ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Drought stress response in two Algerian durum wheat genotypes: physiological and molecular analysis of two stress responsive genes

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Manuscript Info

Manuscript History:

Received: 22 August 2014
Final Accepted: 26 September 2014
Published Online: October 2014

Key words:

aquaporin, dehydrin, drought, durum wheat, Expression level, Physiological traits

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Abstract

Drought is a worldwide constrains affecting plant growth and productivity. This problem is becoming a more serious threat to the high-quality crop production within a changing climate. Drought triggers a variety of plant responses by modifying a range of genes expression. The aim of this work was to study two durum wheat genotypes (Waha and Beliouni) response to drought stress at physiological and molecular level, by focusing on two stress responsive genes (Dehydrin and Aquaporin). At the physiological level, the characterization of genotypes response was performed by determination of germination rate (GT), relative water content (RWC), leaf temperature (LT) stomatal conductance (g_s), chlorophyll content (SPAD index) and electrolyte leakage (EL). Expression level of an aquaporin gene (TdPIP2,1) and a dehydrin gene (DHN5) was also analyzed in different plant tissues under different drought stress conditions . The DHN5 gene is not expressed in the well watered plants but only in the stressed plants, while the TdPIP2,1 is expressed in both with more higher level in stressed tissues. Moreover, the transcript level of the two genes was more accumulated in the tolerant genotype (B) than the more sensitive one (W). These results confirm our physiological data. Identification of a possible connection between activated stress responsive genes can be useful by providing a starting point to further elaborate biotechnology-based approaches.

Introduction

Wheat production in Mediterranean region is greatly reduced by drought. As the worming of the climate system became unequivocal, drought events are increasing (Cativelli et al., 2010, Bhargava and Sawant., 2013). Understanding the response of plants to their external environment is of importance for developing drought tolerant agriculture species (Bernachia and Furini., 2004). Because of the complexity of abiotic stress tolerance mechanisms, biotechnology need to be complemented with plant physiology and breeding (Vinocur and Altman., 2005, Gupta et al., 2014). Plant tolerance responses rely on the expression of several genes (Shinozaki and Yamaguchi-Shinozaki., 2007). The outcome is a change in transcriptional programs leading to physiological responses. Among these responses, the synthesis of new proteins, including dehydrins and aquaporins (Bhargava and Sawant., 2013). Water movement across membrane is facilitated by aquaporins (AQP), proteins belonging to the conserved family of major intrinsic proteins (MIPs) ((Maurel., 2008, Chaumont and Tyerman., 2014**)**. Fives subfamilies of plants MIPs have been identified, among them the [Plasma membrane Intrinsec Proteins, PIPs]. PIP proteins could also be divided into

two subgroups PIP1 and PIP2 (Almeida-Rodriguez et al., 2010). The AQP appears as tetramers each of the monomers acting as an independent water pore (Tyreman et al., 2002, Maurel., 2007)**.** Over the last decade evidence has accumulated showing that water stress can change the expression patterns of MIPs (Lovisolo et al., 2007). In fact, water transport by AQP does not appear to be only for house-keeping. Various studies revealed a differential expression of mRNA transcripts and/or PIPs proteins upon exposure of plants to stresses such as salt, drought, or cold (Forest and Bhave et al., 2008). The wide variation of plant AQP expression is influenced by the species, developmental stage, growth conditions (Tyreman et al., 2002; Almeida-Rodriguez et al., 2010), and could also be influenced by the intensity and duration of stress (Galmes et al., 2007). Experimentations on the expression patterns of various AQP under water stress have brought extremely complex results **(**Santos et al., 2013**)**. Thus their role is still not completely understood. However, these proteins would be vital for water flow, homeostasis and identification or creation of any stress tolerant genotypes of crop species (Forest and Bhave et al., 2010).
The dehydrins (DHN) or group 2 LEA proteins are by far the most frequently described under stress conditions (Close.,1996, Goyal et al., 2005, Rorat et al., 2006, Brini ., 2007).These peripheral membrane proteins could be located in cytosol, nucleus and near plasma membrane (Qian et al., 2008, Hassan et al., 2013). They are thought to be involved in water binding molecules and stabilizing both macromolecules and membranes (Melloul et al., 2014**)**. This protective effect may be exerted either by direct interaction or by acting as molecular shield (Allagulova et al., 2003, Wise and Tunnaclife., 2004). Actually it has been suggested that DHN might exhibit specialized individual functions instead of one general function (Ildeko., 2013). Nevertheless DHN accumulation is associated with drought stress tolerance in different plant species (Rampino et al., 2006, Vaseva et al., 2013). The aim of the work was to study two durum wheat genotypes responses to drought stress at physiological and molecular level by focusing on two stress responsive genes, Dhn gene (Dhn5) and AQP gene (TdPIP 2,1).

