organize and prepare the information for further analysis (Hosseinpour et al. 2012). The software

8   (MedScan) processes undertaken data by natural language processing (NLP).The language extracts

9   functional relationships between microRNAs, proteins, small molecules, and cellular processes by

10   interpreting logical concepts (Nikitin et al. 2003). Visualization of gene relationships/interaction is carried

11   out by Pathway Studio software.

12   **Mining regulatory elements on the promoter regions of 3 drought responded genes and**

13   **transcription factor prediction**

14   Available whole genome sequences of *Prunus persica* and *Arabidopsis thaliana* were used for

15   promoter identification of up-regulated genes in response to stress (2-deoxyglucose-6-phosphate

16   phosphatase, protein kinase *MK5* (*ACF2*) and Urease) due to unavailability of whole almond genome

17   sequence. At first these genes were blasted against the genomic sequences of *Prunus persica* and

18   *Arabidopsis thaliana* in the Phytozome database (http://www.phytozome.net/) to find the corresponding

19   orthologs in these genomes (Babgohari et al. 2013; Moghadam et al. 2012). Then, the 1500 bp upstream

20   of the transcriptional start point of the corresponding genes were taken and considered as promoters.

21   *In silico* promoter analysis was carried out in two levels: (1) determining regulatory elements

22   (transcription factor binding sites) on promoter regions, and (2) determining transcription factors (TFs)

23   and their modules which can bind to these promoter regions. Firstly, the possible promoter regions of the

24   above mentioned genes were mined with PlantCARE and compared with known cis-regulatory elements

25   in PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002).

26   The cis-regulatory elements were recorded for each promoter. Secondly, to find shared involved TFs, the

27   upstream regions of the 3 genes were analyzed using the PLANTPAN (http://plantpan.mbc.nctu.edu.tw)

28   to predict corresponding transcription factors which can activate the three differentially expressed genes

29   in dehydration (drought) condition. Based on shared TFBs (regulatory elements), PLANTPAN finds

possible transcription factors which can bind to and activate these elements (Chang et al. 2008). The procedures have been described in our previous publications (Babgohari et al. 2013; Moghadam et al. 2012).

Results

The measurement of relative water content in *Prunus dulcis var. Sefied* leaves

The RWC of almond leaves was measured to monitor the induction of the water-deficit conditions after 10 days of water withholding. The leaf RWC in control plants was 85%. When water-deficit stress started, the RWC in the leaf decreased to 65% within 7 days and then gradually, RWC showed a slight increase, but maintained at 68% for the rest of the 10-day treatment.

cDNA-AFLP analysis

In this study, double-stranded cDNA was digested by the restriction enzymes *Pst*I and *Mse*I for cDNA-AFLP method; so we could not rule out the possibility that this combination of restriction enzymes might not give sufficient data for a comprehensive analysis. Interestingly, most of the TDFs generated using 10 primer combinations were constitutively expressed in almond. Therefore, 20 TDFs above 100 bp (between 100-500 bp) were eluted from the dried gel on the basis of difference in band intensities in control conditions versus water-deficit stress conditions. The differential bands were cut from the gel (Figure 1), re-amplified, cloned and sequenced. Comparison of the homologies of these sequences and those in the databases suggested that most of them are homologs of genes involved in metabolism, signal transduction, plant development, photosynthesis, cell signaling and cell division, transcription regulation and protein synthesis (Table 2). We selected three TDFs (#14, #16 and #17) to validate our cDNA-AFLP data through qRT-PCR.

