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Differential expression pattern of transcription factors across annual *Medicago* genotypes in response to salinity stress

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Abstract

Plants respond differently to salinity stress due to their unique gene architectures. Among genes, transcription factors (TFs) regulate many physiological and biochemical processes by modulating the rate of transcription initiation of target genes. Modulation of TFs has been correlated to the salt adaptation of any given genotype. In order to identify the expression of eight TFs (belong to bHLH, CBF, MYB, WRKY, and Zpt2 families) in three annual *Medicago* genotypes (*M. polymorpha* cv. Ieze, *M. laciniata* cv. Shushtar, and *M. laciniata* cv. Gheshm) under salinity stress, the RT-qPCR analyses were performed. Attempts were also made to establish relationships between gene expression profiles and morpho-physiological traits in these genotypes. In response to salinity, cv. Ieze had minimal changes in biomass, the electrolyte leakage, H_2O_2 content, and the higher ratio of reduced to oxidized glutathione than the other genotypes. Furthermore, Ieze had lower accumulation of Na^+ and less decrease in K^+ content. Altogether, it is concluded that Ieze could be regarded as a salt tolerant genotype. Transcriptome profile showed considerable variation across *Medicago* genotypes and among plant tissues. Among five TFs, *Zpt2-2* and *CBF4* had higher expression in salt-tolerant genotypes suggesting these genes as good candidates in genetic improvement programs to produce stress-tolerant plants.

Additional key words: electrolyte leakage, glutathione, H_2O_2 content, potassium, RT-qPCR, sodium, transcriptome analysis.

Introduction

Tolerance to abiotic stresses is a quantitative trait in which numbers of genes work in concert. Interactions among genes themselves and interactions between genes and the environment make it hard to ascertain the genes that are actually responsible for salinity tolerance (Sanchez *et al*. 2011). Nowadays, the development of high throughput omics technologies has provided an opportunity for breeders to understand the molecular basis of complex plant processes and also to search for candidate genes that confer tolerance to abiotic stresses (Vilanova *et al*. 2012, Baloglu *et al*. 2014), which makes marker assisted selection more efficient.

Plants differently respond to salinity stress, which is modulated through a series of signaling molecules involved in the regulation of stress-inducible genes (Wang *et al*. 2014). Transcription factors (TFs) interact with *cis*-acting elements present in the promoter region of various stress-responsive genes and thus activate whole network of processes leading to enhanced tolerance to multiple stresses (Lindemose *et al*. 2013). Accordingly, TFs are considered as an attractive category of genes for manipulation of abiotic stress tolerance, as their overexpression can lead to either up-regulation or down-regulation of the whole array of genes under their control (Rafiei *et al*. 2015).

Legumes are economically important plants that provide grain, forage, and edible oils. They also contribute to sustainable agriculture as nitrogen-fixers in association with rhizobial bacteria. Among legumes, annual *Medicago* species play an important agronomic role in dry land farming (Sheaffer *et al*. 2001, Walsh *et al*. 2001, De Haan *et al*. 2002). *Medicago truncatula*, a salt-sensitive species (glycophyte), has been selected as a model in genetic research (Merchan *et al*. 2007). Though there is a great deal of information on the transcriptom pattern of *M. truncatula* in response to salinity, little information exists about other annual *Medigaco* species, which are well adapted to different environmental

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Abbreviations: EL - electrolyte leakage; GSH - reduced glutathione; GSSG - oxidized glutathione; RT-qPCR - reverse transcription quantitative polymerase chain reaction; TFs - transcription factors; TGSH - total glutathione.
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conditions (Karami *et al*. 2015) and may have unique beneficial genes that *Medicago sativ*a lacks (Tivoli *et al*. 2006). This may confer an excellent implication to plant breeders in order to increase the resistance of plants to different stresses. Identification of transcription factors responsible for salinity tolerance may participate in generating transgenic plants coping with salinity. In addition, they can possibly be used for determining salt-tolerant genotypes in plant breeding programs.