Materials and methods

Plant material and stress conditions

Two Algerian cultivars of durm wheat (Triticum durum Desf L.), Waha (W) and Beliouni (B), were supplied by ITGC, Institut Technique des Grandes Cultures (Station El-khroub Algeria) and used in our experimentations. To test the response of seeds to drought stress, 25 seeds of the two wheat cultivars after being sterilized, were studied using distilled water (controls) and polyethylene glycol (PEG-6000) solutions of 10% (-0,6MPa) and 20% (-1,2MPa). As PEG is frequently used to induce water stress in germination experiments (Vahid., 2009; Jurial Balock et al., 2012). After two weeks of incubation (25°C, 16h-light/8-h dark photoperiod and 60$\pm$ 10% relative humidity), percentage of germination was determined. For other drought test, seeds were surface sterilized and pre-germinated in Petri dishes for 48h at room temperature and then transferred into pots containing a sand/soil mixture and grown under greenhouse conditions (16h photoperiod, 27 $\pm$ 10°C, 50 $\pm$ 10% relative humidity). Drought stress was imposed at the third leaf stage. The pots of each cultivar were divided into two groups: one was left as control and irrigated with tap water and the other was restricted to drought by withholding water up to the end of the experiment (twelve days). Samples were frozen in liquid nitrogen and stored at -80°C.

Physiological analysis

Leaf RWC was measured in control and stressed plants. Leaves were excised and weighted immediately (FW). The turgid weight (TW) was measured after floating on deionized water at 4°C for 24h in darkness. Dry weight (DW) was recorded after drying for 48h at 70°C. The RWC was calculated by the formula as given by Gonzàlez and Gonzàlez-Vilar., (2003): (fresh weight- dry weight)/ (rehydrated weight- dry weight) x100. The physiological status of plants was monitored by RWC. The stomatal conductance was measured at mid-morning on the adaxial leaf surface with a hand-held porometer (AP4). The given values represent the stomatal resistance (r_s), from which we deduced the stomatal conductance (g_S): $g_s = 1/r_s$. In order to find out leaf chlorophyll content, SPAD index was estimated nondestructively using a SPAD chlorophyll meter (Minolta crop, USA). It is a hand held spectrometer which measures light (650nm) absorbed by single leaves and gives an estimation of plant chlorophyll. This index was used preferentially as the strong relationship between readings of the chlorophyll meter and leaf chlorophyll content has been demonstrated by previous works (Neufeld et al., 2006, Silva et al., 2007). For leaf temperature, the evaluation was done using a hand held infrared thermometer. The thermometer was held so that the sensor viewed only the leaf surface within its natural orientation to avoid shade effects. Electrolyte leakage was determined according to the method described by Dkhil and Denden., (2012). Leaf samples were washed with distilled water in order to remove surface adhered electrolyte or tissue particles and then cut into discs of uniform size. After which the samples were immersed in 10 ml distilled water at room temperature. 24 h later, the conductivity of the solution

(C_1) was read. The samples were autoclaved at 120°C for 20 min, cooled to room temperature and conductivity of the solution (C_2) was read again. Electrolyte leakage was calculated as the ratio of C_1 to C_2.

