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Expression analysis and physiological response of *sunTIP7* aquaporin gene to different water regimes in sunflower

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Abstract

The response of a plant to drought stress is positively related to physiological traits and gene expression. Various recent studies suggest that membrane channel proteins, named aquaporin (AQPs), by affecting stomatal conductance behavior could be involved in plant responses to stress conditions. The *sunTIP7* gene is a member of AQPs protein that is included in different environmental stress such as drought stress. In this study in order to investigate the *sunTIP7* gene expression and its relation with relative water content (RWC), stomatal conductance, shoot fresh and dry weight, root area, chlorophyll index (SPAD) and electrolyte leakage (EL), six selected sunflower (*Helianthus annuus* L.) inbred lines were planted in a greenhouse under normal irrigation and water deficit conditions. The water deficit treatments were applied during 4th leaflet to flowering stage. Drought conditions reduced crop growth and physiological traits. The highest stomatal conductance was found in the C138 line under both conditions. Sunflower lines had different fold change expression of *sunTIP7* gene under drought stress. The expression of the *sunTIP7* gene was the lowest in C138, and this downregulation may explain its highest stomatal conductance. However, there was not any clear relationship between physiological traits and expression of *sunTIP7* gene in all six sunflower inbred lines. These results suggest that drought tolerance in sunflower is a complex trait and there is no simple molecular explanation for drought tolerance in sunflower lines.

Keywords: Aquaporin; Drought stress; Stomatal conductance; Sunflower; *sunTIP7*.

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Introduction

Regardless of the comparatively high water permeability of cell membranes, the plants contain the appointed channels of water that can help and regulate water passage. Some water-selective-channels proteins, that are called aquaporins (AQPs), relative to the major intrinsic protein (MIP) family, are present in all kingdoms. In plants, MIPs are sorted into several subfamilies: small basic intrinsic proteins (SIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NIPs) (Johanson *et al*. 2001; Zardoya and Villalba 2001; Forrest and Bhave 2008), GlypF like intrinsic proteins (GIPs) (Gustavsson *et al*. 2005), x intrinsic proteins (XIPs) (Venkatesh *et al.* 2015) and hybrid intrinsic proteins (HIPs) (Danielson and Johanson 2008). AQPs play a critical role in supporting plants to retain water balance and homeostasis under water stress status (Johansson *et al*. 2000; Galmés *et al*. 2007). Two classes of AQPs, the PIPs and the TIPs, have been most considered in water relations of shoots and roots. TIPs subfamily is used in turgor homeostasis and cell elongation with intermediating the transport of small molecules and water across the vacuolar membrane (Sarda *et al*. 1999). TIPs also are involved in response to drought and salt stress in different plants. For example, *rTIP1* in rice (Liu *et al*. 1994), *sunTIP20* in sunflower (Sarda *et al*. 1999) and *BobTIP26* in cauliflower (Barrieu *et al*. 1999) showed overexpression in drought stress. Expression of AQPs in guard cells of stomata have been reported in various species, such as tobacco (*NtAQP1*), *Arabidopsis thaliana* (*AtPIP1***;2**), spinach (*SoPIP1*;1), barley (*HvPIP1***;**6), broad bean (*VfPIP1*), maize (*ZmPIP1*;2; *ZmPIP2*;1/2;2) and the sunflower tonoplast intrinsic proteins *SunTIP7* and *SunTIP20* (Huang *et al*. 2002; Leonhardt *et al*. 2004; Fraysse *et al*. 2005; Wei *et al*. 2007; Hachez *et al*. 2008). According to Sarda *et al*. (1997), *SunTIP20* expression did not show a significant effect on the stomatal movement, but *SunTIP7* mRNA accumulated during stomatal closure. They indicated that *SunTIP* genes are differentially regulated in the same cell. Another *TIP* family expression was found by *in situ* hybridization in guard cells of mature organs and seedlings of *Picea abies* (Oliviusson *et al*. 2001). However, there is little direct evidence for the functional effects of AQPs in stomatal movement. For example, RNA interference-mediated decreases in the expression of the water and CO_2 diffusion facilitator *NtAQP1* (PIP1-like) has lower stomatal conductance in contrast to wild-type tobacco plants (Uehlein *et al*. 2008).