The objective of the present study was to evaluate the possibility of gene expression pattern as a potential tool to select salt tolerant genotype. In order to achieve that, salinity effects on expression profiles of *bHLH*, *CBF4*, *MYB* (*MYB112, MYB14*), *WRKY* (*WRKY70-1*, *WRKY70-2*), and *Zpt2* (*Zpt2-1*, *Zpt2-2*) genes in two annual *Medicago* species (*M. polymorpha* and *M. laciniata*) were studied. The responses of these species to salt stress in terms of vegetative growth, H_2O_2, glutathione, Na^+, and K^+ content, and electrolyte leakage were analyzed.

Materials and methods

Plants and growth conditions: Seeds of *Medicago polymorpha* L. cv. Ieze, *M. laciniata* (L.) Mill. cvs. Shushtar and Gheshm were provided by Iranian Forest, Range and Watershed Organization (www.frw.org.ir). The seeds were soaked in concentrated sulfuric acid for 5 min to facilitate germination and rinsed 4 - 5 times with distilled water. All seeds were then placed in Petri dishes and stored for 1 d at 24 °C to ensure uniform germination. The germinated seedlings were transferred to pots containing a mixture of *Coco-peat*:*Perlite* (70:30) and irrigated with half-strength Hoagland solution (Hoagland and Arnon 1950), pH 6. After a week, the plants were watered with complete Hoagland solution. The plants were grown in a controlled glasshouse with day/night temperatures of 25/21 °C, a relative humidity of 65 %, a 16-h photoperiod, and a photosynthetic photon flux density of 300 μmol $m^{-2}s^{-1}$.

Salinity stress experiment: Four-week-old plants were exposed to salinity treatments in a completely randomized block design, with two NaCl concentrations (0 and 150 mM NaCl), three genotypes, and three replications. After three weeks of salinity treatment, seven plants were sampled of each replicate (21 plants per treatment) and left at 60 °C for 72 h to determine dry mass of shoots and roots.

Electrolyte leakage (EL) was determined as described in details by Lopez-Prez *et al*. (2009). The content of Na^+ and K^+ was measured in dry tissue samples by means of a flame photometer. The content of H_2O_2 was determined by the method described by Alexieva *et al*. (2001). An amount of 0.05 g fresh tissue was ground in a pre-cooled hand grinder in presence of 0.1 % (m/v) trichloroacetic acid. The resulting extract was centrifuged at 12 000 *g* and 4 °C for 15 min. A standard curve was plotted by reading the absorbance at 390 nm of known concentrations of H_2O_2.

Total glutathione (TGSH) content of the roots and shoots was measured according to May and Leaver (1993). In brief, 100 mg of fresh plant tissue was ground in liquid nitrogen and extracted in 0.5 cm^3 of 0.1 M phosphate buffer with 5 mM EDTA (pH 7.6). Following centrifugation at 9 000 *g* for 10 min, 0.1 cm^3 of the supernatant was taken in a 1 cm^3 mixture containing 6 mM dithiobis-(2-nitrobenzoic acid) (*Sigma-Aldrich*, St. Louis, USA), 3 mM NADPH, and 2 units of glutathione reductase from *Saccharomyces cerevisiae* (*Sigma-Aldrich*) and total glutathione was measured spectrophotometrically. Glutathione-dependent reduction of dithiobis (2-nitrobenzoic acid) was monitored at 412 nm. TGSH content was calculated by means of a linear regression equation generated from a standard glutathione (GSH) curve. Oxidized glutathione (GSSG) was determined in the same extracts after derivatization of reduced GSH. Plant extract (0.1 cm^3) was derivatized in 0.5 cm^3 of 0.5 M phosphate buffer solution, pH 7.6, in the presence of 4 cm^3 of 2-vinyl pyridine (*Sigma-Aldrich*) during 1 h at room temperature. After extraction of the GSH-conjugated 2-vinyl pyridine with diethylether, the GSSG was measured by a spectrophotometer. GSH was determined as the difference between TGSH and GSSG.