RNA extraction and RT-PCR assay

Total RNA was extracted from 100 mg leaf tissue using the RNeasy total RNA isolation kit (Qiagen). To remove contaminating DNA, RNA_s (10 mg) were treated with RNase- free DNase (Promega). DNase treated RNA samples (0,5 mg) were reverse transcribed using M-MLV reverse transcriptase (Invitrogen). The reverse transcription (RT) reactions were performed at 37°C for 1h using 2 mM oligo-dT18. Two microlitres of the first strand cDNA was used as templates for PCR amplification. The TdPIP2,1 gene was amplified using specific primers reported in Table 1. Specific primers designed in the 3'UTR region of TdPIP2,1 were used to avoid any possibility of cross hybridization, nevertheless a possible cross-reaction cannot be excluded **(**Ayadi et al., 2011**)**. For the DHN5 gene, we also used the 3'UTR end as a probe. The DHN5 gene was amplified using specific primers reported in Table 1 (Brini et al., 2007). A wheat Actin gene fragment used as an internal control was also amplified with the primers reported in Table 1. Samples were denatured for 3min at 94°C and run for 35 cycles of 30 sec at 94°C, 30 sec at 50 °C and 2 min at 72°C followed by 5 min at 72°C as a final extension. To separate PCR product, an agarose gel electrophoresis was performed, stained with ethidium bromide and analyzed under ultra violet (UV) light.

Statistical analysis

For all collected data of the physiological parameters, standard deviation of means was calculated and analysis of variance was carried out, the means were compared using Student t test.

Results

Physiological analysis

The results revealed that under standard condition, the germination percentage of both cultivars was high (>90%). However, these percentage declined with PEG imposition and with a higher amount under 20% (-1,2MPa). Considering both genotypes, (W) suffered more under the PEG induced drought stress than (B). As expected, leaf RWC differed according to water treatment, the differences were highly significant (P<0.0001). Values ranged from a high of approximately 87 % showed by (B) for well watered condition to a low of approximately 64 % showed by (W) for the none watered. Drought stress can also affect leaf temperature. The values observed under control conditions become significantly (P<) higher under stress conditions, although with a lesser extent in (B). The results for the above parameters are shown in Table 2 and Figure 1.

Under stress conditions, a decrease in stomatal aperture was observed as indicated by lowering of stomatal conductance. The plants with optimum watering had significantly higher stomatal conductance than those under drought conditions (P<0.0001). Considering stressed plants separately, even so, (B) exhibited higher values than (W). As well as for the chlorophyll content, here to, it decreased significantly in all genotypes under drought conditions in comparison with controls (P<0.0001). Among stressed plants, (B) (with 38) had significantly higher SPAD index than (W) (with 35) (P<0.05). Our results also revealed that for both cultivars, electrolyte leakage increased under stress conditions, the lower values were recorded under control condition and significant difference was found between varieties (P<). These results are shown in Table 3 and Figure 2.

Expression analysis of the candidate genes TdPIP2,1 and DHN5

We have studied the expression level of two candidate genes involved in the plant desiccation. RT-PCR analysis of the AQP gene TdPIP2,1 in cultivars (W) and (B) exposed to 10% and 20 % PEG showed a high expression levels of TdPIP2,1 in the sheaths and in the leaves of the two genotypes compared to the roots in stress conditions. In control plants the mRNA levels of the TdPIP2,1 were maintained at low levels (Figure 3). When we compared the two genotypes, it seems that (B) accumulate more transcript than (W). For DHN-5, the expression levels was comparable to TdPIP2,1 except for the control tissues who we noticed the absence of transcripts in all plant tissues (Figure 3). A slightly higher expression of DHN-5 was observed in (B) as compared to (W) (Figure 3).

Table 1. Primers used in PCR expression analysis.

Primer sequence (5' to 3')	Target gene	Gene bank accession number	Amplicon size (bp)
TCCATCCAACACCAACACTAATAAC GGCGTACCACCAGTACATCC	TdPIP2,1	EU182655.1	400

GCGAATTCGAGGACGACGGCATGGGC GAATTCTCAGTGCTGGCCTGGG	DHN5	AY619566.1	344
GTGCCCATTTACGAAGGATA GAAGACTCCATGCCGATCAT	Actin	AB181991	380

Table 2. Result of Variance Analysis for germination rate (GT), leaf relative water content (RWC) and leaf temperature (LT) under control and drought conditions.

