

Differential expression of *TLP*, *ERF1*, and *R2R3MYB* in annual *Medicago* species under salinity conditions

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ABSTRACT. The present study was conducted to evaluate the responses of three annual *Medicago* species (*M. truncatula*, *M. laciniata*, and *M. polymorpha*) to salinity. We analyzed publicly available microarray data in NCBI pertaining to salinity-response genes in *M. truncatula*. Our data search identified Tubby C2 (*TLP*) and ethylene responsive transcription factor 1 (*ERF1*) as genes that potentially respond to salinity. We evaluated morpho-physiological traits and the expression of the genes in three *Medicago* species that had been maintained under control and saline conditions. The analysis of morpho-physiological traits showed that *M. polymorpha* and *M. laciniata* were more tolerant to salinity than *M. truncatula*, as they had lower reductions in biomass and dry root weight and lower increases in anthocyanin concentration. The saline conditions caused a significant increase ($P < 0.01$) in the expression of *TLP* in all *Medicago* species, but caused a significant decrease

in the expression of *ERF1*. Considerable variation in anthocyanin concentrations was found among the three *Medicago* species. To investigate the cause of this variation, we examined the expression of *R2R3MYB*, a gene involved in the biosynthesis of anthocyanins. Our analysis showed that saline conditions induced high over-expression of *R2R3MYB* in all three *Medicago* spp. The high efficiency of the primer pairs used in qRT-PCR enabled us to compare the expression levels of each gene in the three species. We concluded that the more salt tolerant species showed higher expression of *TLP* and *R2R3MYB* under both control and salinity conditions.

Key words: Annual *Medicago*; Gene expression; Microarray analysis; Salinity

INTRODUCTION

Soil salinity or salt stress has a considerable adverse effect on food production by limiting the growth, development, and yield of crop plants (Tester and Davenport, 2003). Salinity is estimated to pose a threat to approximately 25% of agricultural land worldwide. The main contributors to salinity of the soil are the use of saline water for irrigation, poor drainage of the soil, and excessive application of fertilizers (Tester and Davenport, 2003).

Salinity gives rise to both hyper-osmotic and hyper-ionic conditions, which endanger plants through dehydration, ion toxicity, nutritional deficiencies, and oxidative stress (Tester and Davenport, 2003). Plants have evolved complex signaling pathways to overcome salt stress through changes in gene expression patterns in response to salinity and other abiotic stresses (Li et al., 2011; Sanchez et al., 2011). Characterization of the functions of novel genes is crucial to a better understanding of how an organism functions as a whole. Traditionally, investigation of gene functions involved testing of specific hypotheses through well-designed mutagenesis experiments. However, such methods are costly and laborious. Nowadays, a huge amount of protein and nucleic acid sequence data, such as those obtained from microarray analyses, are available in public databases, and provide an excellent resource for reanalysis and investigation of gene functions (Amthauer and Tsatsoulis, 2010).

Legumes are economically important crop species that provide grain, forage, edible oils, and resources for industry. In addition, legumes contribute to sustainable agriculture by fixing nitrogen in soils in association with rhizobial bacteria. *Medicago truncatula* Gaertn. or 'barrel medic', is regarded as a model legume system in genetic research. *M. truncatula* has a short life cycle, can self-pollinate, and has a small diploid genome; efficient methods have also been developed to allow genetic transformation. All of these factors have contributed to making this species a model for the study of legume biology (Cook, 1999; Bell et al., 2001). In recent years, the genome of *M. truncatula* has been fully sequenced. This information, in combination with developments in experimental techniques such as microarray and RNA-Seq, has expanded our capacity to explore regulatory systems in *M. truncatula*. The findings from such studies can be applied for improvement of forage legumes in breeding programs (Young and Udvardi, 2009; Sanchez et al., 2011).

Several genes have been shown to have a role in salt stress responses in *Medicago*. In a previous study, de Lorenzo et al. (2007) compared gene expression patterns of two *M.*

truncatula genotypes, namely, the salt sensitive cultivar 108-R and the salt tolerant cultivar Jemalong A17. Under salt stress conditions, they found overexpression of a homolog of the abiotic stress-related gene *COLD-REGULATED1* and of the *TFIIIA*-related transcription factor *MtZpt2-1* in Jemalong A17 plants compared to 108-R plants. Overexpression of these transcription factors in the roots of 108-R plants resulted in increased root growth under salinity conditions, suggesting a role in the adaptive response pathway to salt stress. Li et al. (2011) carried out a microarray experiment using root samples in plants grown under salinity conditions. They found that the *MtCBF4* transcription factor gene was significantly overexpressed and suggested this gene might be a good candidate for genetic improvement programs to increase stress-tolerance. Zahaf et al. (2012) performed a transcriptomic analysis of the roots of TN1.1t1 (a salt-adapted genotype) and Jemalong A17 plants in response to salinity. They found that the *bHLH*-type transcription factor showed the greatest difference in expression level between the genotypes and enabled significantly improved root growth under salt stress. They proposed that an increase in expression of the *bHLH* transcription factor may be linked to the adaptation of *M. truncatula* to saline soil environments.