Sunflower is one of the most important sources of plant oil in the word that mainly is grown in warm to moderate semi-arid climatic areas. However, water deficit stress is a limiting factor in sunflower performance (Blanchet *et al*. 1981) and has significant impact on grain yield and composition of fatty acids (Alberio *et al*. 2016). Water deficit stress also stimulates many plant responses such as relative water content (RWC) and stomatal conductance (Ghobadi *et al*. 2013) and the expression of drought stress-inducible genes (Lim *et al*. 2012). In this study, considering the role of AQPs in plant response to water stress, we focused on some physiological traits and expression of *sunTIP7* gene under drought stress and regular irrigation in six inbred lines of sunflower.

Materials and Methods

Plant materials and water stress treatments

The sunflower inbred lines in this experiment were provided by Seed and Plant Improvement Institute (SPII), Karaj, Iran. The seeds were planted in 3.5 L pots filled with 50% sand and 50% field soil. The inbred lines and irrigation levels formed a complete factorial experiment. The pots were arranged in a randomized complete block design with four replications in a greenhouse of University of Zanjan, Iran. After determining the field capacity (FC) of the soil, 50% FC was considered as drought stress and 90% FC as the normal irrigation treatments. Drought stress was applied from the 4-leaf to flowering stages.

Measurements of morpho-physiological traits

All traits were measured at the flowering stage. Stomatal conductance was measured by leaf porometer (Nunes Instruments Co., India) as mM m^{-2} s^{-1} (Poormohammad Kiani *et al.* 2007).

RWC was determined based on the following formula:

RWC = [(FW – DW)/TW – DW)] × 100

where, FW is fresh weight, TW is turgid weight after 24 h of rehydration at 4 ºC in a dark room by placing the petioles in a container with distilled water, and DW is dry weight after drying up for 24 h at 80 ºC (Poormohammad Kiani *et al*. 2007). Shoot fresh weight was measured just after harvesting, and shoot dry weight was determined after drying the plants in oven at 72 ºC for 72 h. To measure the electrolyte leakage (EL), leaves were washed with the sterilized distilled water to remove surface contamination of leaves (Blum and Ebercon 1981). Leaf segments, containing 2 gr were placed in the vials with 10 ml of deionized water and incubated at 25 °C on a rotary shaker with 100 rpm. Then, the electrical conductivity of the solution (L_t) was measured after 24 h. Samples were then dried at 100 °C for one hour and the last conductivity reading (L_0) was gathered upon equilibration at 25 °C. The EL was finally calculated as (L_t/L_0) × 100 (Lutts *et al*. 1996). Chlorophyll index (SPAD) was assessed by using a portable chlorophyll meter (CCM-200, Opti-Sciences, England). For this purpose, measurements were made at upper, middle and lower points of each leaf (three leaves per plant). For the measurement of root area, the pots washed with water. The roots were separated from the soil and stained with black color, and then the root area was determined by the scanner (Mardani *et al*. 2012).

RNA extraction and gene expression analysis

To analyze the *sunTIP7* gene expression, leaf samples were harvested at the flowering stage and immediately frozen in the liquid nitrogen. Total RNA was extracted from each individual plant leaves by the GeneAll solution and the genomic DNA was removed by DNase I treatment. Reverse transcription **reactions were** performed using DNA-free RNA with cDNA Synthesis Kit (Pishgam, Iran) following the manufacturer's instructions. Real time PCR was performed in Rotor Gene Q using the real time SYBR Green MasterMix (Pishgam, Iran), following the manufacturer's instructions. Primers were designed by the primer3 site (http://primer3.ut.ee/) and OligoAnalyzer (https://eu.idtdna.com/). For normalization of gene expression, we used two housekeeping genes, 18s rRNA and actin. The results showed that the expression stability of the two reference genes was similar. The reference gene was 18s rRNA (Table 1).

