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Dehydration stress memory genes of two wheat landraces (*Triticum turgidum* L. var*. durum*).

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Pre-exposing plants to diverse a biotic stresses including drought may alter their physiological and transcriptional responses to a subsequent stress, by improving resistance to future exposures, these observations have led to a concept of “stress memory”, implying that during subsequent exposures plants provide responses that are different from those during their first encounter with the stress. To asses epigenetic modulation of drought responsive genes (DRGs) in durum wheat (*Triticum turgidum* L. var.*durum*), the landraces Hourani27 and Omrabi5 seedlings were exposed to two sequential dehydration treatments S1(first dehydration stress) and S3(second dehydration stress). Differential DRG expression was revealed by quantitative PCR (qPCR). The data was calibrated by the house keeping gene actin. In Hourani 27, the ERF/AP2memory gene was up-regulated later in S3 (4.12 fold) compared to S1, while expression level was similar between S1 and the control ([=/+] W= S1 < S3). This is a type of memory DRGs called the later-responsive. In the contrary, Omrabi5 showed gene induction in S1 (1.76 fold) followed be decline in S3 (1.13 fold) compared to S1 ([+/−], which is a classical memory DRG (W< S1 > S3).The second DRG gene LEA3 showed the same trend as ERF/AP2 in Hourani 27, where a similar expression level in S1 compared to the control followed by a dramatic increase in S3 (13.71 fold) compared to S1([=/+] (W = S1 < S3). The expression behavior of LEA3 in Omrabi 5 followed the same trend as ERF/AP2, where the expression increased in S1 compared to the control (9.93 fold) followed a down regulations in S3 compared to S1 (-6 fold) ([+/-] (W < S1 > S3).The results demonstrate an evidence of epigenetic memory of DRGs in durum wheat as it modulates gene expression patterns. The gene expression behavior varies from a genotype to another. Omrabi 5 is presumably more drought resilient than Hourani 27 for the investigated DRGs.

Keywords: *Triticum turgidum* L. var.*durum*, drought, drought responsive genes (DRGs), memory gene

INTRODUCTION

Durum wheat (*Triticum turgidum* L. *var.durum*) is one of the most important staple food crops grown under dry land conditions, mainly in the Mediterranean region and particularly in Jordan, where its productivity is drastically affected by drought.

Durum in Latin means "hard", and the species is the hardest of all wheats. Its high protein content, as well as its strength, make durum good for special uses, the most well-known being pasta. Durum wheat is used extensively in bread making. However, it is unusual in that, despite very high protein content, it is low in desirable gluten needed to form a glutinous web necessary for bread to rise (Zilic et al. 2011).

Jordanian durum wheat landraces have high variation; this variation indicates different level of diversity. The presence of this wide range of variability among landraces possesses high levels

of variability for biological yield, fertile tillers, number of seeds per spike, seed weight per spike and the thousands grains weight. These landraces must be considered as a reservoir of genes that plant breeders need in their wheat improvement programs and should be conserved (Jaradat, 2013; Rawashdeh et al. 2007; Rawashdeh and Rawashdeh, 2011).

Drought is one of the major limiting factors affecting plant productivity worldwide and it is well documented that accessibility of water for plant growth is a key aspect determining plant distribution in natural ecosystems. The severity and duration of the drought stress are both critical (Moaveni, 2011; Rawashdeh and Rawashdeh, 2011; Dura et al. 2013).

In many regions of the world, water is scarce and agriculture is dependent on rain. The major factors which determine the productivity and adaptability of wheat under rainfed areas are yearly precipitation and distribution over the growing season. Drought-triggered dehydration stress is one of the most common environmental stresses endured by plants, and their responses to dehydration stress are extensively studied at organism, cellular, and genome levels. Durum wheat is known to tolerate drought stress. Therefore, understanding important factors and genes involved in drought tolerance in durum wheat is important (Nezhadahmadi et al. 2013; Hassan et al. 2015).