These studies provided the first insights into the genetics of salt stress responses in annual *Medicago* species. However, genomes are complex networks in which the genes interact to maintain homeostasis and survival of plants under stress conditions. To identify further aspects of the network associated with responses to salt stress, we analyzed publicly available microarray data in the NCBI database. This search showed that the Tubby gene and an “ethylene-responsive transcription factor 1” gene were candidates for the response of *M. truncatula* to salinity. We then carried out an experimental study to test the role of these candidates in *M. truncatula* and two other annual pasture legumes, *Medicago laciniata* and *Medicago polymorpha*, which are widespread in rangelands around the world. The expression patterns of *R2R3MYB*, a gene involved in anthocyanin biosynthesis was also examined, and an attempt was made to establish the relationship between gene expression profiles and morpho-physiological traits in annual *Medicago* species.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of the three *Medicago* species used here were scarified in concentrated sulfuric acid for 5 min and rinsed 4-5 times with distilled water. To ensure uniform germination, scarified seeds were placed on Petri dishes and kept at 24°C in a greenhouse for 1 day. Seedlings were transferred to trays containing a mixture of cocopeat:perlite (70:30) and placed in a greenhouse. After one week, the plantlets were transferred to 40 x 50 x 25 cm containers filled with Hoagland solution. The strength of the Hoagland solution was increased on a weekly basis: in week 1, the plants were kept in 1/4 strength solution and this was increased to reach full-strength in week 3. In the salt stress experiments, the plants were exposed for a four-week period. Salt (NaCl) was dissolved in the Hoagland solution to obtain a saline solution with a concentration of 100 mM. Control plants were exposed only to normal Hoagland solution. At the end of the exposure, seven plants were randomly sampled from each replicate experiment (21 per treatment) and used to measure dry weight biomass (aerial part), dry weight of roots, Na⁺ and K⁺ contents in root and shoot, and anthocyanin concentration. Digested samples of dried tissue were used to measure the concentration of Na⁺ and K⁺ in a flame photometer.

Anthocyanins were extracted from oven-dried and ground leaf samples. In brief, 0.1 g dried powdered leaves were placed into 10 mL acidified methanol (methanol:H₂O:HCl, 79:20:1 by volume) and auto-extracted at 0°C for 72 h. After centrifugation, absorbance was measured at 530 and 657 nm for each supernatant. Anthocyanin content was calculated as $Ab_{530\text{ nm}} - 1/3 Ab_{657\text{ nm}}$ mg/g dry matter (Lutts et al., 1996).

Internet accessible data and microarray analysis

The GSE13921 set of microarray data, derived from *M. truncatula*, was downloaded from NCBI. These data were produced in a series of experiments by Li et al. (2009) and include data from a transcriptome analysis of *M. truncatula* cv. A17 under salt stress conditions. In their study, three-day-old *M. truncatula* seedlings were exposed to salinity; RNA was extracted from roots at 0, 6, 24, and 48 h post treatment. We used the data from this analysis to examine transcriptional responses to salt stress.

The raw data were normalized by the robust multiarray normalization method using the Expression Console Software (version 1.3.1); the results were exported to the FlexyArray (version 1.6.1) software and overexpressed genes were identified using a Bayesian *t*-test analysis. Overexpressed probe sets with symmetric fold change greater than 6 and a significant P value less than 0.05 were selected. These probe sets were then exported to Microsoft Excel and 23 co-overexpressed entries were obtained. In order to identify the relevant TAIR annotation (homologous genes in *Arabidopsis*), the probe sets were analyzed using the Plant Expression Database (www.plexdb.org/mod-ules/gl_Suite/gl_main.php; Dash et al., 2012). Three of the probe sets, Mtr.17903.S1at, Mtr.20233.1.S1_s_at, and Mtr.2333.1.S1, were selected and used to design primers and to verify function in laboratory experiments; the probe sets were highly induced after salinity treatment but had limited information on their protein function or their role in salinity tolerance. Mtr.17903.S1_at is part of a 558-nt mRNA sequence and is orthologous to “At5g01750.2” of *Arabidopsis*. The function of the protein translated from At5g01750.2 is unknown (DUF567). Also, the whole mRNA sequence of Mtr.17903.S1 is included in that of XM_003588822.1 (MTR_1g014180) from *M. truncatula* (NCBI database). Tubby C2, known as Tub_2, is a protein domain encoded by XM_003588822.1. Therefore, we designated XM_003588822.1 as Tubby-like protein, *TLP*, throughout the manuscript (Table 1).

