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Research article

The constitutive expression of alfalfa *MsMYB2L* enhances salinity and drought tolerance of *Arabidopsis thaliana*

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ABSTRACT

MYB-type transcription factors are known to participate in the response of plants to a number of stress agents. *MsMYB2L* is an alfalfa member of this large gene family. Its transcription in alfalfa seedlings was found to be rapidly and strongly induced by salinity, moisture deficiency and exogenously supplied abscisic acid. An analysis based on a yeast one hybrid assay indicated that its product is able to activate transcription, consistent with its function as a transcription factor. When the gene was constitutively expressed in *Arabidopsis thaliana,* both germination and seedling growth were more sensitive to ABA treatment than wild type, and growth was less strongly compromised by salinity and moisture deficiency stress, presumably as a result of the induction of certain stress-related genes active in ABA-dependent pathways. The transgenic seedlings' enhanced the synthesis of many osmotic regulatory substances such as proline and soluble sugar, and decreased the lipid peroxidation. In all, *MsMYB2L* represents a potential candidate gene for manipulating the salinity and drought tolerance of alfalfa.

1. Introduction

Soil salinity and moisture stress represent major constraints over crop productivity worldwide (Agarwal et al., 2012). Plants have evolved numerous mechanisms to adapt to these and other abiotic stressors. Many of the genes acting in this context are regulated by transcription factors (TFs), represented in plants by members of the NAC, WRKY, bHLH and MYB families (Lindemose et al., 2013). The MYBs, which comprise the largest plant TF family (Butt et al., 2017), have been organized into four subfamilies (1R-MYB, R2R3-MYB, R1R2R3-MYB and 4R-MYB), based on the number of imperfect repeats encoded within their MYB domain (Dong et al., 2017). Over 100 R2R3-MYB encoding TF genes have been identified in *Arabidopsis thaliana* (Stracke et al., 2001), and many of them have been shown to be central to the defense response to environmental stress (Du et al., 2009; Lindemose et al., 2013). Thus, for example, *AtMYB2*, *AtMYB14*, *AtMYB15*, *AtMYB96*, *AtMYB70*, *AtMYB44*, *AtMYB77* and *AtMYB73* all participate in the response to one or more of low temperature, salinity and moisture stress (Abe et al., 2003; Chen et al., 2013; Ding et al., 2009; Seo et al., 2009; Jung et al., 2008; Kim et al., 2013), as do the rice homologs OsMYB91, OsARM1, OsMYB30, OsMYB2P-1, OsMYB3R-2 and OsMYB4 (Zhu et al., 2015; Wang et al., 2017a,b; Lv et al., 2017; Dai et al., 2012; Ma et al., 2009; Vannini et al., 2004).

The growth of alfalfa (*Medicago sativa*), a widely cultivated forage legume species, is particularly sensitive to soil salinity (Chao et al., 2009; Wang et al., 2013). However, only a handful of alfalfa MYB genes involved in stress responses have been previously reported. Comparisons between the transcriptomes of the two alfalfa cultivars 'Dryland' (DL) and 'Sundory' (SD) have implicated a number of MYBs in their differential response to salinity in our previous study (Dong et al., 2018). Among these MYB TFs, the effect of heterologously expressing *MsMYB15* (A phylogenetic analysis revealed that MsMYB15's most closely related *A. thaliana* homolog was AtMYB2. For this reason, *MsMYB15* has here been referred to as *MsMYB2L* in *A. thaliana* is described). We found that overexpression of *MsMYB2L* in *Arabidopsis* could increase both salt and drought tolerance by promoting the expression of several genes belonging abscisic acid dependent pathway, indicating that MsMYB2L is an ideal candidate gene for genetic breeding of stress-tolerant crops.