qRT-PCR analysis and expression patterns of the TDFs

qRT-PCR was performed on three of the TDFs (#14, #16 and #17). The expression patterns of these cDNA fragments were similar in both cDNA-AFLP and qRT-PCR experiments and there was a good agreement between the cDNA-AFLP data and the qRT-PCR results. TDF #14 exhibited homology with Urease of *Morus alba* with 85% identity. The size of this TDF was 182bp. cDNA-AFLP revealed that the expression of this clone significantly increased under stress conditions compared to the control. qRT-PCR analysis disclosed that the expression of this transcript under stress conditions was 4.87-fold higher than that under control conditions (Figure 2 A). TDF #16 showed homology to 2-deoxyglucose-6-

phosphate phosphatase of *Ricinus communis* (91% sequence identity). The size of this TDF was 345 bp. cDNA-AFLP displayed that the expression of this clone was increased in stress conditions compared to the control. qRT-PCR analysis validated that the expression of this transcript under stressed conditions was 1.61-fold higher than that in the control (Figure 2 B). TDF #17 showed homology with protein kinase *MK5* of *Mesembryanthemum crystallinum* (92% identity). The size of this TDF was 419bp and cDNA-AFLP indicated that the expression of this clone was increased under stress conditions compared to the control. qRT-PCR analysis revealed that the expression of this transcript under stress conditions was 2.39-fold higher than that in the control (Figure 2 C). Regarding error bars, had no overlapping between two conditions, there were significant differences in stress conditions compared to the control (Figure 2).

Network analysis

Predicted network of drought stress response in cultivated almond is presented in Figure 3, and its underlying interactions are documented in Table 3. Architecture of this network highlights the central role of phosphorylation performed by protein kinase *MK5* (*AFC2*) in activation of ABA responsive transcription factors and histone alteration. Histone alteration results in epigenetics-based gene expression. In addition, predicted network (Figure 3) suggests that induction of drought stress is accompanied with decrease in free acid phosphatase and subsequent increase in Urease (*URE*) and protein resistance protein (*PR1*). The network architecture shows that ABA-dependent transcription factor (*ABI3*), located at nucleus, is a major inducer/owner of regulatory network, which positively regulates overexpression of 2-deoxyglucose-6-phosphate phosphatase (*ABI2*) and *PR1*.

Interestingly, protein kinase *MK5* (*AFC2*) show protein-protein interaction with nuclear RNA splicing proteins (including *SR45, SR33, SRZ-22,* and *RSZP21*), which are involved in sugar mediated signaling. To validate the effect of drought stress on activation of subnetwork governed by *AFC2, SR45, SR33, SRZ-22,* and *RSZP21* in leaves, we studied the expressions of these genes encoding during drought stress. qRT-PCR demonstrated that the serine/arginine-rich (*SR*) proteins genes *SR45, SR33, SRZ-22,* and *RSZP21* were significantly (*t* test; P < 0.01) downregulated (2.9-, 4.8-, 4.2- and 2.6-fold, respectively) by drought stress in almond leaves of cultivar *Sefied* (Figure 4). Altogether, our results show that drought stress in almond is accompanied with significant up-regulation of *AFC2* protein kinase and subsequent down expression of RNA splicing proteins (including *SR45, SR33, SRZ-22,* and *RSZP21*) perhaps through *AFC2* protein phosphorylation.

9

ABA-response regulatory elements and TFs involved in ABA signaling are shared on promoter regions of almond drought responsive genes

In average, we found 2-3 MBS (*MYB* binding site involved in drought-responses) and 1-2 *ABRE* (cis-acting element involved in abcisic acid responsiveness) on promoter regions of all 3 up-regulated genes, revealed by regulatory element mining of promoter regions using PlantCARE (data not shown). Confirming this finding, TF survey using PlantPAN discovered co-binding of *MYB2*, *ABI*, and *ARR* transcription factor on promoter regions of up-regulated genes. MYB and ABI ARE the most prominent ABA-based activated TFs in drought. *ARR* is a transcription activator which binds to 5'-[AG] GATT-3' and is involved in His-to-Asp phosphorylation signal. Interestingly, *ARR* interacts with histones and is a possible epigenetics regulator (Chang et al. 2008). These findings suggests the involvement of ABA signaling pathway, possible epigenetics processes, and phosphorylation as underlying regulatory mechanism of cultivated almond response to water deficiency.

Discussion

The molecular responses involved in drought tolerance are largely unknown in woody plants (Chaves et al. 2003). Although many genes induced by dehydration have been identified in various plant species, there is little evidence for the contribution of a specific master-switch gene in desiccation protection (Campalans et al. 2001).