The Mtr.20233.1.S1_s_at, and Mtr.2333.1.S1 sequences have been classified as “ethylene-responsive transcription factor 1” (*ERF1*) genes. Mtr.20233.1.S1_s_at has high similarity with XM_003607929.1 (abbreviated as *ERF1-1* through the manuscript), and Mtr.2333.1.S1_at has high similarity with XM_003607932.1 (abbreviated as *ERF1-2* through the manuscript) in the NCBI database (Table 1).

Gene-specific primers for use in qRT-PCR were designed with the Primer-BLAST tool available in NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For each gene, three different primer pairs were designed. Temperature gradient PCR was used to find the optimum annealing temperature and primer efficiency was determined using a standard curve. Only those primer combinations, which had efficiency higher than 90% in all *Medicago* species, were used in the qRT-PCR analysis (Table 1). Reactions lacking a cDNA template were also performed at the optimized conditions as a control.

The considerable variation among *Medicago* species for anthocyanin concentration led us to measure expression of *R2R3MYB*, which is involved in anthocyanin biosynthesis (Hancock et al., 2012).

Table 1. Sequences of primers used for *TLP*, *ERF1*, *R2R3MYB*, and *Actin*.

(5'→3') ¹	Primer	Sequence ID in NCBI	Probe set ID
AGAGAGGACTGTGAGAGA	<i>TLP</i> -F	XM-003588822.1	Mtr.17903.S1_at
CTACGGAGATGAAGAATG	<i>TLP</i> -R		
ATAATACGACTGAATGTG	<i>ERF1-1-F</i>	XM_003607929.1	Mtr.20233.1.S1_s_at
TAGAATGAGAAGAATCCA	<i>ERF1-1-R</i>		
GGATAGTATCGGAGAAGC	<i>ERF1-2-F</i>	XM_003607932.1	Mtr.2333.1.S1_at
CATAGGTGATAAGGCAGC	<i>ERF1-2-R</i>		
GAAGACTTCCAGGGCGAACA	<i>R2R3MYB-F</i>	JN157820.1	-
GTGGTGAGTTGGGAGGTGAG	<i>R2R3MYB-R</i>		
CCCCTGGATGTCTGTAGGT	<i>Actin-F</i>	JQ028731.1	-
AGAATTAAGTAGCAGCGCAA	<i>Actin-R</i>		

¹These primers had high efficiency (92-95%) in all *Medicago* species.

RNA extraction, cDNA synthesis, and RT-PCR

Root and leaf tissues for transcriptome analysis were sampled 24 h after initiation of salinity treatment. The samples were obtained from two biological replicates. Total RNA was extracted using Total RNA isolation kit (TRIzol, Invitrogen™, USA) following the manufacturer instructions. The purified total RNA was quantified by a spectrophotometer (WinAsPECT PLUS, Analytikjena). The quality of RNA was checked on an RNA MOPS gel. DNase treatment was carried out using a Fermentas Kit (Fermentas, Hanover, MD, USA). For real-time RT-PCR, 1.5 µg total RNA was reverse transcribed for 1 h at 42°C using the SUPERSRIPT II first-strand synthesis system (Vivantis) and subsequently denatured for 10 min at 75°C. The specificity of the primers and product lengths were confirmed by checking the melting temperature, and running the qRT-PCR products on an agarose gel.

Before the RT-PCR, cDNAs were diluted to 1:8. Four microliters diluted cDNA was used as the template in a 10-µL PCR mixture. *MtActin*, which shows stable expression across different conditions, was used as the internal control in RT-PCR.

Statistical analysis

Morpho-physiological data were analyzed using a randomized complete block design in a split plot layout using the SAS software (Version 9) according to the following model: $Y_{ijk} = \mu + R_i + A_j + AR_{ij} + B_k + AB_{jk} + e_{ijk}$. In this model, Y_{ijk} is observation; μ is the general mean; R_i is the effect of the block; A_j is the effect of the salinity treatment; AR_{ij} is the main plot error (salinity); B_k is the effect of genotype; AB_{jk} is the interaction; e_{ijk} is the sub-plot error. In order to represent real-time PCR data as individual data points, the $2^{-\Delta\Delta Ct}$ method was used, where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{internal control}}$ (Schmittgen and Livak, 2008) and referred to “expression” throughout the manuscript.