Abbreviations: ABA, abscisic acid; PEG6000, polyethylene glycol; GFP, green fluorescent protein; qPCR, quantitative real-time polymerase chain reaction; WT, wild-type

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2. Materials and methods

2.1. Plant materials and growing conditions

Alfalfa seeds were germinated on moist filter paper in the dark for 24 h at 4 °C, then in the light for 48 h at 20 °C. Germinated seeds were potted into a 1:1 mixture of perlite and sand, and irrigated with half strength Hoagland's solution three times a week. The plants were maintained in a cabinet delivering a relative humidity of 60%, a constant temperature of 25 °C and a photoperiod of 12 h (light intensity 300 μmol m^{-2}·s^{-1}). Different stress treatments were given to three-week old seedlings by including one of 200 mM NaCl, 20% w/v PEG6000, or 100 μM ABA in the irrigation solution. The first sampling of the roots was taken prior to the commencement of the stress treatment and subsequent ones after 1, 3, 6, 12 and 24 h. The root samples were snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. Gene isolation and sequence analysis

The published sequence in our previous study (Dong et al., 2018) was used to design a pair of PCR primers (Table S1) able to amplify the full length cDNA of *MsMYB2L*. These amplicons were introduced into the pEASY-T vector (TransGen, Beijing, China) and sequenced. Polypeptide sequences homologous to MsMYB2L were obtained from GenBank, and were aligned using the MegAlign program implemented within DNAStar software (www.dnastar.com/).

2.3. Transcriptional analysis using quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen, USA) and treated with RNase-free DNase (Promega) to remove any contaminating genomic DNA. The cDNA first strand was synthesized using an M-MLV kit (Invitrogen), following the manufacturers protocol. Each 20 μL qRT-PCR contained 10 μL 2× SYBR Premix Ex Taq mix (TaKaRa), 0.2 mM of each primer (Table S1) and 1 μL of a 1:10 dilution of the cDNA first strand reaction; the cycling regime comprised an initial denaturation (95 °C/2 min), followed by 45 cycles of 95 °C/10 s, 60 °C/20 s, 72 °C/20 s. A melting curve analysis was performed over the range 80–95 °C at 0.5 °C intervals. The MsACTIN gene was used as the reference (Wang et al., 2015), and relative transcript abundances were derived using the $2^{-\delta\delta Ct}$ method (Livak and Schmittgen, 2001). Three technical replicates were run for each of the three biological replicates. In comparing mean values, statistical significance was determined using the Student's *t*-test, implemented in the SPSS statistical package.

2.4. A. thaliana transformation and the assessment of the stress response of transgenic plants

The MsMYB2L coding region was inserted into the vector (De et al., 2007), placing it under the control of the CaMV 35S promoter. The resulting construct was transformed into *A. thaliana* ecotype Col-0 using the floral dip method (Clough and Bent, 1998). Seeds harvested from transgene homozygotes (OE lines) and from lines were surface-sterilized and plated on solidified medium containing half strength Murashige and Skoog (MS) salts. The plates were held at 4 °C in the dark for two days, and then moved to a chamber delivering a 16 h photoperiod and a constant temperature of 22 °C. After three days, the seedlings were transferred to a fresh plate containing the same medium, but supplemented with various concentrations of either NaCl or mannitol or of ABA for ten days. Each experiment was run in triplicate. For the germination assay, about 100–150 seeds of each of wild type (WT), and the two selected OE lines were surface-sterilized and plated on solidified medium containing half strength MS salts containing 10% (w/v) sucrose plus a variable concentration of salt, mannitol and ABA. The plates were held in the dark at 4 °C for three days, and then moved to the light at 21 °C. Germination was deemed as successful when the radical had visibly emerged, and was scored at various time points. Drought stress was induced in 3-week-old plants in soil by halting watering. Watering was reinitiated after 21 days then survival rates were calculated for each group of plants at 7 day. Three independent measurements of 20 seedlings were averaged.

2.5. Sub-cellular localization and transactivation assay

The coding sequence of MsMYB2L (lacking its stop codon) was fused to the N terminus of GFP represented in the construct pCaMV35S::MsMYB2L. The fused vector was transformed into *A.thaliana*, and transgene homozygous progenies were selected. The sub-cellular localization of GFP activity was monitored using a confocal laser scanning microscope (Leica) equipped with a 488 nm filter. To characterize the transactivation of MsMYB2L in yeast, the full length coding sequence of *MsMYB2L* was amplified and cloned into pGBKT7 vector, and then transformed into the yeast strain AH109. The empty pGBKT7 (BD) and fusing the P53 vectors were used as negative and positive controls, respectively. The transactivation activity was evaluated according to the growth on SD/−Trp and SD/−Trp−His−Ade media at 30 °C for 3 days.