Plants respond to dehydration stress through physiological adjustments presumably regulated by the expression of specific genes involved in the dehydration stress response (Virlouvet et al. 2018). Pre-exposure to diverse types of stresses, including dehydration stress, may alter subsequent responses, suggesting a form of “stress memory”, which was proved in other model plant, e.g. maize (Ding et al. 2013; Ding et al. 2014; Hazen et al. 2003; Lämke and Bäurle 2017). This experiment conducted to find evidence of epigenetic memory of DRGs in durum wheat as it modulates gene expression patterns.

Historically, AP2/ERF proteins containing at least one DNA binding domain – named the AP2 domain – have been divided into three separate families, namely the ERF, AP2 and RAV families. Most proteins with a single AP2 domain and whose genomic sequence contains a small amount of introns are assigned to the ERF family (Nakano et al. 2006). In the AP2/ERF super family, AP2 TFs act primarily in the regulation of developmental programs, while ERF proteins mainly affect these processes in the frame of responses to environmental stimuli or hormones. Among the ERFs that regulate ethylene-dependent transcription, ERF members positively or negatively regulate the expression of ethylene inducible genes downstream of EIN3 (Solano et al., 1998; Yang et al. 2005). A number of ERF genes from various plants have been shown to confer multiple stress tolerance when expressed ectopically (Yi et al. 2004; Seo et al. 2010; Fukao et al. 2011; Mito et al. 2011). This ‘un specific’ effect can be explained by the activation of tolerance pathways that alleviate a generic stress status, such as oxidative bursts produced as consequences of the primary stresses. Alternatively, constitutive ERF expression could set the plant in a general alert state which fasten or magnify the response when a specific stress is applied.

The increase in world population, estimated to reach 9 billion by 2050, poses a serious challenge for crop production (Xu et al. 2011). It demands a concomitant increase in food production that is unlikely to be achieved only by improving agricultural practices. Yield is strongly affected by environmental cues such as water deficit or excess, high soil and water salinity, cold and drought stresses, and hence it is of the utmost importance to develop crop varieties able to withstand such adverse condition but at the same time limiting yield decreases. Transcription factors represent ideal targets for traditional or genetic engineering-assisted breeding of plants with specific traits related to stress tolerance or higher yield. In particular, ERF genes are among the most interesting TFs because they have been selected through evolution to regulate a series of stress-response pathways (Bi et al. 2016; Xu et al. 2006).The importance of AP2 and ERF proteins in the regulation of physiological processes in plants has been already established by several authors. In general, following a common ancestral origins, AP2 and ERF genes seem to have diverged to orchestrate developmental programs and responses to environmental factors, respectively (Nakano et al., 2006). With the advancement of molecular techniques and increased sensitivity of proteomic assays, the increment or reduction of transcription factor activity and abundance can also be assessed (Kaufmann et al. 2010; Smaczniak et al. 2012). At the same time, the occurrence of post-transcriptional regulation of the ERF transcription factor is emerging as a crucial factor in the control of their activity (Qin et al. 2007; Bailey-Serres et al. 2012; Cheng et al. 2012).

The late embryogenesis abundant (LEA) proteins constitute of a family of hydrophilic proteins that are presumed to play a protective role during exposure to different a biotic stresses (Cuevas et al. 2016; Huang et al. 2016). They were first described to highly accumulate during the late stages of cotton seed development, when the embryo becomes desiccation tolerant (Dure et al., 1981). They were not only found in the seeds of many other plants, but also detected in vegetative organs. More importantly, they are usually induced under stress conditions such as cold, drought, or high salinity (Ingram and Bratels, 1996; Thomas, 1999). LEA proteins were initially classified to six subgroups on the basis of specific domains; many studies have been performed to characterize their functions, especially the roles in stress responses (Dure et al. 1989). Results from these studies suggest that LEA family could be considered as a reservoir for stress-responsive genes, which have great potential in genetic improvement of stress tolerance in plants.

Keeping in view the objectives of this experiment is to study the transcriptional responses of drought responsive genes of selected durum wheat landraces obtained from NARC, which exposed to multiple dehydration stress which conducted at the Plant Biotechnology Laboratories, Department of Horticulture and Crop, Faculty of Agriculture at the University of Jordan, during 2014 -2016.