A *t*-test analysis was also performed to identify significant differences in comparisons of morpho-physiological traits and gene expression profiles within each genotype.

RESULTS

Morpho-physiological criteria

The biomass of salt-stressed *M. truncatula* plants was significantly reduced ($P < 0.01$)

compared to controls; plants under salt stress showed a 2.3-fold reduction in dry shoot weight and a 1.5-fold reduction in dry root weight (Table 2). Dry weights of root and aerial tissues were highest in *M. polymorpha* and lowest in *M. laciniata*. The *M. laciniata* plants were significantly smaller and had less leaf and root mass than other *Medicago* plants at the end of the experiment. Although both *M. laciniata* and *M. polymorpha* showed reductions in dry weights after salt stress treatment, the effect was not significant in either species.

In all three species, the shoot Na⁺ content increased significantly, and shoot K⁺ content decreased significantly under salt stress conditions (Table 2). However, the changes were greater in *M. truncatula* than in *M. polymorpha* and *M. laciniata* (Table 2).

A similar effect was observed in the root tissues of all three species, namely that the salt stress significantly increases Na⁺ content and significantly reduces K⁺ content (Table 2). Again, the stress-induced changes were relatively greater in *M. truncatula* than in the other two species (Table 2).

Salinity treatment increased anthocyanin contents in all three species. However, *M. truncatula* showed a 200% increase, *M. polymorpha* showed a 21.4% increase, and *M. laciniata* showed a 23% increase.

Table 2. Responses of annual *Medicago* species to salinity (100 mM NaCl).

	<i>M. truncatula</i> A17		<i>M. laciniata</i>		<i>M. polymorpha</i>	
	Control	Treated	Control	Treated	Control	Treated
Dried shoot (g/plant)	1.11 ± 0.072	0.49 ± 0.009**	1 ± 0.14	0.8 ± 0.09	1.5 ± 0.18	1.3 ± 0.08
Dried root (g/plant)	0.14 ± 0.007	0.095 ± 0.007**	0.1 ± 0.01	0.09 ± 0.018	0.19 ± 0.03	0.16 ± 0.001
Na ⁺ shoot (mmol/g DW)	0.014 ± 0.001	0.2 ± 0.005**	0.016 ± 0.002	0.174 ± 0.08**	0.018 ± 0.001	0.2 ± 0.04**
K ⁺ shoot (mmol/g DW)	2.74 ± 0.004	1.12 ± 0.003**	3.1 ± 0.08	1.47 ± 0.09**	2.91 ± 0.008	1.43 ± 0.09**
Na ⁺ root (mmol/g DW)	0.14 ± 0.04	1 ± 0.01**	0.15 ± 0.04	0.85 ± 0.005**	0.16 ± 0.007	0.95 ± 0.003**
K ⁺ root (mmol/g DW)	1.61 ± 0.02	0.94 ± 0.04**	1.62 ± 0.08	1.45 ± 0.03*	1.73 ± 0.08	1.4 ± 0.06*
Anthocyanins (Ab/g DW)	0.06 ± 0.005	0.13 ± 0.003**	0.13 ± 0.006	0.16 ± 0.007**	0.14 ± 0.003	0.17 ± 0.007**

Data are reported as means ± S.E (number of observations for each trait = 21). *Comparisons within each genotype are significantly different at P ≤ 0.05. **Comparisons within each genotype are significantly different at P ≤ 0.01.

qRT-PCR analysis of genes selected

The primer pairs used in qRT-PCR showed high efficiency across all *Medicago* species indicating a high degree of genetic conservation. Consequently, we were able to compare the expression level of each gene among the three species.

Salinity increased the levels of expression of the *TLP* and *R2R3MYB* genes in roots and leaves of all three species (Figure 1 and 2). Conversely, expression of *ERF1* transcription factors declined in roots and leaves in plants exposed to salinity (Figure 3). *ERF1-1* and *ERF1-2* showed similar trends under control and salinity conditions.

The levels of expression of *TLP*, *R2R3-MYB*, and *ERF1* in roots and leaves of *M. polymorpha* and *M. laciniata* were greater than in *M. truncatula* under control and salinity conditions (Figures 1, 2, and 3).