Statistical analysis

Analysis of variance was performed for the measured data and means were compared by Duncan's multiple range test. Furthermore, Pearson's correlation coefficients among traits were calculated using the SPSS software. The expression fold change of the *sunTIP7* gene was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Because the relative expression data in the real-time PCR analysis was based on a small sample size and data were not normally distributed, test of significance for the relative expression of the *sunTIP7* gene was carried out by applying the bootstrap resampling method with 1,000 resamples using the REST software (Pfaffl *et al*. 2002).

Table 1. Oligonucleotides used for the real time PCR analysis.

Primer	Sequence	Gene ID
18s_H.a	F: TACCGTCCTAGTCTCAACCAT	AF107577.1
18s_H.a	R: TCAGCCTTGCGACCATAC	AF107577.1
Actin_H.a	F: CCCGTTCTTCTTACTGAGGCA	AF28264.1
Actin_H.a	R: TCCAGAATCCAGCACAATACC	AF28264.1
SunTIP7	F: CAACTTCTTGGCTCAACCGT	x95950.1
SunTIP7	R: GTAAACAGTGTAAACCATTGCGA	x95950.1

Results and Discussion

The higher resistance to water-deficit stress conditions can be achieved by altering some traits that help plants to cope with the drought stress effectively (Skoric 2009). In this study, analysis of variance and comparison of means showed significant differences among the lines for the physiological traits and the *sunTIP7* gene expression in both water regimes. Drought stress reduced stomatal conductance, RWC, shoot fresh weight, shoot dry weight, root area, SPAD and increased EL in all inbred lines, but the amount of change between lines was different (Tables 2 and Table 3). Because the *sunTIP7* gene is expressed in sunflower guard cells, we focused on the stomatal conductance in the sunflower inbred lines. C138 showed significantly higher stomatal conductance than other lines under both conditions. Although C138 had higher RWC and shoot dry weight than other lines in both conditions, it was not significantly different from C111 and C148 for RWC under drought stress and from A19 and R217 for shoot dry weight under normal irrigation (Tables 2 and 3). Moreover, this inbred line demonstrated smaller changes of stomatal conductance (16.92%) from normal irrigation to drought stress conditions compared with other lines (Tables 2 and 3). Although drought tolerant plants were often known by low stomatal conductance in drought stress condition, because they attempt to loose less water caused by transpiration (Poormohammad Kiani *et al*. 2007); however, Blum *et al.* (1981) have indicated that genotypes which have ability to retain more water without closing their stomata, are suitable for arid regions. Regarding these results, the genotypes such as C138 can be considered as drought-tolerant, because, despite the high stomatal conductance, its relative water content was higher than other inbred lines. The high RWC in C138 means that it has the ability to preserve more water under drought stress. The plant's root system is essential for optimizing water uptake and has an important role in drought stress, because root traits such as root length, root biomass and lateral root density determine the plant efficiency in water absorption from the soil (Rauf 2008). Reports showed that sunflower roots were affected by water shortage (Pekcan *et al*. 2016). Our results revealed that the root area is dramatically affected by drought stress. C138 showed significantly higher root area than other inbred lines under drought conditions (Table 3). The studies have indicated that genotypes with the extensive root systems access more water and maintain stomatal conductance under stress conditions (Wasson *et al*. 2012). Therefore, high stomatal conductance and RWC in C138 may be due to its higher root area,

Table 2. Mean values for growth and physiological traits in inbred lines of sunflower under normal irrigation.

Inbred line	Stomatal conductance	Relative water content	Shoot fresh weight	Shoot dry weight	Root area	SPAD	Electrolyte leakage
A19	210.50±2.64^{c}	71.42±1.01^{c}	123.16±1.40^{a}	14.14±1.33^{a}	221.97±3.57^{ab}	29.57±1.09^{a}	46.46±0.55^{d}
C111	173.00±7.74^{e}	77.25±1.03^{b}	110.91±1.91^{b}	9.86±0.81^{b}	184.68±8.35^{b}	18.95±1.03^{c}	41.86±0.92^{b}
C123	189.50±3.69^{d}	71.08±0.49^{cd}	126.35±1.65^{a}	10.17±0.81^{b}	212.33±2.97^{ab}	19.10±1.97^{c}	44.39±1.15^{c}
C138	263.00±3.36^{a}	85.30±1.07^{a}	123.12±2.27^{a}	14.77±1.23^{a}	240.94±9.11^{a}	20.05±0.78^{bc}	38.08±1.54^{a}
C148	176.25±6.34^{e}	69.61±0.90^{d}	117.51±0.91^{ab}	9.84±1.53^{b}	185.73±8.78^{b}	21.15±1.13^{b}	41.11±1.61^{b}
R217	222.00±3.16^{b}	70.84±0.30^{cd}	122.19±3.37^{a}	13.07±0.85^{a}	204.22±6.91^{ab}	21.32±0.90^{b}	48.21±1.37^{d}