TDF #14 is homologous with the Urease of *Morus alba*. The main function of the Urease in plants is recycling nitrogen from urea. Most of the endogenously generated urea comes from arginine, which constitutes 18% of storage-protein N and is actively degraded to urea and ornithine upon germination. Urease catalyzes N reconversion from urea to ammonia, which is subsequently assimilated via glutamine synthetase. A potential Urease role in maintaining N_2 fixation under water stress was suggested in a model by Purcell et al. (2000) and Vadez and Sinclair (2001). It is shown that direct production of Urease from ureides guaranties stable N fixation during water deficiency. In this research, in agreement with Purcell et al. (2000) and Vadez and Sinclair (2001), cDNA-AFLP and qRT-PCR techniques confirmed significant upregulation of this clone in water-deficit stress conditions by 4.87-fold change, which indicates its potential role in maintaining N homeostasis in water deficit stress.

TDF #16 is homologous with 2-deoxyglucose-6-phosphate phosphatase of *Ricinus communis* (91%). In many signal transduction pathways, reversible phosphorylation of proteins is a major form of

signal relay. The enzymes that catalyze reversible phosphorylation processes are protein kinases and protein phosphatases. Protein phosphatases dephosphorylate proteins and thereby attenuate and reverse the function of protein kinases (Rai and Takabe 2006). By controlling the phosphorylation status of other proteins, protein kinases and phosphatases play a fundamental role in coordinating the activity of many known signal transduction pathways in both prokaryotic and eukaryotic organisms. The major gene families known to encode protein kinases and phosphatases are involved in stress-related signal transduction in plants (Pareek et al. 2010). In this research, cDNA-AFLP and qRT-PCR analyses showed significant upregulation of this clone under water-deficit stress conditions compared to control by 1.61-fold change. The activation of 2-deoxyglucose-6-phosphate phosphatase suggests its possible role in modifying phosphorylation status of proteins in water-deficit stress.

TDF #17 showed homology with protein kinase *MK5* of *Mesembryanthemum crystallinum*. Protein kinase *MK5* is a transcription factor and a secondary messenger that regulates the expression of other genes in response to stress (Mishra et al. 2007). Changes in cytoplasmic Ca^{2+} concentration are likely to mediate the integration of different signaling pathways. Some of the most abundant regulatory protein kinases involved in abiotic stress signaling are Ca^{2+} dependent (*CDPK*) (Knight and Knight 2001) and mitogen activated protein kinase (*MAPK*) (Kutz and Burg 1998). *MAPK*s are mediators in several signal transduction pathways in eukaryotic cells, including responses to a variety of environmental stresses. The *MAP* kinase cascade comprises of three protein kinases. *MAPKKK*s are the primary signal receivers, which upon activation phosphorylate and activate *MAPKK*s, which in turn relay the signal to *MAPK*s. Several *MAPK*s and *CDPK*s have been identified in plants subjected to water stress and shown to be involved in transducing the dehydration signals sensed at the plasma membrane to the nucleus (Manuela et al. 2003).

In this research, cDNA-AFLP and further qRT-PCR analysis showed a sharp increase in expression of protein kinase *MK5* (*AFC2*) in response to water-deficit stress by 2.39-fold in cultivated almond (*Prunus dulcis* var. Sefied). Altogether, it seems that activation of *MK5* protein kinase, in response to water-deficiency, plays a significant role in abiotic stress signaling in almond. Further investigations such as phosphorylation(kinase) based proteomics can provide a comprehensive catalogue of kinase-based alteration and its pattern during almond resistance to stress.

Little information is available regarding the possible role of RNA splicing proteins (*SR45, SR33, SRZ-22*, and *RSZP21*) in stress response. However, some reports indicate a link between alternative splicing and abiotic stress responses in plants (Duque 2011). For example, genomewide studies in Arabidopsis have identified different patterns of splicing during a range of stress responses (Iida et al. 2004; Filichkin et al. 2010). The discovered stress-response subnetwork in this study is a significant finding, which highlights the negative regulatory effects of kinase *MK5* on RNA splicing proteins. It should be noted that during stress, plant does not spend a lot of energy for completing the splicing and spends much of its energy in overexpression of stress response genes. Consequently, shorter proteins with incomplete splicing are more frequent. The negative regulation of protein kinase *MK5* on RNA splicing proteins (*SR45, SR33, SRZ-22*, and *RSZP21*) can explain increasing incomplete RNA splices.