DISCUSSION

Salt tolerance is a complex phenomenon in which many genes are involved. In le-

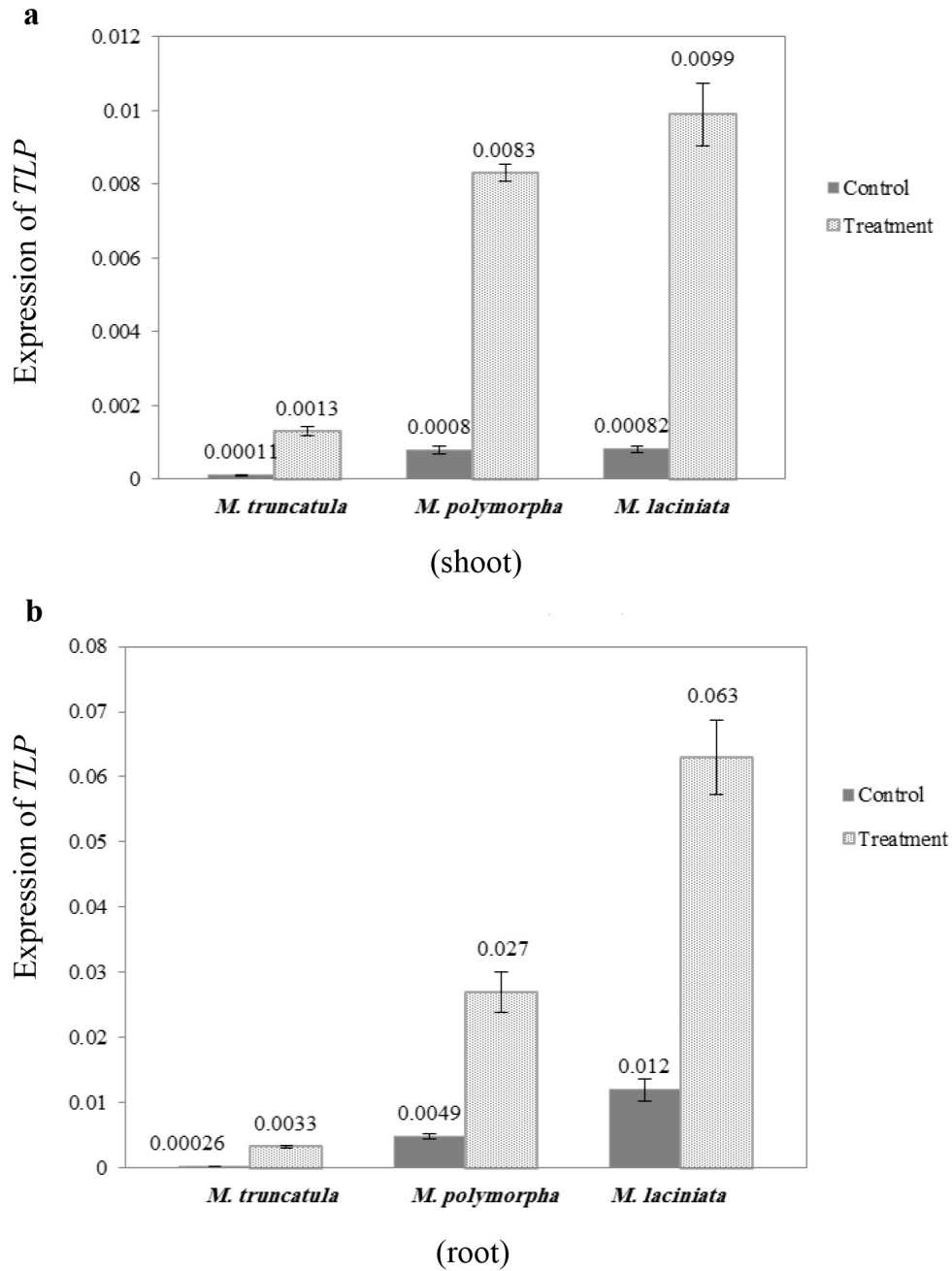


Figure 1. Real-time RT-PCR analysis of changes in *TLP* expression in response to salt stress. Gene expression was analyzed under control and salt stress (treatment) conditions (after 24 h at 100 mM NaCl). Values on the x-axis indicate the relative level of *TLP* expression calculated using the $2^{-\Delta\Delta Ct}$ method.