2.6. The quantification of proline, soluble sugar and malondialdehyde (MDA)

Whole 2-week-old *Arabidopsis* seedlings were analyzed for the content of the soluble sugar, proline, and MDA following the instructions of the measurement kit (Category number: A145/A107/A003, Jiancheng, Nanjing) respectively. All the measurements were repeated three times, and the Student's t-test was used to compare means.

3. Results

3.1. MsMYB2L transcription profiling

As estimated from our previous RNA sequence experiment (Dong et al., 2018), the abundance of *MsMYB2L* transcript in cv. DL seedlings exposed to 200 mM NaCl for 1 h was 2.4 fold greater than in control seedlings, and this difference rose to 6.25 fold after 24 h, while in cv. SD seedlings the corresponding fold change were 1.14 for 1 h and 2.5 for 24 h (Fig. 1A). Here, three week old seedlings of both cultivars were exposed to either 200 mM NaCl, 20% polyethylene glycol 6000 (to impose moisture stress) or 100 μM ABA for up to 24 h, and the detailed transcriptional response of *MsMYB2L* was measured using quantitative real-time PCR (qRT-PCR). The experiment confirmed that *MsMYB2L* was induced by salinity in both cultivars, but more strongly so in cv. DL (Fig. 1B). When the stressor was polyethylene glycol, once again the gene's induction was rapid (within 1 h) in both cultivars, and seedlings of cv. DL proved to be more responsive than those of cv. SD. The abundance of *MsMYB2L* transcript remained high throughout the treatment period, peaking around 24 h after the imposition of the stress at, respectively, 5.4 and 4.2 fold the background level in DL and SD (Fig. 1C). The effect of exposure to ABA was to rapidly raise the abundance of *MsMYB2L* transcript, after which the level declined to somewhat above the pretreatment one (Fig. 1D).

3.2. The MsMYB2L sequence and its phylogeny

The length of the *MsMYB2L* coding sequence was 993 nt, and the gene's predicted translation product was a 330 residue protein of molecular weight 37.2 kDa and a predicted pI of 5.89. Alignment of the translation product's sequence with those of other plant MYBs identified that it featured a conserved SANT domain at its N terminus, allowing it to be classified as a member of the R2R3 subfamily; the sequence also included the WxPRL motif, which is common to R2R3-MYB proteins of subgroup 20 (Fig. 1E). The phylogenetic analysis revealed that