RNA extraction, cDNA synthesis, and reverse transcription (RT)-qPCR: For transcriptome analysis, samples of roots and leaves were collected 24 h after salinity treatment and immediately stored in liquid nitrogen at -80 °C until further analyses. The reason for collecting the samples after 24 h was due to the fact that TFs are early sensitive elements to any kind of stress. Such early response could be exploited in marker assisted selection, which enables us to select tolerant genotype in early stage of development. Each sample had two biological replicates. Total RNA was extracted by *Dena Zist Asia*, Mashhad, Iran according to the manufacturer's instructions. DNase treatment was conducted by *Fermentas* (Hanover, Germany) kit. For RT-qPCR, 1.5 μg of total RNA was reverse transcribed at 42 °C for 1 h using the *SUPERSCRIPT II* first-strand synthesis system (*Vivantis*, Malaysia) and subsequently denatured at 75 °C for 10 min. The PCR reaction mixture contained 4 mm^3 of 8 × diluted cDNA, 5 mm^3 of *SYBR Green Master Mix* (*TaKaRa*, Japan), 0.5 mm^3 of forward primers, and 0.5 mm^3 of reverse primers (Table 1 Suppl.). Each biological replicate had four technical replications in RT-qPCR. The specificity of the primers and the product length were confirmed by checking the melting temperature, and running the RT-qPCR products on agarose gel.

In the current study, the TF genes including *bHLH* (JN833714) (Zahaf *et al*. 2012), *CBF4* (XM_003592274.2)

(Li *et al*. 2011), *Zpt2-1* (XM_013606316), and *Zpt2-2* (XM_003592454.2) (De Lorenzo *et al*. 2007) were selected based on literature mining. Also, WRKY, and MYB transcripts were selected based on analysis of the microarray datasets (GSE13921 and GSE14029) available in *NCBI*. These microarray data were used by Li *et al*. (2009) and included transcriptome analysis of *M. truncatula* cv. A17 in salt stress condition. The relevant *TAIR* annotation (homolog genes in *Arabidopsis*) for these genes, were obtained using *Plant Expression Database* (www.plexdb.org/mod-ules/gl Suite/gl_main. php; Dash *et al*. 2012). Mtr.15010.1.S_s1_at (*TAIR* annotation: AT1G48000; gene name: *AtMYB112*) and Mtr.37886.1.S_at (*TAIR* annotation: AT2G31180; gene name: *AtMYB14*) were selected as candidate transcripts for MYB family. Mtr.23616.1.S1_at (*TAIR* annotation: AT3G56400; gene name: *AtWRKY70*) were selected as candidate transcripts for WRKY family. *FASTA* sequence of the selected WRKY and MYB transcripts were downloaded from *Plexdb* and blasted against *M. truncatula* genome in *NCBI*. *BLASTN* results indicated that *M. truncatula* had two homologs for entire mRNA sequence of Mtr.23616.1.S1_at, which were XM_003623590.1 (designated as *WRKY70-1* throughout the manuscript) and XM_003623586.1 (designated as *WRKY70-2* throughout the manuscript). Despite having very high homology, these sequences were not identical and their protein ID was distinct. Therefore, authentic primers were designed based on their domain. *Actin* gene was selected as internal control (housekeeping). The sequences of designed primers are summarized in Table 1 Suppl.

Statistical analysis: A randomized complete block design was used to analyze the morpho-physiological data by *SAS* software (*v. 9*). The $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008) was used to represent RT-qPCR data. Significant differences were identified according to a *t*-test analysis for comparing the morpho-physiological traits. The high efficiency of the same primer pairs used in RT-qPCR across all *Medicago* genotypes enabled us to compare the expression of each gene among them. The mean comparisons of greater than twofold change having *P* value less than 0.05 were expressed as significant result.

Results

Salinity resulted in a significant reduction in dry masses of shoots and roots, however, *M. polymorpha* had the greatest performance under salinity among the three genotypes (Table 1). The content of Na^+ in the shoots and roots of *Medicago* species significantly increased and the content of K^+ significantly decreased in all stressed plants. Under salinity treatment, *M. polymorpha* consistently had less Na^+ accumulation and less K^+ reduction compared to *M. laciniata* genotypes (Table 1). Electrolyte leakage (EL) was significantly increased in the shoots and roots of all salinity-treated plants. EL was lower in *M. polymorpha* than *M. laciniata*. In general, EL was higher in the roots than in shoots of all studied genotypes (Table 1). The content of H_2O_2 in the shoots and roots of all genotypes was increased by salinity, which was more profound in *M. laciniata* genotypes.