MATERIALS AND METHODS

Plant growth and treatments

Durum Wheat (*Triticum turgidum* L. var*. durum*) landraces where obtained from the National Agricultural Research Center (NARC). Hourani 27 is Jordanian improved selection from the Hourani landrace, well adapted to local ecological conditions, Omrabi5 is a cross between the landrace "Hourani" and the improved cultivar "Jori-C69" (Nachit, 1998), it is released in Jordan, Turkey, Algeria, Morocco, Iran and Iraq for commercial production; it is drought tolerance cultivar (Dura et al. 2013).

Seeds were surface sterilized and germinated in MS medium (*Murashige and Skoog, 1962*) then placed in a growth chamber at 24±1ºC with 16:8 light: dark photoperiod under cool-white fluorescent illumination of 40 - 45 µmol/m2/sec. of photosynthetic photon flux density (PPFD).

The roots of two weeks Seedlings were blotted using sterilized towel, and they left for 2 hours at room temp (22 ± 2 °C, dry air) (first dehydration stress, S1), followed by a period of re-hydration recovery for 22 hours (watered, W). Repeated stress was performed as described above (second dehydration stress, S3). For control plants, seedling doesn't expose to air drying (Control, C). Leaf tissue collected from stressed (S1, S3) and unstressed (control) plant then they were put in liquid nitrogen and stored in - 80 °C refrigerator direct in the same day(Ding et al. 2013, Ding et al. 2014).

BLAST analysis

Memory and non-memory drought responsive genes for durum wheat were retrieved from genbank utilizing homologous from Arabidopsis, *Zea mays* and *Oryza sativa* (Ding et al. 2013, Ding et al. 2014).

The homology search was performing using basic local alignment searching tool (BLAST). Blast search was performed for both nucleotides and amino acid using BLASTn at NCBI server (NCBI, 2015). BLAST was specifically designed to search nucleotide and protein databases (Stephen et al. 1990)

Primer Design

The primers" design was done on the basis of the five gene sequences. Comparison of sequences of drought memory genes of durum wheat and other sequences of reference genes from Arabidopsis, rice and, maize were performed and new specific primers were designed. The selections of primers were made using „Primer 3" software, and were prepared commercially. The details of primer sequences and target genes have been given below in table (1). Primer parameters including annealing temperature, GC content and length were considered. To assess the designed primers, serial dilutions were prepared to use in the PCR mixture.

New primers were designed specific for DRG, length of primers was 20 bp, melting temperature(Tm) ranged from 58 of 60 ◦C with an expected product size of 270 to 383 bp. All primers were screened, some were showed amplification, because primers developed through next generation sequence. Some not amplified because there were assembly errors.

Table 1: Primer sets designed for detection drought memory genes in Durum wheat (*Triticum turgidum* L.var*. durum*) landraces.

Oligo Name	Target Gene	Primer Sequences (5' to 3')	Tm	Expected size
T.d_F01	Actin F	CCGAACGGGAAATTGTAAGG	55.4	374
T.d_R01	Actin R	TCTCTGCCCCAATGGTGATC	57.4	
T.d_F02	ERF/AP2 F	ACACGCAGTGTAAAGTTGTCGATAG	58.9	340
T.d_R02	ERF/AP2 R	GGAGCAGAGCAGTCCCAAAC	59.5	
T.d_F05	LEA3 F	CGTCCGAGACGGCCCAGGCCG	69.5	284
T.d_R05	LEA3 R	GCTGTCTCCCCCCATCCCCAGC	67.4	
T.d_F10	LEA3 F	CGGCGAGCAAGTGAAGAAC	57.1	283
T.d_R10	LEA3 R	ATGCAACCGCGACAGGTATAT	5.9	

RNA extraction

Leaf tissues were grounded with liquid Nitrogen for total RNA extracted, by adopting RNA kit procedure (Qiagen, USA according to the manufactures constructions). Quality and quantity of the RNA were assessed by agarose gel (1%) electrophoresis.