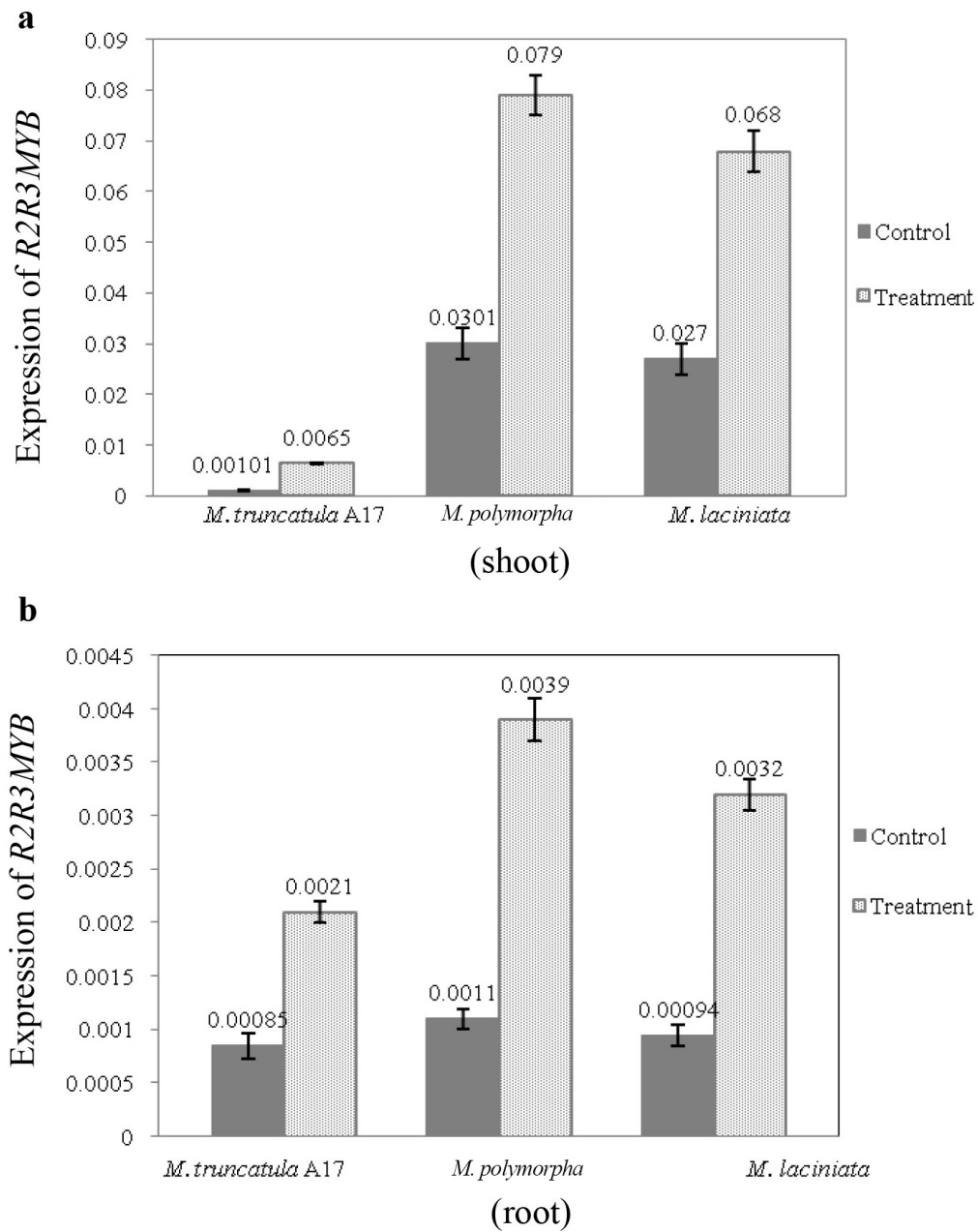


Figure 2. Real-time RT-PCR analysis of changes in *R2R3MYB* expression in response to salt stress. Gene expression was analyzed under control and salt stress (treatment) conditions (after 24 h at 100 mM NaCl). Values on the x-axis indicate the relative level of *R2R3MYB* expression calculated using the $2^{-\Delta\Delta Ct}$ method.