Table 1. Different responses of annual *Medicago* genotypes to salinity. Means ± SE, n = 21 (for biomass) or 9 (for other data); *, ** - significant differences among genotypes at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Genotypes	*M. polymorpha* (Ieze)		*M. laciniata* (Gheshm)		*M. laciniata* (Shushtar)	
Treatment/parameters	control	salinity	control	salinity	control	salinity
Shoot dry mass [g]	0.46 ±0.009	0.43 ±0.002*	0.12 ±0.002	0.06 ±0.004**	0.14 ±0.02	0.08 ±0.003**
Root dry mass [g]	0.072±0.002	0.069±0.004	0.02 ±0.000	0.013±0.000**	0.021±0.001	0.014±0.002**
Na^+, shoot [mmol g^{-1}(d.m.)]	0.016±0.001	0.25 ±0.03**	0.017±0.007	0.29 ±0.001**	0.016±0.002	0.26 ±0.06**
K^+, shoot [mmol g^{-1}(d.m.)]	2.75 ±0.005	1.18 ±0.08**	2.80 ±0.005	1.01 ±0.05**	2.60 ±0.08	1.07 ±0.05**
Na^+, root [mmol g^{-1}(d.m.)]	0.14 ±0.005	0.96 ±0.01**	0.14 ±0.004	1.36 ±0.02**	0.12 ±0.04	1.14 ±0.01**
K^+, root [mmol g^{-1}(d.m.)]	2.10 ±0.06	0.98 ±0.06**	2.30 ±0.02	0.80 ±0.007**	2.01 ±0.04	0.86 ±0.02*
EL, shoot [%]	2.39 ±0.04	5.29 ±0.67**	2.46 ±0.28	8.37 ±0.12**	3.31 ±0.15	8.89 ±0.06**
EL, root [%]	23.30 ±0.58	38.10 ±1.76**	33.19 ±1.55	77.22 ±3.05**	25.09 ±0.23	66.98 ±2.95**
H_2O_2. shoot [µmol g^{-1}(f.m.)]	2.59 ±0.16	6.06 ±0.16**	6.20 ±0.58	17.02 ±0.99**	4.51 ±0.22	11.69 ±0.66**
H_2O_2, root [µmol g^{-1}(f.m.)]	2.59 ±0.16	5.77 ±0.28**	2.45 ±0.24	7.88 ±0.41**	2.36 ±0.03	8.13 ±0.46**
TGSH, shoot [µmol g^{-1}(f.m.)]	85.07 ±2.06	119.11 ±1.58**	65.80 ±2.08	83.80 ±0.00**	79.04 ±1.09	98.57 ±0.82**
TGSH, root [µmol g^{-1}(f.m.)]	56.80 ±3.65	81.20 ±3.36*	52.10 ±0.41	63.80 ±0.27**	52.80 ±2.47	60.71 ±2.70*
GSSG, shoot [µmol g^{-1}(f.m.)]	29.92 ±3.98	16.67 ±0.79**	24. 90 ±1.96	20.57 ±0.00**	29.42 ±2.16	22.61 ±1.23**
GSSG, root [µmol g^{-1}(f.m.)]	20.85 ±1.00	10.66 ±1.08**	19.52 ±0.54	12.85 ±0.82**	17.38 ±1.22	10.76 ±1.08**
GSH, shoot [µmol g^{-1}(f.m.)]	55.15 ±2.14	102.44 ±0.93**	40. 90 ±2.51	63.23 ±0.00**	49.61 ±2.77	75.95 ±0.41**
GSH, root [µmol g^{-1}(f.m.)]	36.01 ±2.75	70.58 ±2.36**	32.61 ±0.13	50.95 ±1.09**	35.47 ±1.35	49.95 ±1.09**