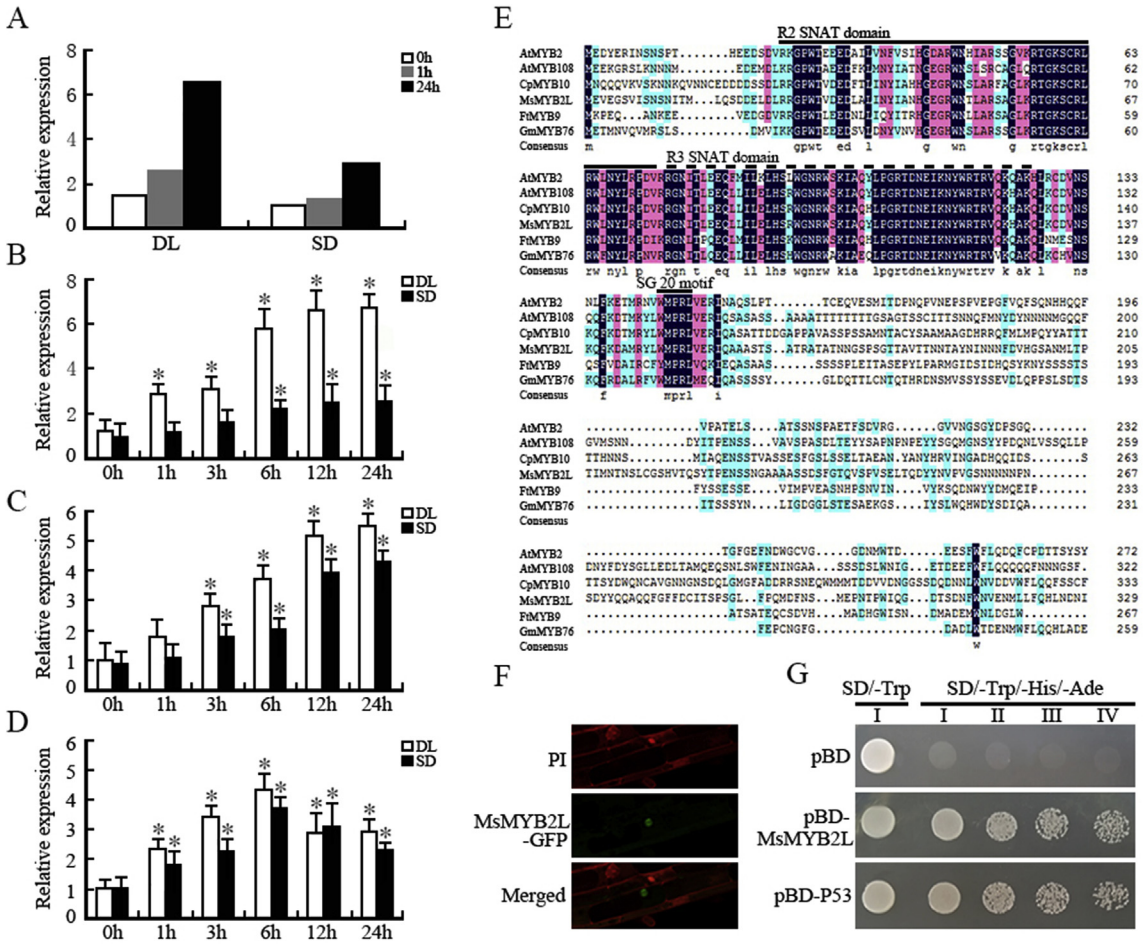

Fig. 1. The character of alfalfa gene *MsMYB2L* and its translation product MsMYB2L. (A) The transcriptional response to exposure to 200 mM NaCl, as determined by microarray analysis. The abundance of *MsMYB2L* transcript, as measured by qRT-PCR, in alfalfa plants exposed to (B) 200 mM NaCl, (C) 20% w/v PEG 6000, (D) 100 μM ABA. Values displayed in the form of mean ± SE (n = 3). Columns headed by an asterisk indicate means which differed significantly ($P < 0.05$) with the 0 h control. (E) Alignment of the MsMYB2L sequence with that of other MYB proteins. (F) The transient expression of *MsMYB2L* in *A. thaliana* demonstrates that the transgene product is deposited specifically in root cell nuclei. (G) Transactivation assay of *MsMYB2L* in yeast. Transformed yeast cells were grown on both SD-Trp and SD-Trp-His-Ade media.

MsMYB2L's most closely related *A. thaliana* homolog was AtMYB2, which known to be responsive to ABA and to be involved in the drought stress response (Abe et al., 2003).The next most closely related AtMYBs were AtMYB62 which participates in the response to phosphate starvation (Devaiah et al., 2009).

3.3. *MsMYB2L localizes to the nucleus and acts as a transcriptional activator*

Confocal microscopy analysis of the roots of *A. thaliana* plants harboring the *MsMYB2L* coding sequence revealed that the MsMYB2L protein was deposited exclusively in the nucleus (Fig. 1F). When a construct comprising the *MsMYB2L* sequence fused to the sequence encoding the P53 DNA-binding domain was expressed in yeast, cells were able to grow on a selective medium (Fig. 1G), indicating that MsMYB2L was able to activate transcription in yeast.

3.4. *A. thaliana plants expressing MsMYB2L were more tolerant of moisture stress than wild type*

The *MsMYB2L* coding sequence was constitutively expressed in *A. thaliana* by placing it under the control of the *CaMV 35S* promoter. Five independent transgene homozygous lines (OE lines) were generated, and the level of *MsMYB2L* transcription in each was estimated using qRT-PCR. As expected, there was no detectable *MsMYB2L* transcript present in the wild type (WT) control plants, while there was some variation in the intensity of *MsMYB2L* transcription among the OE lines, with OE#1 and OE#2 showing the highest abundance of the transgene transcript (Fig. 2A). The effect of the transgene on drought tolerance was tested by depriving three week old plants of OE#1, OE#2 and WT of water. There was no detectable effect of the transgene in well-watered plants, but after withholding water for 21 days, the WT plants became strongly wilted and only ~60% of them were able to recover after re-watering. A number of the OE line plants maintained green leaves throughout the stress period, and ~82% of them were able to recover after re-watering (Fig. 2B and C). Under well-watered conditions, detached leaves of the OE plants retained their level of hydration more effectively than those harvested from WT plants (Fig. 2D). The OE seedlings accumulated 40% more proline and 30–40% more soluble sugar in their leaves than WT seedlings managed (Fig. 2E and F), and featured a significantly lower malondialdehyde content (Fig. 2G).