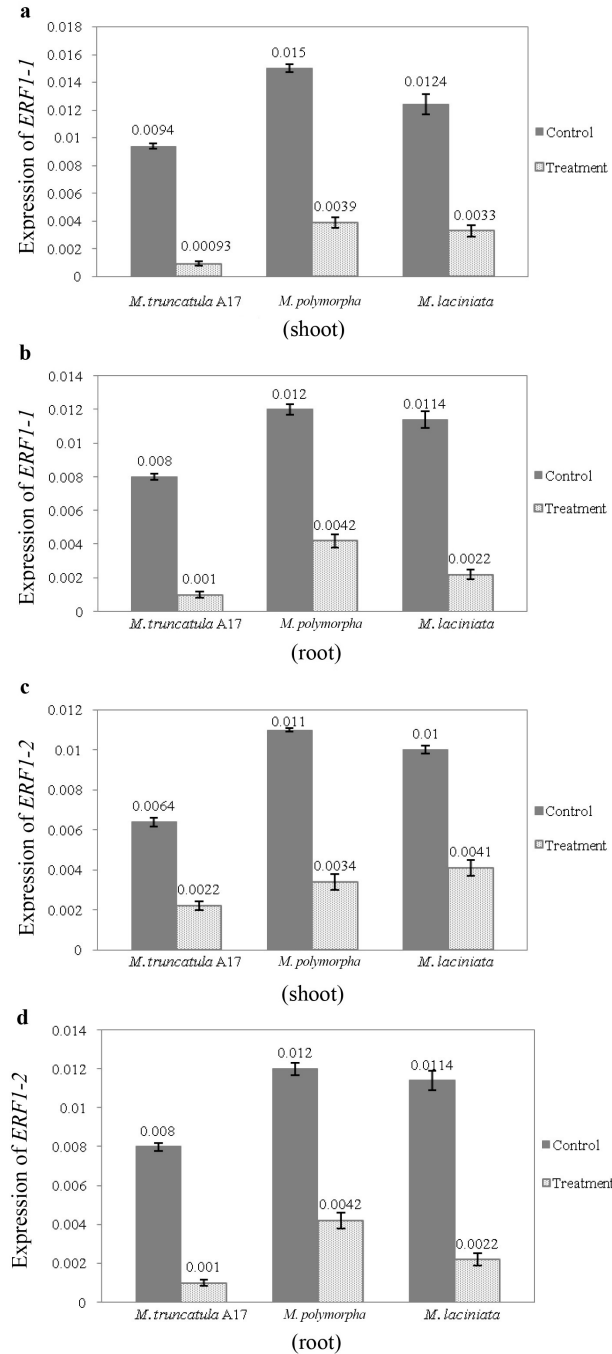


Figure 3. Real-time RT-PCR analysis of changes in *ERF1* (*ERF1-1* and *ERF1-2*) expression in response to salt stress. Gene expression was analyzed under control and salt stress (treatment) conditions (after 24 h at 100 mM NaCl). Values on the x-axis indicate the relative level of *ERF1* expression calculated using the $2^{-\Delta\Delta C_t}$ method.

gumes, salt stress significantly limits productivity because of its adverse effects on the growth of the host plant and on its symbiotic interactions. Root growth, nodule development, and nitrogen-fixation efficiency are particularly affected (Cardovilla et al., 1994). Breeding strategies to improve salt tolerance include exploitation of natural genetic variation and generation of transgenic plants, in which novel genes are introduced or in which the expression levels of existing genes are altered (Austin, 1993; Davletova et al., 2005).

Here, we found that plants of all three *Medicago* species showed higher concentrations of Na⁺ and concomitant decrease in K⁺ compared to controls when subjected to salinity conditions. However, the salt-tolerant *M. laciniata* and *M. polymorpha* showed lower accumulation of Na⁺ in shoots and roots, and a lower decrease in K⁺ contents compared to *M. truncatula*. These results are consistent with previous observations that tolerant glycophytes accumulate lower levels of salt ions than sensitive species (Tester and Davenport, 2003; Sanchez et al., 2011).

Our microarray analysis demonstrated a potential role for Mtr.17903.S1_at in the adaptation of *M. truncatula* to salt stress. Using analytical bioinformatic tools, we showed that Tubby C2, known as Tub_2, is the protein domain encoded by the whole Mtr.17903.S1_at sequence. The Tubby gene was first identified from obese mice through positional cloning (Lai et al., 2004; Kou et al., 2009). Boggon et al. (1999) found that the Tubby protein is a bipartite transcription regulator and that its N-terminal segment is involved in modulating transcription. In plants, Tubby proteins contain a conserved F-box domain (Liu, 2008; Yang et al., 2008). F-box proteins regulate diverse cellular processes, including cell cycle transition, transcriptional regulation, and signal transduction. The plant genome harbors more tubby genes than that of humans indicating that tubby genes may have a wider range of roles in plants than in humans and animals. Our understanding of the roles of plant tubby genes is very limited. *TLPs* are believed to participate in the ABA signaling pathway in *Arabidopsis* (Lai et al., 2004; Cai et al., 2008) and the *OsTLP* family is involved in host-pathogen interactions in rice (Kou et al., 2009).