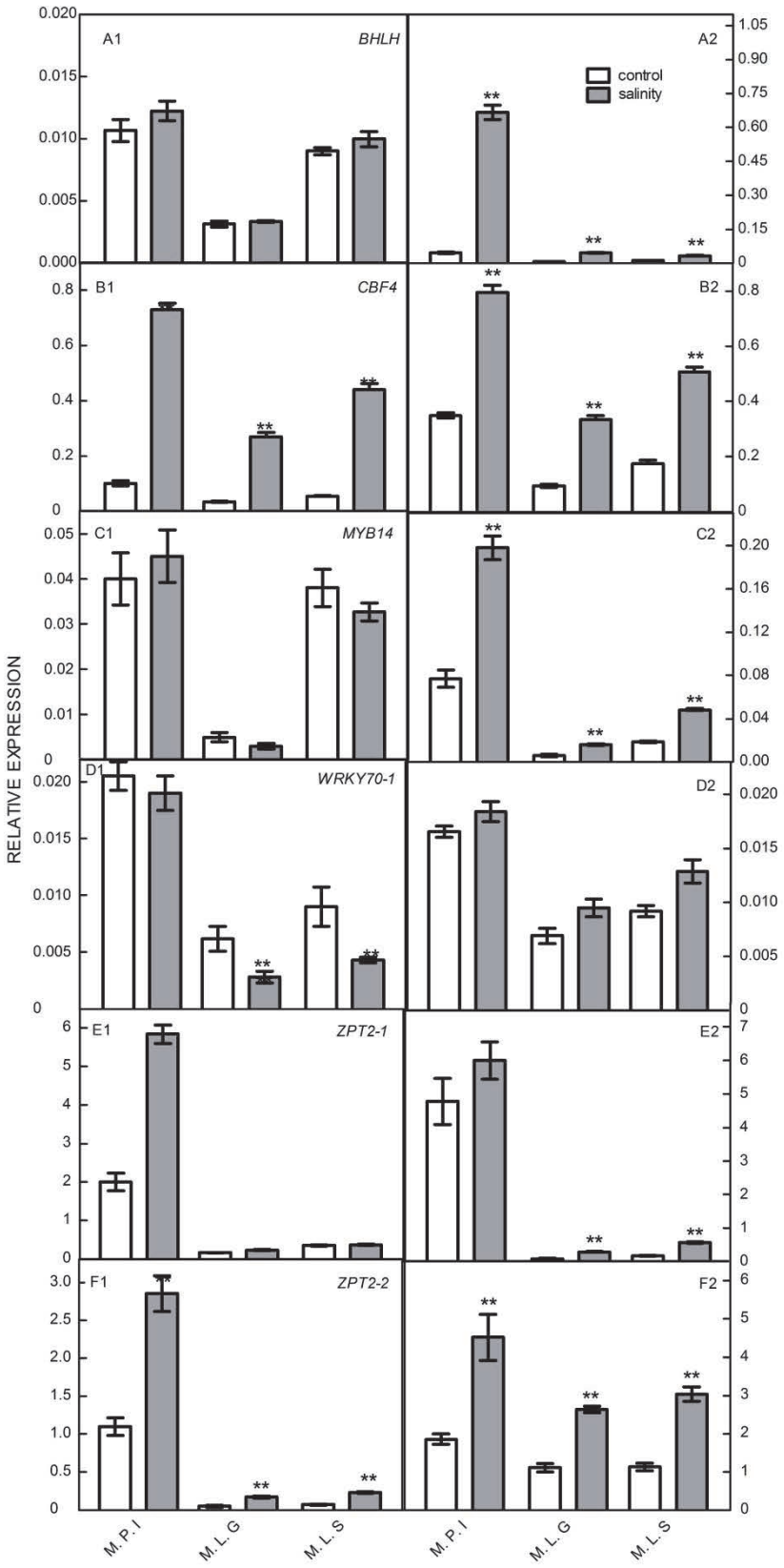

Fig. 1. RT-qPCR analysis of *bHLH* (*A*), *CBF4* (*B*), *MYB14* (*C*), WRKY70-1 (*D*), *Zpt2-1* (*E*), and *Zpt2-2* (*F*) expression in the shoot (*1*) and the root (*2*) of *Medicago* species (M.P.I. - *M. polymorpha* Ieze; M.L.G - *M. laciniata* Gheshm; M.L.S. - *M. laciniata* Shushtar) in response to salt stress (150 mM NaCl, 24 h). ** - significant differences among genotypes at $P \leq 0.01$.

H_2O_2 content was generally higher in the shoots than the roots of all genotypes (Table 1). The content of GSH and TGSH, and GSH/GSSG ratio were increased in the shoots and roots of all genotypes under salinity stress, the effect was more pronounced in *M. polymorpha* (Table 1). However, the GSSG content was declined by salinity in both shoots and roots of all genotypes.