Conclusion

In this study, some water-deficit inducible genes were identified for the first time in the leaves of almond *Prunus dulcis var. Sefied*. Most genes were involved in various functions in response to abiotic and biotic stresses. Three genes that disclosed significant similarity to genes with known function including Urease, 2-deoxyglucose-6-phosphate phosphatase and protein kinase *MK5* were confirmed by both cDNA-AFLP and qRT-PCR. Overall, there was a good agreement between the cDNA-AFLP data and the qRT-PCR results.

Some of the TDFs in this study did not have any homology with other known genes, indicating that further work is required to understand the role of these genes in stress-response pathways and to elucidate the mechanism of water-deficit response. This study highlighted the existence of subnetwork consisting of protein kinase *MK5* and splicing proteins (*SR45, SR33, SRZ-22*, and *RSZP21*) in stress response and opens a new avenue for gene discovery through bioinformatics-based network predictions in almond.

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9 **Table legends**

10 Table 1. Primer used in quantitative real-time PCR.

11 Table 2. Characteristics of transcript-derived fragments (TDFs) expressed during the drought stress given

12 to almond *Prunus dulcis var. Sefied.*

13 Table 3. Relationships in predicted gene interaction network of drought resistance in almond.

2 Figure 1. cDNA-AFLP polyacrylamide gel showing the TDFs with differential expression in control (C)

3 and drought stress (S) conditions in the almond *Prunus dulcis var. Sefied*. Three genes that were selected

4 for qRT-PCR analysis are TDF# 14, 16, 17. M: Size Marker VIII (Roche, Germany). The replications of

5 the control (C) and the stressed (S) treatments are indicated in gel for each plant. The following primer

6 combinations were used: A: *Pst*I-ACT/*Mse*I-CAG; B: *Pst*I-ACT/*Mse*I-CGA; C: *Pst*I-ATT/*Mse*I-CAC; D:

7 *Pst*I-AAT/*Mse*I-CAC; E: *Pst*I-AAT/*Mse*I-GGT; F: *Pst*I-GCG/*Mse*I-CTT.
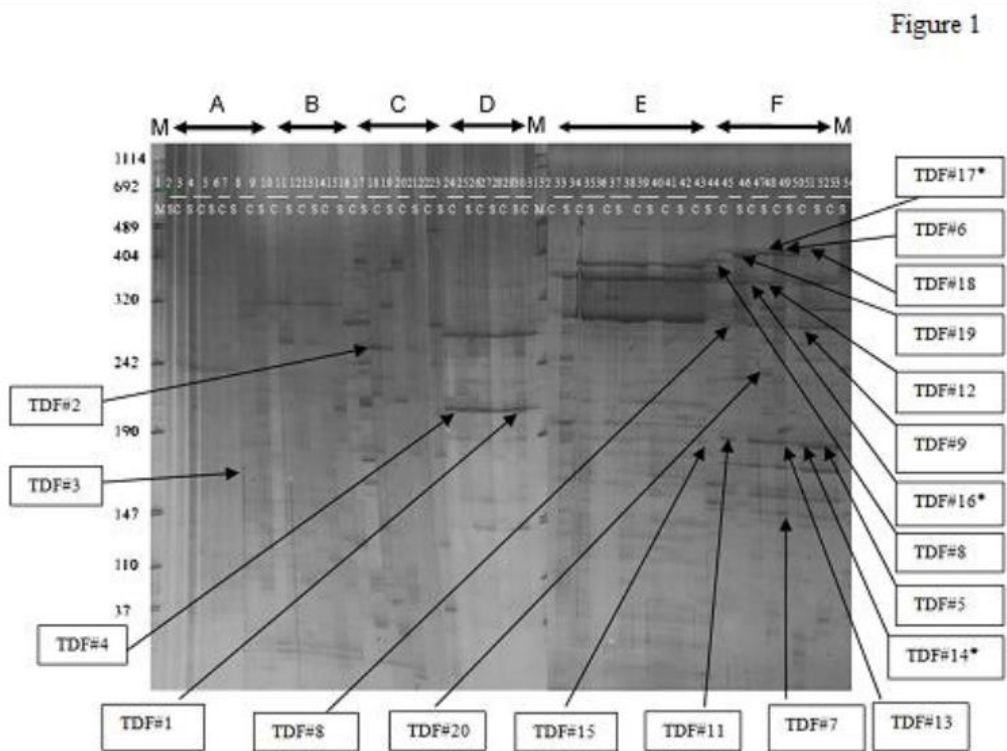
Figure 1