A survey of the literature did not produce any evidence of a role for tubby genes in plant responses to salinity. Under saline conditions, an increased concentration of Na⁺ and a greater decrease in K⁺ content was seen in the roots and shoots of *M. truncatula* compared to *M. laciniata* and *M. polymorpha*. Interestingly, the latter two salt tolerant species had higher levels of expression of *TLP* compared to *M. truncatula*. Similarly, Moghadam et al. (2013) showed that expression of a 6-kDa subunit of *ATP6* was higher in salt-tolerant wheat and wild genotypes compared to a salt-sensitive genotype. Zamani-Babgohari et al. (2012) showed that expression of the *HKTI;5* gene follows a tissue- and genotype- specific pattern and that wheat species with a D genome showed the highest level of *HKTI;5* expression compared to the A genome.

In this study, transcription of *R2R3MYB* gene was found to be induced by salinity in all *Medicago* species. The MYB family of proteins is large, functionally diverse and has representatives in all eukaryotes. Most MYB proteins function as transcription factors with varying numbers of MYB domain repeats conferring the ability to bind DNA. In plants, MYB family proteins are involved in abiotic stress tolerance and response (Dubos et al., 2010). Most plant MYB genes encode proteins of the *R2R3MYB* class. Numerous *R2R3MYB* proteins have been characterized by genetic approaches and found to be involved in the control of plant-specific processes (Dubos et al., 2010). Several *R2R3MYBs* are involved in the anthocyanin biosynthetic pathway (Lin-Wang et al., 2010). In legumes, Hancock et al. (2012) provided evidence that the *R2R3MYB* transcription factor, *TaMYB14*, is involved in

the regulation of proanthocyanin biosynthesis in *Trifolium arvense*. It has also been reported that the biosynthesis of anthocyanins in plant leaves is induced during senescence and by different environmental stresses (Lichtenthaler, 1987; Ashraf and Harris, 2013). In our study, considerable variation was seen among *Medicago* species with respect to anthocyanin content. We postulated that expression of genes involved in biosynthesis of anthocyanins might be induced under salinity treatment. Therefore, we measured the expression of *R2R3MYB* as a candidate gene for anthocyanin biosynthesis in the three *Medicago* species. Interestingly, *M. laciniata* and *M. polymorpha* had higher concentrations of anthocyanins, and higher levels of expression of *R2R3MYB*. Therefore, the higher concentration of anthocyanins can, at least in part, be attributed to higher expression of *R2R3MYB* in *Medicago* species. The higher expression of *R2R3MYB* in shoots than roots is consistent with the fact that leaves are the main site of anthocyanin biosynthesis.

The ERF gene family encodes plant-specific transcription factors, which usually regulate plant responses to biotic and abiotic stresses (Nakano et al., 2006). Several groups have reported that salinity-responsive gene expression is modulated by the antagonistic interaction between JA-ET and ABA signaling pathways (McGrath et al., 2005; Pré et al., 2008; Cheng et al., 2013). ERF1, a member of the ERF family, is an upstream component in jasmonate (JA) and ethylene (ET) signaling and is involved in pathogen resistance. Cheng et al. (2013) reported the induction of *AtERF1* by salinity treatment. However, we found that *ERF1* was highly suppressed by salinity in all *Medicago* species. Cheng et al. (2013) revealed that salt stress induction of *ERF1* expression was promoted by ET and JA signaling. In our study, however, the evidence suggested a role for ABA signaling. Contrary to our microarray results, we found that *ERF1* was highly suppressed by salinity in all *Medicago* species. This discrepancy may be due to the differences in developmental stages and stress levels used in the different studies. In agreement with our results, Ellouzi et al. (2013) also showed that *ERF1* was strongly downregulated after 72 h of salt stress treatment.

CONCLUSION

Analysis of qRT-PCR using specific primers showed that the transcription levels of *TLP* and *R2R3MYB* increased within 24 h in roots and shoots of all three *Medicago* species. *M. truncatula* responded differently from *M. polymorpha* and *M. laciniata*, with very low expression of *TLP* and *R2R3MYB*. Increased expression of both genes in *M. truncatula* as a result of salinity was not adequate to combat the adverse impact of salinity on plant biomass. In conclusion, augmented expression of *TLP* and *R2R3MYB* in *Medicago* species is associated with higher tolerance against salinity.

Conflicts of interest

The authors declare no conflict of interest.

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