Polymerase Chain Reaction (PCR) amplification

In order to detect the presence of the desired DNA. Different sets of primers were used. PCR conditions were adjusted for each set of primers. PCR amplification reactions were performed in a thermal cycler (BIORAD my cycler and Eppendorf Master cycler). Briefly, 10µl of 2 X PCR master mixtures was prepared and adjusted as needed. The amplification conditions were as follows: Initial denaturation step at 95°C for 3min, 35 cycles of denaturation 95°C for 30sec, annealing temperature 50°C for 30sec, extension at 72°C for 30sec, followed by a final extension step at 72°C for 10 min. The gels were visualized and photographed. The primers were screened and used for RT-PCR. PCR products were separated with gel electrophoresis for detection of amplified bands. HyperLadder™ 100bp used run on a 1.5% TAE/agarose gel and stained with ethidium bromide.

Analysis of Drought response memory genes using real-time PCR

Drought responsive genes, both memory and non-memory, were assessed in both a presumably drought tolerant durum wheat cultivar Omrabi 5 and Hourani 27. Gene expression was measured with quantitative real-time PCR (qPCR) and three biological replicates using SYBR Green mix (Qiagen, USA) and Applied Biosystems 7500 thermal cycler (ABI, USA). Primer stringency was verified with melting curves following qPCR. Expression of Drought responsive genes was calibrated against expression of reference wheat gene *actin 1*. The fold change in expression (Stressed compared to control) was achieved using comparative C_T method (*Livak and Schmittgen, 2001*). The means with error bars of 95% confidence interval (95% C.I., with Z score = 1.96) were calculated using the following equations (Sadder and Al-Doss 2014): F = Fold change in expression = $2 \wedge \Delta\Delta C_T$

Statistical analysis

For qPCR experiments, data was as fold change in expression (stressed compared to control), which will be determined using the comparative CT method (*Livak and Schmittgen, 2001*), means with error bars of a 95% confidence interval (95% CI) will be calculated (Sadder and Al-Doss, 2014).

RESULTS AND DISCUSSION

This study was conducted for the first time as a new study for selected durum wheat landraces exposed to multiple dehydration stress memory gene that was not previously examined, while DRG was studied for other crops such as maize.

The specificity of utilized primers was investigated using melting curves for both wheat cultivars (Fig 1). They showed single and clean peaks indicating the amplification of single DNA products in the PCR. Comprehensive analyses of the transcriptome data to revealed the existence of distinct transcription patterns and levels of DRG. Genes that respond to a first stress by up-regulating or down-regulating their transcription but in a subsequent stress provide a significantly different response define the 'memory genes' category, [+/+] (W < S1 < S3), [−/−] (W > S1 > S3), [+/−] (W < S1 > S3), [−/+] (W > S1 < S3). Genes responding similarly to each stress form the 'non-memory' category, either Induced [+/=] (W < S1 =

S3) or Repressed [−/=] (W > S1 = S3). Genes that didn't respond to a first stress but in a subsequent stress provide a significantly different response by up-regulating [=/+] (W = S1 < S3) or down-regulating their transcription [=/-] (W = S1> S3) define the Late-response genes.

In this experiment ethylene responsive factor (ERF/AP2) drought gene in landrace Hourani 27 were up-regulated late in the S3 level with 4.12 fold increase compared to S1 and control with 1 fold, [=/+] (W= S1 < S3), 1=1<4.12 folds.

In Omrabi5 there was induced in the first stress with 1.76 fold increase compared to the watered non stressed, and then it was revised down regulating in the second stress with 1.13fold decrease ERF/AP2 memory gene as follow [+/-], (W < S1 > S3), 1<1.76>1.13.

For the second gene late embryogenesis abundant protein (LEA3) in Hourani 27 there was 1.06 fold increase in S1 compared with 13.71 fold increase in S3 with repetitive induced pattern in the two stresses as follow ,[+/=] (W = S1 < S3), 1<1.06 fold<13.71 folds. The LEA3 revised memory gene in Omrabi 5 there was 9.93 fold increase in S1than C, compared with 0.16 fold decrease in S3 with repetitive induced pattern in the two stresses as follow, [+/-], (W < S1 > S3) 1<9.93>0.16 folds. LEA3 and ERF\Ap2 genes were up regulated in the S3 in Hourani 27 landrace but the trend was revised in Omrabi 5 (figure 2, 3).