Treatments	Parameters		
	GT	RWC	LT
Water conditions (W)	2384,397***	780,531***	83,741***
Genotype (G)	515,205***	159,287***	21,068**
Interaction (GxW)	118,795***	46,571**	8,501*

Values are mean squares. *(P<0.05); **(P<0.01); ***(P<0.0001).

Table 3. Result of Variance Analysis for stomatal conductance (g_s), estimated chlorophyll content (SPAD index) and electrolyte leakage (EL) under control and drought conditions.

Treatments	Parameters		
	g_s	SPAD index	EL
Water conditions (W)	19201,600***	59,703***	1247,256***
Genotype (G)	1728,480**	14,297**	29,324**
Interaction (GxW)	560,060**	6,410*	12,526*

Values are mean square. *(P<0.05); **(P<0.01); ***(P<0.0001).

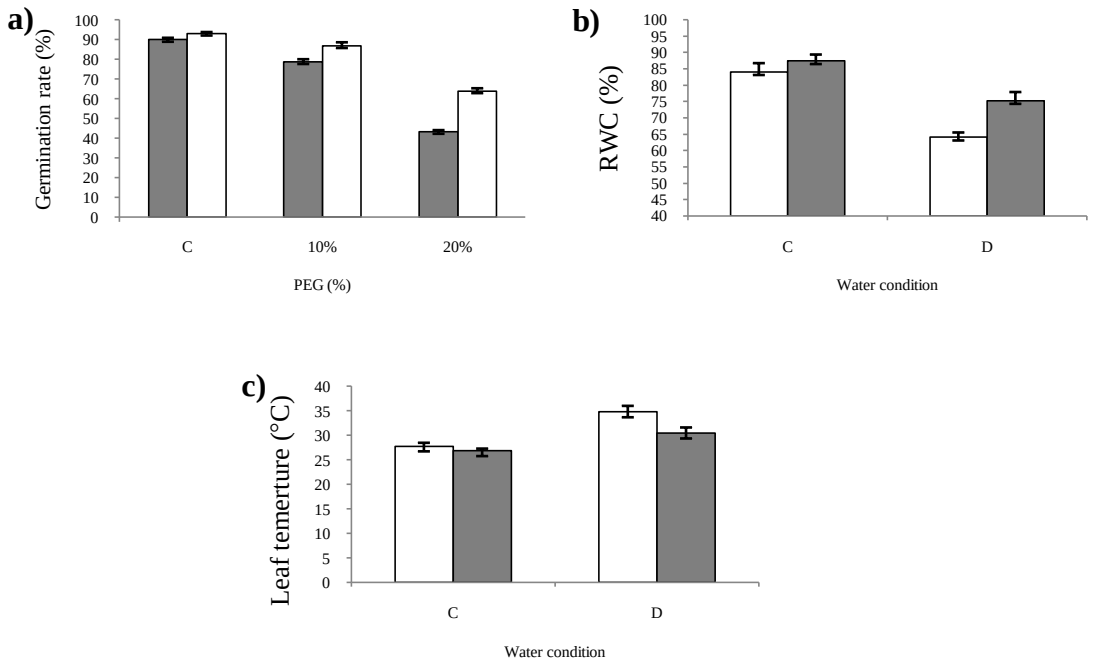

Figure 1.

Figure1. Effects of drought stress on a) Germination rate (GT), b) leaf Relative water content (RWC), c) Leaf temperature (LT) of two durum wheat genotypes (W) (white bars), (B) (grey bars) under control (C) and drought (D) conditions. Values are means ± SD (n=3).