8

1   Figure 2. qRT-PCR analysis of the TDFs expression in response to drought stress in almond *Prunus*

2   *dulcis var. Sefied.* Expression patterns of Urease TDF#14 (A), 2-deoxyglucose-6-phosphate phosphatase

3   TDF#16 (B) and protein kinase *MK5* TDF#17 (C) have been given. For comparison, the amount of

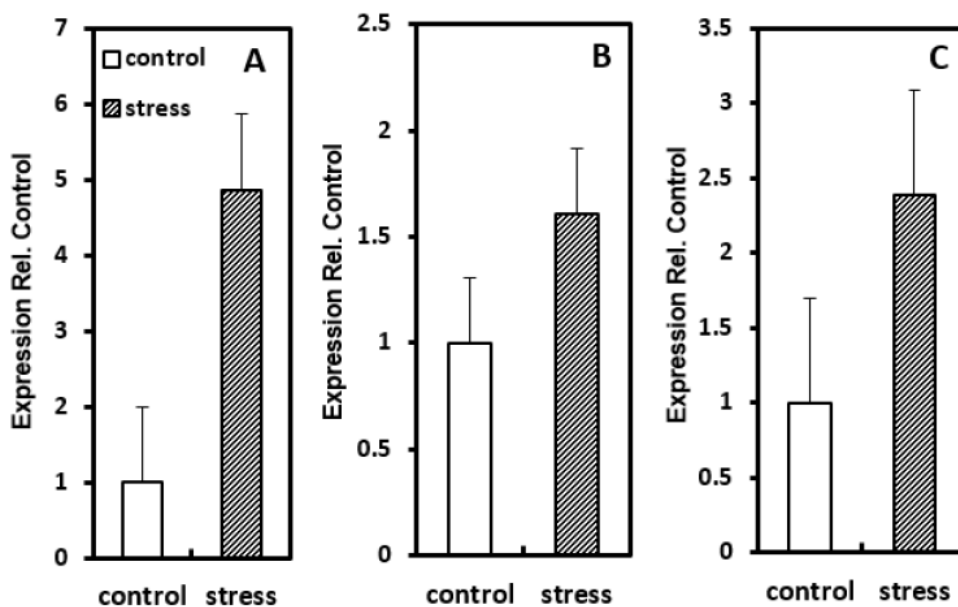4   transcripts obtained for each target gene under control condition was designated 1.0.