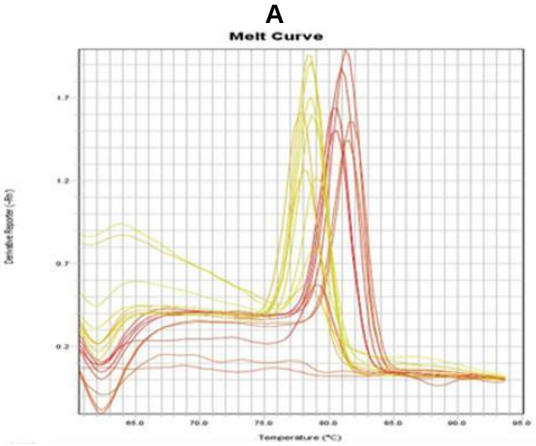
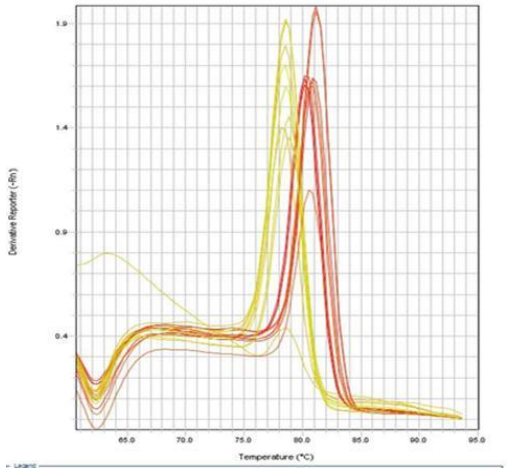


Figure 1: (a) q PCR amplification curve for Hourani 27. Melting curve for amplified products.

b



(b) qPCR amplification curve for Omrabi 5.) Melting curve for amplified products

The response mechanisms of plant to drought are complex and include molecular and physiological changes which influence the morphology and phenology of whole plants (*Blum, 1996*). One of the important results of this study is the evidence that wheat display dehydration stress memory and modify its transcriptional responses by similar transcriptional memory patterns. The RNA-Seq transcriptome analyses allowed identification of genes that function similarly in the two lineages, as well as genes that function in species-specific ways (Ding et al. 2014).

To asses epigenetic modulation of drought responsive genes (DRGs) in durum wheat, both Hourani 27 and UmRabi5 seedlings were exposed to two sequential dehydration treatments (S1 and S3). In such a scenario, epigenetic modulation can be detected when genes display the ability to remember dehydration stress and modify their transcriptional response.

The qPCR data showed that in Hourani 27, the ERF/AP2 memory gene was up-regulated later in S3 (4.12 fold) compared to S1, while expression level was similar between S1 and the control. This is a type of memory DRGs called the later-responsive. In the contrary, Omrabi5 showed gene induction in S1 (1.76 fold) followed be decline in S3 (1.13 fold) compared to S1, which is

a classical memory DRG.