Values are the mean of four measurements per line and ± indicates the standard error. Values followed by different letters indicate significant difference at the 0.05 probability level using Duncan’s multiple range test.

Table 3. Mean values for growth and physiological traits in inbred lines of sunflower under drought stress.

Inbred line	Stomatal conductance	Relative water content	Shoot fresh weight	Shoot dry weight	Root area	SPAD	Electrolyte leakage
A19	154.25±1.70^{c}	57.57±1.91^{b}	89.72±2.07^{c}	7.48±0.98^{b}	166.58±8.61^{b}	15.24±0.41^{a}	65.23±0.79^{e}
C111	124.33±3.21^{e}	61.65±1.12^{a}	105.40±0.65^{a}	5.09±1.10^{c}	128.30±3.07^{d}	12.70±1.25^{b}	56.96±0.46^{bc}
C123	134.00±4.16^{d}	56.83±0.98^{b}	78.39±2.68^{d}	6.19±1.56b^{c}	153.80±6.39^{c}	10.10±0.74^{c}	62.47±1.31^{d}
C138	218.50±2.64^{a}	62.10±0.56^{a}	99.09±2.82^{ab}	9.55±0.93^{a}	185.56±9.53^{a}	14.27±0.89^{a}	52.84±1.75^{a}
C148	113.75±3.50^{f}	61.07±1.61^{a}	64.89±3.75^{e}	6.25±0.77^{bc}	132.83±5.40^{d}	11.51±0.56^{b}	56.23±1.61^{b}
R217	162.00±5.47^{b}	56.13±0.96^{b}	96.05±4.26^{bc}	6.21±1.21^{bc}	144.60±8.23^{c}	14.55±0.82^{a}	59.12±4.36^{c}

Values are the mean of four measurements per line and ± indicates the standard error. Values followed by different letters indicate significant difference at the 0.05 probability level using Duncan’s multiple range test.

because this trait had positive correlation with stomatal conductance and RWC in the sunflower lines (Table 4).

The cell membrane is vulnerable to abiotic stresses. Thus, there is an agreement that maintenance of stability and integrity of cell membranes under drought stress can be the main component of drought tolerance in different plants (Bajji *et al*. 2002). Our results indicated that EL was increased by water shortage. Ghobadi (2013) also reported that EL is increased with drought. Among the sunflower lines, C138 had the lowest EL (Tables 2 and 3). Since lower leaf EL is a criterion of membrane stability of the cell (Ghobadi *et al*. 2013), thus C138 line had the highest membrane stability among lines. EL had negative correlation with other traits under study (Table 4).

Since biomass positively correlates with chlorophyll content and RWC (Zaharieva *et al.* 2001), it seems that SPAD can be a beneficial criterion for selecting the genotypes with more tolerance to drought stress. Furthermore, SPAD value is regarded as an efficient and none-destructive method for screening of the genotypes. We also found a positive and significant correlation of SPAD with shoot fresh weight, shoot dry weight and RWC (Table 4). In this study, SPAD significantly decreased under drought stress. Among the lines, only A19 reserved a high SPAD value in both conditions; however, it was not significantly different from C138 and R217 under drought stress (Tables 2 and 3). Our results were consistent with those from other studies (Ghaffari *et al*. 2012).

A large variation was observed in the relative expression of the *sunTIP7* gene among sunflower lines. As shown in Figure 1, the fold changes in three lines (C138, C111, C123) by using the bootstrap resampling method were significant and negative. Whereas, none-significant positive fold

Table 4. Pearson correlation coefficients between physiological and growth traits of sunflower.