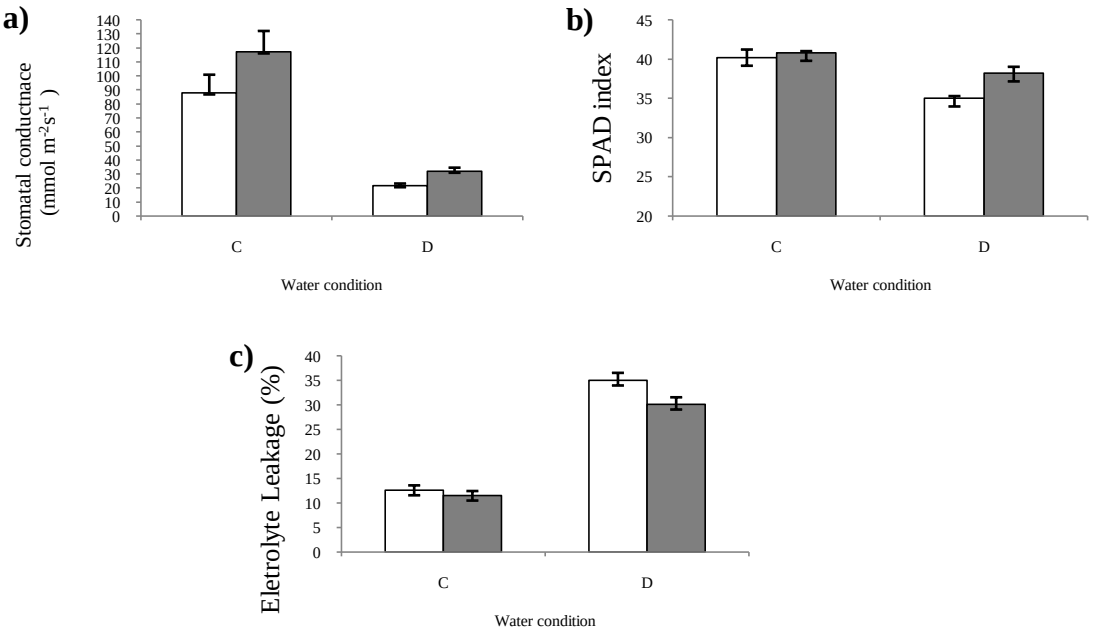

Figure 2.

Figure 2. Effects of drought stress on a) Stomatal conductance (g_s), b) estimated chlorophyll content (SPAD index), c) Electrolyte leakage (EL) of two durum wheat genotypes (W) (white bars), (B) (grey bars) under control (C) and drought (D) conditions. Values are means ± SD (n=3).

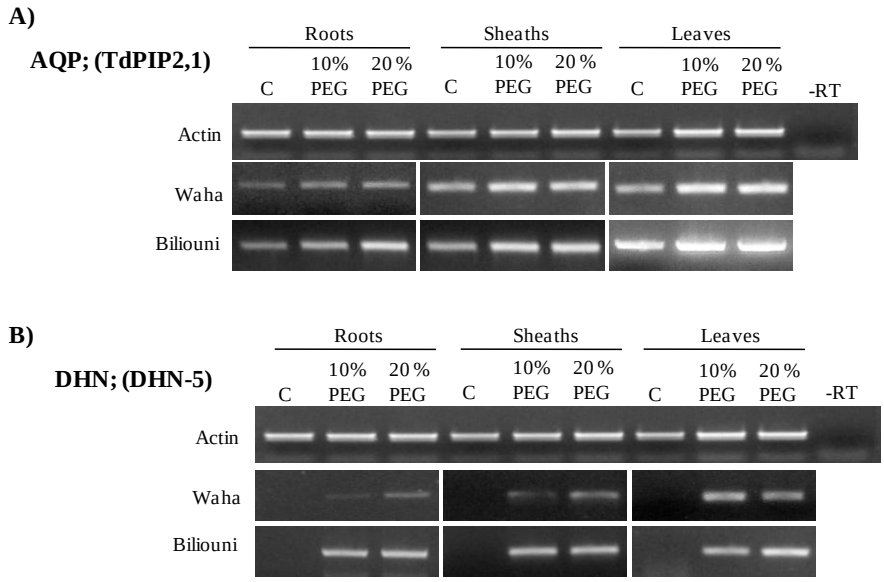

Figure 3.