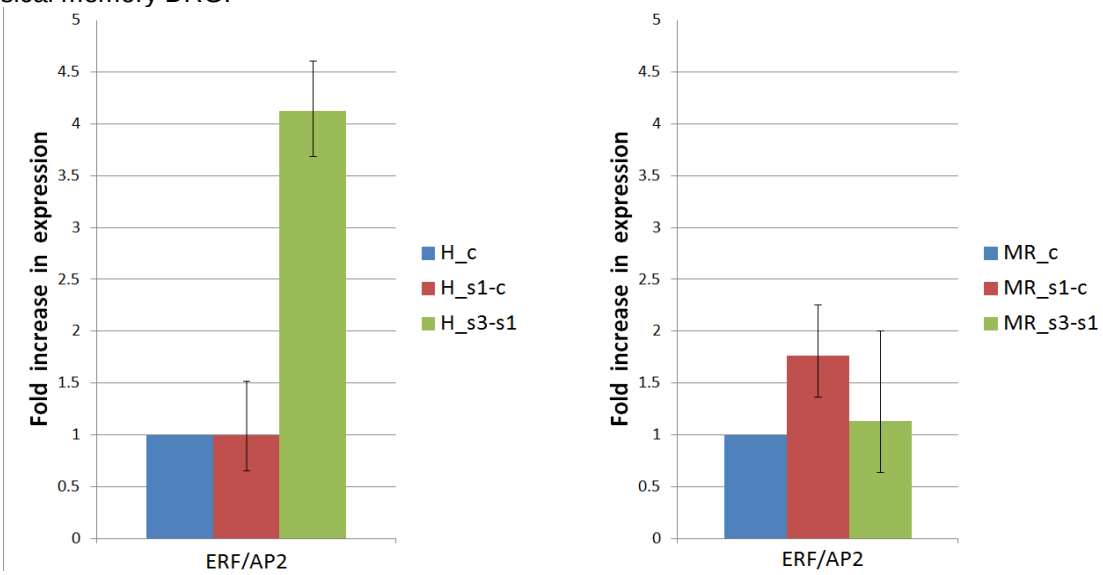


Figure 2: Transcription patterns of studied durum wheat landraces Hourani 27and Omrabi 5dehydration stress ERF\AP2 responding genes under initial pre-stressed watered (C) conditions, upon the first stress (S1), and upon a subsequent stress (S3) measured by real time qRT-PCR.

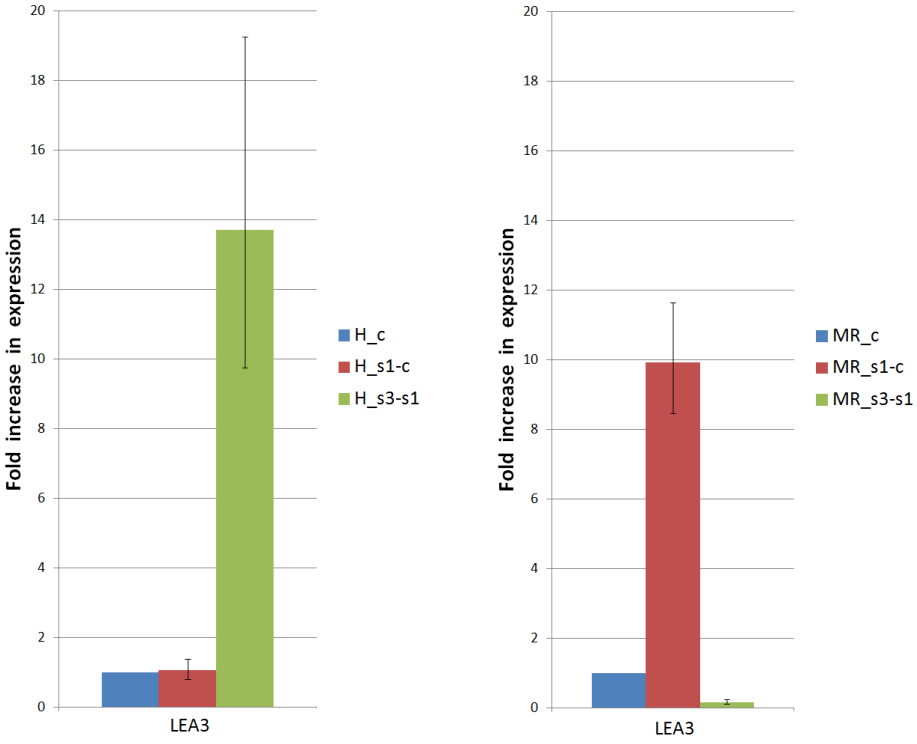


Figure 3:Transcription patterns of studied durum wheat landraces Hourani 27and Omrabi 5dehydration stress LEA3 responding genes under initial pre-stressed watered (C) conditions, up on the first stress (S1), and upon a subsequent stress (S3) measured by real time qRT-PCR.

The second DRG gene LEA3 showed the same trend as ERF/AP2 in Hourani 27, where a similar expression level in S1 compared to the control followed by a dramatic increase in S3 (13.71 fold) compared to S1. The expression behavior of LEA3 in Omrabi 5 followed the same trend as ERF/AP2, where the expression increased in S1 compared to the control (9.93 fold) followed a down regulations in S3 compared to S1.