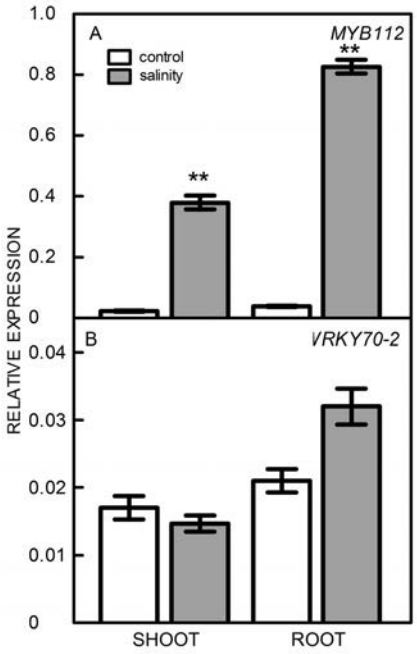

Fig. 2. Real-time RT-PCR analysis of *MYB112* (*A*), and *WRKY70-2* (*B*) differentially expressed between shoots and roots of *Medicago polymorpha* in response to salt stress. Gene expression was analyzed in control and salt stressed plants (24 h in 150 mM NaCl). Numbers on the y-axis indicate the expression of each specific transcription factor calculated using 2-ΔCt method. The mean comparisons exceeded two-fold change and *P*-value less than 0.01 (**) are significant.

The transcriptome profile among species and tissues showed considerable variation. The expression of *bHLH* in the leaf showed no significant change in response to salinity (Fig. 1) but salinity resulted in up-regulation of *bHLH* in the roots of all genotypes, the effect was greater in *M. polymorpha* than in *M. laciniata* genotypes (Fig 1). An overexpression of *CBF4* was found in the shoots and roots of all *Medicago* genotypes after salinity treatment. *M. polymorpha* had higher expression of *CBF4* than *M. laciniata* genotypes (Fig. 1).

An overexpression of *MYB112* was only detected in the shoots and especially in roots of *M. polymorpha* in response to salinity (Fig. 2). Although, the expression of *MYB14* did not show significant change in leaves of all genotypes, it was increased in the roots of all plants after salinity treatment (Fig. 1).

Salinity increased overexpression of *WRKY70-1* in the roots of all *Medicago* genotypes, however its expression in the shoots of *M. laciniata* genotypes was significantly decreased (Fig. 1). Under salinity stress, *WRKY70-2* was only expressed in the roots of *M. polymorpha*, whereas its expression in the leaves was reduced (Fig 2).

Salinity imposition caused higher over expression of *Zpt2-1* in the leaves *M. polymorpha* compared with *M. laciniata* (Fig. 1). Despite no significant change in the roots of *M. polymorpha*, *M. laciniata* genotypes showed a significant over expression of *Zpt2-1* in their roots in response to salinity treatment. The *Zpt2-2* was over expressed in the leaves and roots of all *Medicago* genotypes in response to salt stress. The expression of *Zpt2-2* in roots was greater than in the leaves (Fig. 1).

It is interesting to mention that *M. polymorpha* had the greater expression for most of genes studied than *M. laciniata* genotypes. In addition, all *Medicago* genotypes had greater expression of studied genes in the roots than in the leaves.

Discussion

In response to salinity, *M. polymorpha* had less changes in biomass, EL, and H_2O_2 content, lower accumulation of Na^+ and less decrease in K^+ content as well as a higher ratio of GSH/GSSG in comparison with *M. laciniata* genotypes. Mansour and Salama (2004) reported that EL and biomass are markedly changed in salt-sensitive cultivars upon salt exposure. Mansour (2013) indicates a direct relationship between maintenance of plasma membrane permeability and salt tolerance. In addition, the EL as a measure of plasma membrane injury has been used as an efficient screening technique for identifying salt tolerant genotypes (Munns 2010). Consequently, *M. polymorpha* had likely less plasma membrane injury upon salinity exposure, which contributed to its tolerance to salt imposition. In terms of intra-species difference, *M. laciniata* Shushtar appeared to be more salt tolerant genotype compared with *M. laciniata* Gheshm as having less biomass loss, less elevation of H_2O_2 and lower EL. Stomatal closure, Na^+ and Cl^- exclusion, biosynthesis of compatible solutes, osmotic adjustment, and regulation of membrane permeability are reported as the main mechanisms through which plants counteract salinity stress (Mansour and Salama 2004, Zang and Komatsu 2007).