Trait	SPAD	RWC	EL	Shoot FW	Shoot DW	Root area	Stomatal cond.
SPAD	1						
RWC	0.66**	1					
EL	-0.65**	-0.92**	1				
Shoot FW	0.76**	0.73**	-0.73**	1			
Shoot DW	0.78**	0.79**	-0.72**	0.71**	1		
Root area	0.71**	0.73**	-0.69**	0.70**	0.80**	1	
Stomatal Cond.	0.63**	0.73**	-0.67**	0.72**	0.87**	0.82**	1

RWC: relative water content; EL: electrolyte leakage; Shoot FW: shoot fresh weight; Shoot DW: shoot dry weight; Stomatal cond.: stomatal conductance; **significant at the 0.01 probability level.

changes in the expression of the *sunTIP7* gene were observed in the other lines (C148, A19, R217). The highest downregulation of *sunTIP7* was observed in C138. Thus, the highest stomatal conductance in this line may be explained by downregulation of the *sunTIP7* gene in the guard cells. Nevertheless, the same relation was not observed in the other lines with negative fold change (C111, C123) (Figure 1). Also, in other studies, the overexpression of an Arabidopsis *AQPs* (*PIP1b*) in the transgenic tobacco (*Nicotiana tabacum*) plants did not have useful effects and even had a negative impact on efficiency of the plants under drought stress (Aharon *et al*. 2003). Downregulation of *sunTIP7* was previously reported in leaves of sunflower in response to drought stress (Aguado *et al*. 2014). Also, investigations on the model halophytic plant *Mesembryanthemum crystallinum* showed a temporary and coordinated decrease in the expression of three aquaporin genes in salinity stress (Yamada *et al*. 1995). Some studies have reported the effect of AQPs expression on stomatal behavior and RWC; for instance, expression of *Vicia faba PIP1* (*VfPIP1*) gene in transgenic Arabidopsis plants prevents water loss because of the induction of stomatal closure (Cui *et al*. 2008). This is inconsistent with our results, as we mentioned above, C138 had high stomatal conductance and RWC with low *sunTIP7* expression in the drought condition. The higher RWC and stomatal conductance in C138 can be explained partly by its extensive root system. Martre *et al*. (2002) indicated that the PIPs play an important role in the recovery of Arabidopsis from the water-deficient condition. Some studies have reported that AQPs genes are down- or up-regulated by osmotic stress and drought stress in several plant species, such as sunflower, Arabidopsis, rice and cauliflower (Yamaguchi-Shinozaki *et al*. 1992; Liu *et al*. 1994; Barrieu *et al*. 1999; Sarda *et al*. 1999). Overexpression of a member of PIP1 subfamily in the transgenic rice improved its water status in drought condition (Lian *et al*. 2004). Furthermore, the *AQPs* expression of drought-adapted and non-adapted species was different (Lian *et al*. 2004). Due to AQP sensitivity to various factors, such as cell water status and temperature, plant water status and hydraulic conductance, they are responsive to the environmental variation (Moshelion *et al*. 2015). The different results about the role of *AQPs* in drought tolerance could be also explained by their complex nature (Alexandersson *et al*. 2005) and

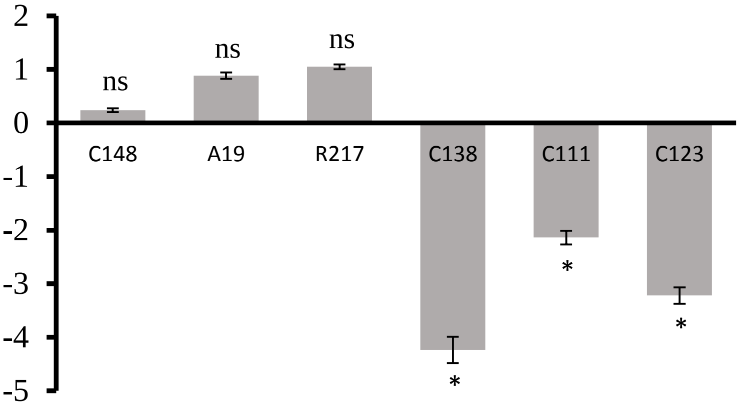

Figure 1. The fold change expression of the *sunTIP7* gene in leaf tissues of six inbred lines of sunflower from normal irrigation to drought stress conditions. Vertical bars represent standard errors. Significance levels were determined by a bootstrap test.

also variation among and within species.