Therefore, we found for the first time, an evidence of epigenetic memory of DRGs in durum wheat as it modulates gene expression patterns. In addition, gene expression behavior varies from a genotype to another. In our case, Omrabi5 is presumably more drought resilient than Hourani 27 for the investigated DRGs. However, it is worth investigating additional DRGs to get a more reasonable conclusion. The previous four transcription patterns indicate that the transcriptional behavior of dehydration stress responding genes under repeated stresses is more complicated than the behavior involved in a single dehydration stress. Ding et al. (2014, 2013) assigned simple classifications to the types of response during the first stress (+ or -) and the second stress (+, -, or =), combining them into six classes: [+ +], [—], [+ −], [− +], [+ =], and [− =]. Two additional classes [=/+] and [=/-] contain genes that in S1 did not change significantly expression compared to pre-stressed levels in W but significantly changed transcription in S3. Formally, these genes do not belong to the S1 dehydration-stress responding fraction.

The repetitive dehydration stress system employed in this study has similarities to the natural diurnal stress. We hypothesize that dehydration stress memory helps Plants to prepare for the next day stress if they were stressed the day before, despite alleviated stress signals during the night. Genes displaying transcriptional memory among the genes are responding to the first dehydration stress. Altering their expression levels in subsequent stresses, presumably, allows the plant to finely tune its responses to ongoing/recurring dehydration stress (Ding et al.2014; Virlouvet et al. 2018).

The possible biological relevance of the genes displaying transcriptional memory is considered of four overlapping strategies generally employed by a plant to improve its stress tolerance and/or survival: 1) increased synthesis of membrane protecting, damage-repairing, and detoxifying functions; 2) coordinating photosynthesis and growth under repetitive stress; 3) re-adjusting osmotic and ionic equilibrium to maintain homeostasis; and 4) readjusting interactions between dehydration and other stress/hormone regulated pathways.

LEA (late embryogenesis abundant) proteins functioning as molecular chaperones to maintain membrane structures, ion balance and homeostasis and enzymes for the synthesis of iso leucine (toxin degradation), serine (redox responses), and proline (an osmolyte) are encoded by [+/+] memory genes. Thereby, [+/+] memory genes ensure elevated synthesis of factors critical for cell survival under multiple dehydration stresses.

The ethylene responsive factor (ERF) family is a large family of plant-specific transcription factors that share a well-conserved DNA-binding domain. Proteins have been subdivided into five subfamilies (*Sakuma* et al. 2002): AP2subfamily, DREB subfamily, ERF subfamily, RAV subfamily and others. Members of the AP2 subfamily contain two AP2/ERF domains connected by a conserved linker of 25 amino acid such as AP2, ANT, Glossy15, At BBM and Bn BBM.Members of the DREB, ERF and other subgroups contain a single AP2/ERF domain, such as ZmDBFs, NtERFs, AtDREBs, AtCBFs, LePtis, AtEBP and AtERFs (Riechmann et al. 2000; Sakuma et al. 2002). The functions of the AP2/ERF-type transcription factors in plant biotic stress responses, with special emphasis on the regulations and functions of two major types of DREBs, DREB1/CBF and DREB2.

This research was the first to asses epigenetic modulation of DRG in 2 genotypes of Durum wheat, we found for the first time, an evidence of epigenetic memory of DRGs in durum wheat as it modulates gene expression patterns. An addition, gene expression behavior varies from a genotype to another. While Ding et al.2014 studied DRG between different varieties, *Zea mays* comparison with *Arabidopsis thaliana*. This study forms the basis for futures investigation on additional DRGs for different genotypes.

CONCLUSION

Transcriptional memory, like defense gene priming, can provide the benefits of a more robust or modified stress response while reducing the costs of the state of preparedness. The behavior of transcriptional memory genes in Um Rabi5 adds a new dimension to our understanding of plants' responses to dehydration stress and to current models for interactions between different signaling systems.

Different sets of designed primers varied in their specify in detection DRGs were found to be more efficient.

In addition, these tools can improve the breeding methodologies and strategies for the dryland in the Mediterranean region, such as in the case for dry areas in Jordan.

Therefore, we found for an evidence of epigenetic memory of DRGs in durum wheat as it modulates gene expression patterns.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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AUTHOR CONTRIBUTIONS

The experiment was designed by all authors. MA performed, maintained the experiment and collected the data. MA and MS conducted the analysis. All authors reviewed the manuscript, contributed to and approved the paper.

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