M. polymorpha had the lowest increase in H_2O_2 content relative to other *Medicago* genotypes when subjected to salinity. In accordance with that, salt tolerant cultivars of bread wheat had lower H_2O_2 content than sensitive counterparts (Rao *et al*. 2013). Furthermore, salinity caused an increase in the content of GSH, total glutathione, and the GSH/ GSSG ratio in the shoots and roots of all studied genotypes under salinity exposure. Elevation of GSH as a result of salinity stress was more pronounced in *M. polymorpha*. Similar trend of results has been demonstrated under different environmental stresses (Polle and Rennenberg 1992, Mittova *et al*.

2003). In agreement with our finding, Tausz *et al*. (2004) indicated an increase in the GSH/GSSG ratio in well acclimated plants under stress condition. Accordingly, high content of GSH observed in *M. polymorpha* demonstrated that this species was more effectively acclimated to salinity stress compared with other genotypes. Taken together, *M. polymorpha* can be regarded as more salt tolerant species than *M. laciniata*.

In the present study, high efficiency of the primers designed based on *M. truncatula* transcriptome (De Lorenzo *et al*. 2007, Li *et al*. 2011, Zahaf *et al*. 2012) confirmed that TFs are highly conserved across annual *Medicago* species. The TFs in our study including *bHLH*, *CBF4*, *MYB*, *WRKY*, and *Zpt2* were differently expressed among *Medicago* species and tissues under salt stress condition. Similar conclusions have been drawn for *Populus* genotypes subjected to drought (Wilkings *et al*. 2009), *Lotus* genotypes (Sanchez *et al*. 2011), wheat species (Zamani Babgohari *et al*. 2012), and *Medicago* species (Karami *et al*. 2015) in response to salinity stress.

The expression of *MYB14* exhibited up-regulation in the root and no change observed in leaves of *Medicago* genotypes. However, *MYB112* was only expressed in *M. polymorpha* exhibiting up-regulation in the roots and leaves. In support to our finding, Li *et al*. (2011) identified an over-expression of *MYB112* in root of *M. truncatula* under salinity treatment. The MYB family of proteins is enormous and in all eukaryotes these proteins play role in regulatory networks controlling development, metabolism, and responses to biotic and abiotic stresses (Dubos *et al*. 2010).

The similar trend was observed for *bHLH* expression in the studied *Medicago* genotypes. Zahaf *et al*. (2012) used a salt-adapted *M. truncatula* genotype TN1.1t1 along with a reference genotype Jemalong A17 to analyze behavior of roots in response to salinity. They proposed that increased expression of *bHLH* TF may contribute to the adaptation of *M. truncatula* to saline soil. Based on the above evidence, *M. polymorpha* can be considered as a salt tolerant genotype.

Also the expression of *CBF4* in *M. polymorpha* was greater than in *M. lacinata* genotypes under salinity. *CBF4*, which belongs to the *AP2-EREBP* transcription factor family, plays important roles in plant growth, development, and responses to biotic and abiotic stresses (Li *et al*. 2011). Overexpression of *CBF4* in *M. truncatula* was highly linked to lower electrolyte leakage and tolerance to salinity stress (Li *et al*. 2011). Therefore, we propose that decreased EL in *M. polymorpha* in response to salinity in the preset work might be correlated with higher expression of *CBF4* in this genotype.

Though *WRKY70-1* was insignificantly up-regulated in the roots of all *Medicago* species, its expression in the leaves was down-regulated in response to salinity. WRKY70 has been demonstrated to play a critical role in plant defense against pathogens (Li *et al*. 2006, Rushton *et al*. 2010). Moreover, in the study of Li *et al*. (2013) the *wrky54wrky70 Arabidopsis* double mutant exhibited enhanced tolerance to osmotic stress, and it was concluded that TFs co-operate as negative regulators of stomatal closure and hence contributing to osmotic stress tolerance in *Arabidopsis*. It was also shown that *WRKY70* and *WRKY54* act as negative senescence regulators in *Arabidopsis* (Besseau *et al*. 2012). It is, therefore, inferred that down regulation of *WRKY70* in the leaves of annual *Medicago* genotypes enhance stomatal closure as a tolerance mechanism. Subsequently, down-regulation of *WRKY70-1* in *M. laciniata* genotypes caused by salinity stress may be translated to earlier senescence. Insignificant down-regulation of this transcription in *M. polymorpha* may suggest that 150 mM NaCl has not been able to cause any change in the expression of *WRKY70-1* within 24 h. It can be concluded that tolerant genotypes in comparison with salt-sensitive genotypes can withstand the stress and continue to their normal functions including photosynthesis during stress conditions.