Figure 3. RT-PCR analysis of (A) the aquaporin gene (TdPIP 2,1) and (B)the dehydrin gene (DHN5) in plant tissues of the two wheat genotype (B and W) under different stress conditions. The expected size of cDNA DHN5 is 344pb and TdPIP2,1 is 400pb. A 380pb Actin was amplified by RT-PCR as internal control.

Discussion

Drought tolerance under germination phase can be considered as an important criterion for plant survival and adaptation (Sassi et al., 2012). In fact, water stress may reduce or even prevent germination completely. Genotypic differences have been observed under PEG induced drought stress in particularly at -1,2MPa, (W) appeared to be more sensitive to water stress at germination phase than (B). Similar results have been reported previously (Khayatnezhad and Golamin., 2011). Under non stressed conditions, the analyzed samples exhibited RWC values compatible with a non stressed status. The values declined after water stress application. RWC is often used to investigate cell and tissue hydration which is highly required for optimum physiological function (Silva et al., 2007). Many studies have shown a reduction in leaves RWC, when subjected to drought (Talamè et al., 2006, Parwata et al., 2012, April et al., 2013, Melloul et al., 2014). Water stress affected stomatal conductance as well. The plants with optimum watering had higher values than those under stress conditions. This decrease had been mentioned by many authors (ykhlef and Djekoun., 2000, Baloch et al., 2012, Parwata et al., 2012). Plants adopt different strategies to avoid drought. Stomatal closure is one of them (Parwata et al., 2012). At severe stress, in order to optimize plant water statues, an increase in AQP expression with low stomatal conductance may boost membrane permeability and thus water transport (Galmes et al., 2007). When plants are subjected to drought stress (transpiration decreases), a lower stomatal conductance result in reduced evaporative cooling and thus in leaf temperature increase (Rizza et al., 2012; April et al., 2013). The genotype showing a relatively lower leaf temperature may be able to preserve a more favorable leaf water status (Silva et al., 2007). In addition to the previous parameters, leaf chlorophyll content was also affected by water deficit. Leaf chlorophyll amount could be a good indicator of plant physiological performance (Neufeld et al., 2006). The reduction of chlorophyll may be a consequence of chlorophyll degradation resulting from a prolonged photo-inhibition (Silva et al., 2007). Previous investigations have reported a reduction in chlorophyll concentration in water stressed durum wheat (Bousba et al., 2013), barley (Guo et al., 2009) and sugarcane (Silva et al., 2007). Studies have shown that, more tolerant wheat varieties tend to keep higher relative water content and higher chlorophyll content. (Atteya., 2003, Parwata et al., 2012). Cell membrane could also be targeted by drought and its stability is of a great importance (Rana et al., 2013). The range of membrane damage was assed indirectly by measurement of cells solute leakage which is inversely proportional to cell membrane stability (CMS) (Farooq and Azam., 2001). Our results revealed that membrane integrity was more conserved for (B) than (W). Drought induced electrolyte leakage has also been reported by others (Farissi et al., 2013). In fact the rate of injury to plasma membrane can be evaluated through electrolyte leakage from cells (Rana et al., 2013), and the proportion of leakage is relative to cell membrane damages (Sayar et al., 2008). AQP genes are important for many aspects of plant adaptation to drought (Forrest and Bhave., 2010). However, their role in leaf water transport under drought conditions remains unclear **(**Almeida-Rodriguez et al., 2010). The TdPIP2;1 mRNA was detected in leaves of both (B) and (W), which is consistent with previous reports showing the expression of a PIP2 AQP in leaves (Galmes et al., 2006, Aroca et al., 2006, Ayadi et al., 2011). The pattern followed by the expression profile of the TdPIP2,1 in leaves showed an increase under water stress with higher extent in (B) than (W). During drought stress, evidence for strong regulation of PIPs was found on transcriptional level **(**Zhang et al., 2007). According to previous studies, the AQP genes expression under water stress is dual and follows two phases: a moderate decrease in water availability results in down-regulation, while the more drastic decrease results in up-regulation **(**Yamada et al., 1997, Galmes et al., 2004). Our observations are consistent with the second phase. The down regulation of AQP gene expression is most commonly observed in Arabidopsis, with exception for: AtPIP1,4 and AtPIP2,5 (Alexenderson et al., 2005). However, Kawasaki and coworkers (2001) suggested that the initial down-regulation is followed by a subsequent up-regulation of AQP gene expression. Likewise, some MIP (Yamada et al., 1997, Lian et al., 2004) and PIP2 genes (Suga et al., 2002, Aroca et al., 2006, Zhang et al., 2007, Galmes et al., 2007, Lovisolo et al., 2007, Almeida-Rodriguez et al., 2010 and Santos et al., 2013) have been reported to be up-regulated under drought. The up-regulation is thought to increase membrane permeability to water transport when water is less available (Yamada et al., 1997) to facilitate water flux (Smith and Bhave., 2007**)** and thus to control water status **(**Santos et al., 2013**)**.