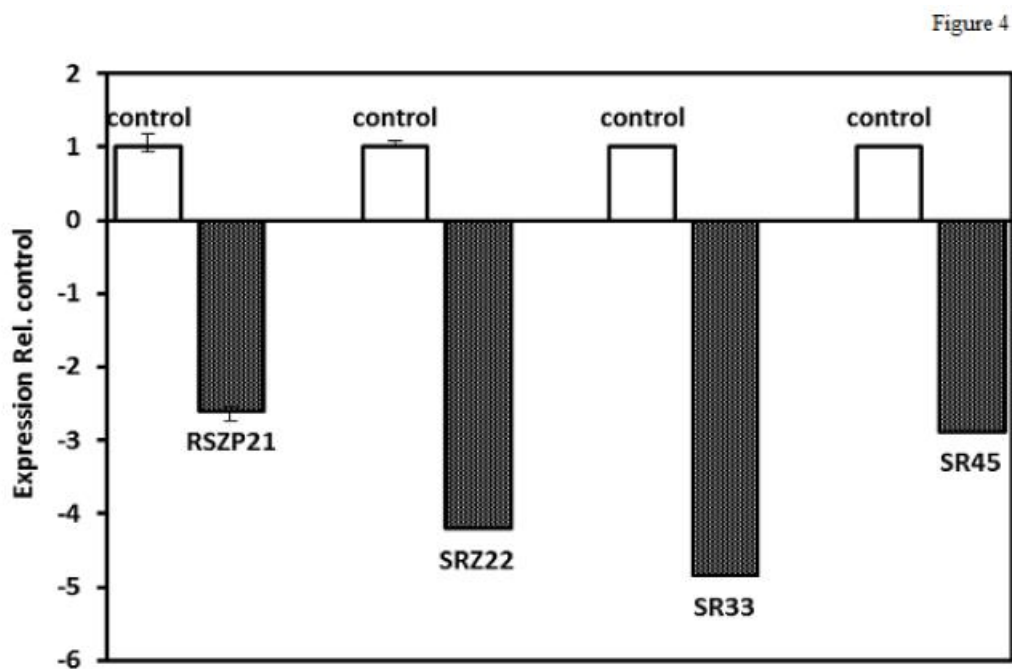
5

1   Figure 3. A network for water-deficit gene response in almond (*Prunus dulcis var. Sefied*) produced by

2   Pathway studio software. The *ACF2* protein kinase plays a central role in network by interacting with SR

3   proteins and histones. The network indicates possible activation of ABA responsive genes including

4   ABI3 transcription factor.
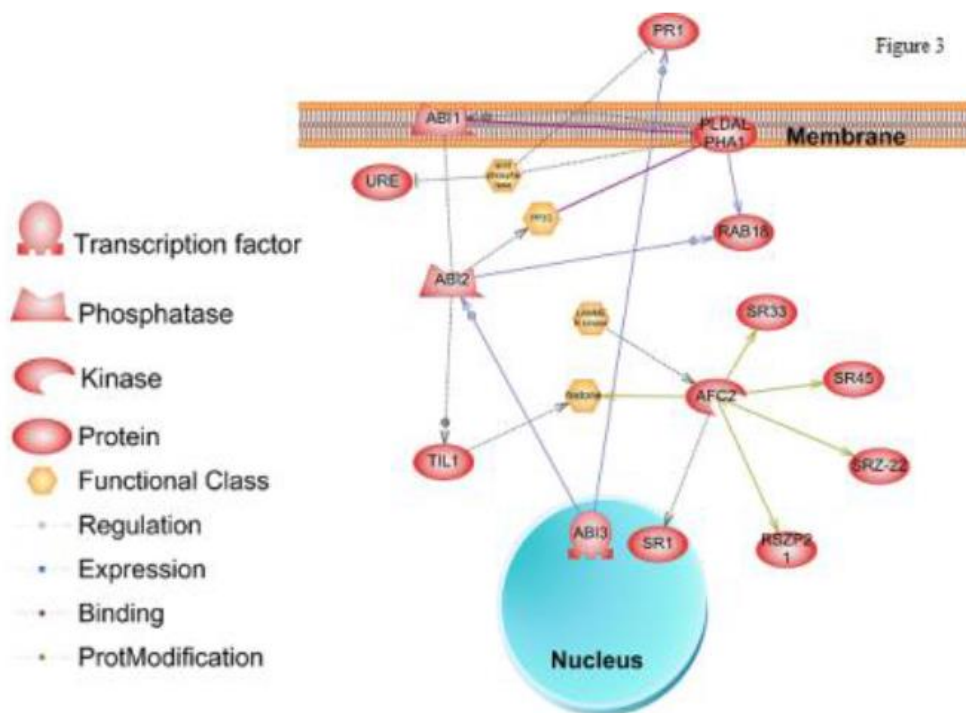
5

1   Figure 4. qRT-PCR analysis of the expression of *SR45, SR33, SRZ-22*, and *RSZP21* in response to

2   drought stress in almond *Prunus dulcis var. Sefied.*

Figure 4

3