According to Sarda *et al*. (1997), *sunTIP7* expression in sunflower increased during stomatal closure and *VfPIP1* expression in broad bean (*Vicia faba*) induced stomatal closure (Huang *et al*. 2002).

These regulations may help to the efficient flux of water in tissues (Kirch *et al*. 2000) or they may cause the extensive change in tissue morphology and plant cells under stress conditions (Barrieu *et al*. 1999).

Conclusion

In this study, the amount of growth and physiological traits, and *sunTIP7* expression in sunflower inbred lines was different at both well-watered and drought conditions. Drought stress decreased all growth traits and increased EL. Expression analysis of *sunTIP7* aquaporin gene showed its down-regulation in three sunflower inbred lines C138, C111 and C123. The highest downregulation of *sunTIP7* belonged to C138, which had also the highest stomatal conductance. However, this was not true for the lines C111 and C123. Thus, down-regulation of AQPs could not play a definite role in stomatal behavior in all sunflower lines under water stress. The different results about the expression of *sunTIP7* gene and its relationship with physiological traits in response to water stress can be attributed to the complex nature of AQPs patterns. The higher stomatal conductance, RWC and photosynthesis of C138 as compared to other sunflower inbred lines resulted in the highest biomass of this line, which can be partly attributed to the down-regulation of *sunTIP7* in the leaves and its higher root area. Therefore, C138 is recommended as a suitable inbred line to be used in hybrid seed production programs in dry zones.

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تجزیه بیان و پاسخ فیزیولوژیک ژن آکواپورین *sunTIP7* تحت رژیم‌های مختلف آبیاری در آفتابگردان

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چکیده
پاسخ گیاه به تنش خشکی با صفات فیزیولوژیکی و بیان ژن رابطه مثبتی دارد. مطالعات متعدد اخیر نشان می‌دهد که پروتئین‌های کانال غشایی به نام Aquaporin (AQPs) با تأثیر بر رفتار هدایت روزنه می‌توانند در پاسخ گیاهان به شرایط تنش نقش داشته باشند. ژن *sunTIP7* عضو خانواده AQPs می‌باشد که در تنش‌های مختلف محیطی از جمله تنش خشکی درگیر می‌باشد. در این مطالعه به منظور بررسی بیان ژن *sunTIP7* و ارتباط آن با صفات محتوای آب نسبی (RWC)، هدایت روزنه‌ای، وزن تر و خشک بخش هوایی گیاه، سطح ریشه، شاخص کلروفیل (SPAD) و نشت الکترولیتی در شرایط تنش خشکی، شش لاین اینبرد انتخابی آفتابگردان (*Helianthus annuus* L.) در شرایط آبیاری نرمال و کم‌آبیاری در گلخانه کشت شدند. تیمار کم‌آبیاری در مرحله چهار برگی تا زمان گلدهی اعمال گردید و موجب کاهش صفات فیزیولوژیک و رشدی گیاه شد. بیشترین میزان هدایت روزنه‌ای در هر دو شرایط در لاین C138 مشاهده شد. مقدار بیان ژن *sunTIP7* در لاین‌های آفتابگردان در شرایط تنش متفاوت بود. کمترین میزان بیان این ژن در لاین C138 مشاهده شد که این کاهش بیان احتمالا با هدایت روزنه‌ای بالا در این لاین مرتبط است. با این حال رابطه مشخصی بین بیان صفات فیزیولوژیک و بیان ژن *sunTIP7* در لاین‌های مورد بررسی مشاهده نشد. این نتایج نشان می‌دهد که تحمل به خشکی در آفتابگردان یک صفت پیچیده بوده و یک راهکار مولکولی ساده قادر به توضیح تحمل خشکی در لاین‌های اینبرد آفتابگردان نیست.

واژه‌های کلیدی: آفتابگردان؛ آکواپورین؛ تنش خشکی؛ هدایت روزنه‌ای؛ *sunTIP7*.