The expression of *Zpt2-1* was much higher in the leaves of *M. polymorpha* than *M. laciniata* genotypes. We observed a significant overexpression of *Zpt2-1* in the roots of *M. laciniata* genotypes in response to salinity but no change in *M. polymorpha*. However, the *Zpt2-2* was significantly overexpressed in all *Medicago* genotypes exposed to salinity. Previous research on *M. truncatula* showed that *Zpt2-1* and *Zpt2-2* were involved in recovery after salinity (De Lorenzo *et al*. 2007, Merchan *et al*. 2007). It has been reported that overexpression of the orthologs of *Zpt* genes in *Arabidopsis* was associated with up-regulation of the oxidative stress-responsive genes (Mittler *et al*. 2006), and down-regulation of genes involved in the metabolism of sugars and lipids (Kodiara *et al*. 2011). Furthermore, Guilfoyle and Hagen (2007) reported that overexpression of *AZF2* was associated with down-regulation of a number of auxin-responsive genes in transgenic plants. Therefore, we presumed that decreased growth performance of annual *Medicago* genotypes in response to salinity could be related to induction of *Zpt2* genes.

Tolerance to abiotic stresses is a quantitative trait with a multigenic nature. This, in turn, is affected by complex interactions with the environment (Salentijn *et al*. 2007). Therefore, it is difficult to ascertain specific genes that are involved in tolerance in plants to be used as selection markers. Transcriptome analysis has opened a new avenue in plant breeding for identifying genes, which play critical role in adaptation of plants to different stresses. Transcription factors are very important in responses to abiotic stresses. Many transgenic plants over-expressing TFs show improved abiotic stress tolerance (Saibo *et al*. 2009 and references therein). Our attempt has been to distinguish between tolerant and sensitive genotypes of annual *Medicago* to salinity stress. To address if gene expression pattern has potential to differentiate salt tolerant genotypes from susceptible ones, gene expression response of five TF families were evaluated under salinity and control conditions. Our results indicated that the expression of *CBF4* and *Zpt2-2*

was highly induced in the roots and shoots of all annual *Medicago* species in response to salinity. *M. polymorpha* showed higher expression of *CBF4* and *Zpt2-2*under control conditiond and salinity stress and was regarded as salt tolerant genotype. The higher expression of TFs in *M. polymorpha* in comparison with *M. laciniata* genotypes can be explained by a greater number of stress-responsive elements in related promoter sequence. This confers salt tolerance to *M. polymorpha* through the expression of downstream stress-responsive genes (Wang *et al*. 2014). There is evidence that certain stress-responding genes have higher expression in tolerant genotypes/species compared to sensitive counterparts even under control conditions (Becher *et al*. 2004, Taji *et al*. 2004, Weber *et al*. 2004, De Lorenzo *et al*. 2007). Baloglu *et al*. (2014) evaluated the effects of salt and drought stresses on expression of different *TF* genes in wheat species and concluded that the expression of *TaMYB33* and *TaWLIP19* genes could be used for distinction of drought or salinity-tolerant and susceptible genotypes. These findings can be used in breeding programs. Our findings also confirmed that gene expression pattern has the potential to distinguish stress tolerant genotypes from sensitive ones. The expression of these genes needs to be studied in a wide range of tolerant and susceptible genotypes in order to be suggested as a potential genetic marker in selection against stresses.

In conclusion, all *Medicago* genotypes showed greater expressions of TF genes in the roots than in the leaves presumably because the roots were directly exposed to NaCl. The expression pattern of five TFs revealed that *Zpt2-2* and *CBF4* had higher expressions in salt-tolerant genotype *M. polymorpha* than in less tolerant *M. laciniata* genotypes. These genes have potential to be used as genetic markers in plant breeding program.

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