Dehydrins accumulation, have been previously associated with drought stress tolerance. Our results showed that DHN-5 transcript was not detected in plant tissues under standard conditions. However, upon water stress treatments there were highly accumulated in both genotypes, which is consist with the results previously reported by others (Brini et al., 2007; Medini et al., 2009, Iskandar et al., 2011, Melloul et al., 2014). Thus, plants subjected to cellular dehydration show substantial increase in the amount of most DHNs (Rorat et al., 2006). The fact that DHN5 gene was not expressed in well watered plants but only in the stressed ones, is consistent with a protection role adopted by plants in response to drought stress (Rampino et al., 2006). The DHN genes expression in drought stressed tissues may pull along tolerance mechanisms by inducing an appropriate cellular response, in order to prevent dehydration

damages (Melloul et al., 2014). The mechanism of action of DHNs is not known in detail (Brini et al., 2011). Due to their intrinsically unstructured character, DHNs can change their conformation and so they can exhibit versatile functions (Hanin et al., 2011). Thus, they can be implicated in protecting proteins and membrane integrity (Close., 1999; Wise and Tunnacliffe., 2004). DHNs can be transported between the nucleus and the cytoplasm suggesting a role in the transcriptional machinery (Carjuzaa et al., 2008). The upper expression of DHN5 coincide with the upper expression of TdPIP2,1 especially in (B). This is suggesting specific functional role of these proteins especially in drought stress tolerance. According to a recent study, a tomato AQP gene (AtPIP1,4 ortholog) was targeted by an ASR1 protein (LEA superfamily) and so up-regulated during drought stress (Ricardi et al., 2014). Moreover, transcripts levels of this AQP gene were found lower in ASR1 silenced plants **(**Ricardi et al., 2014**)**. In fact, Brini and coworkers, (2011) consider that through its potential chaperon property it's not excluded that the dehydrin (DHN-5) can be implicated in regulating the expression of other stress responsive genes

Conclusion

Plants breeding efforts to improve drought tolerance would be aided by a better understanding of the stress response on the cellular and molecular level. Our finding suggests that durum wheat can be able to adjust the expression of DHN5 and TdPIP2,1 according to environmental conditions. The upper expression of these two stress responsive genes may be valuable by setting up stress tolerance mechanisms in the different tissues and especially in leaves. Thus conduct to an appropriate cellular response and prevent dehydration damages. This investigation could be a starting point for more elaborate analysis of the regulation network DHN-stress responsive genes and to establish if the DHN5 has as a target an AQP gene and what are the others. Identification of a possible connection between activated stress responsive genes can be useful by providing a starting point to further elaborate biotechnology-based approaches.

Acknowledgements

This work was supported by the Ministry of Higher Education and Scientific Research of Algeria and Tunisia. Authors are grateful to the ITGC (Institue Thechnique des Grandes Culture) for providing seeds. Authors thank also Mrs Mouellef Adra for her help.

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