3.5. *The constitutive expression of MsMYB2L enhances tolerance to multiple abiotic stressors*

The length of the roots formed by ten day old seedlings of the two OE lines when germinated on agar plates was no different from that of WT seedlings (Fig. 3A). However, when the agar was formulated to

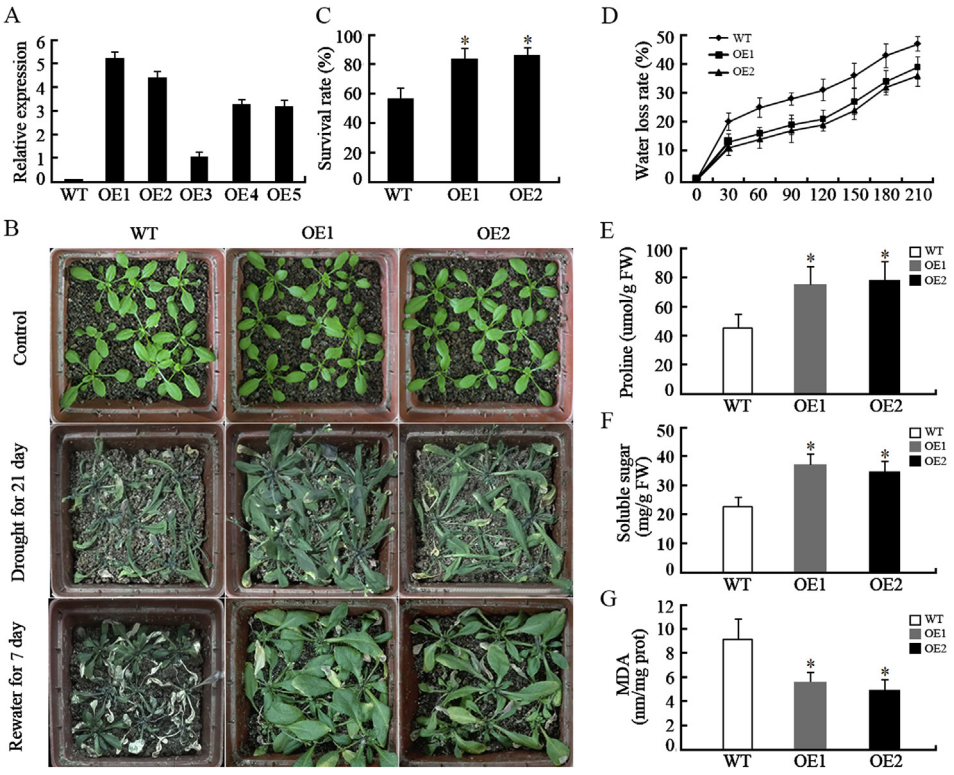

Fig. 2. The constitutive expression of *MsMYB2L* in *A. thaliana* enhances the plants' tolerance of drought stress. (A) The transcription of *MsMYB2L* in *MsMYB2L* transgenic (OE) plants: OE#3 exhibited the lowest transcript abundance so was used as the basis for calculating relative transcript abundances in the other OE lines. (B) The phenotype of moisture-stressed OE line and WT plants. (C) The survival of moisture-stressed OE and WT plants. Each experiment comprised 20 plants. (D) The rate of water loss from detached leaves of WT and OE line plants. The leaf content of (E) proline, (F) soluble sugars, (G) malondialdehyde in WT and OE line plants grown in absence of stress. Values shown in the form mean ± SE (n = 3). Asterisks indicate a significant difference ($P < 0.05$) between the performance of OE line and WT plants.

contain 125 mM NaCl, the length of the primary OE line root was 40–50% longer than those formed by WT seedlings (Fig. 3A and B). In a similar assay testing the effect of the stressors 10 μM ABA or 150 mM mannitol, the presence of ABA inhibited root elongation more strongly for OE than for WT seedlings (Fig. 3A and B), while the opposite was the case in the presence of mannitol (Fig. 3A and B). Germination of WT seed in the presence of either 150 mM NaCl or 200 mM mannitol was more severely restricted than that of the OE seed (Fig. 3C and D), while in the presence of 0.25 μM ABA, the opposite was the case (Fig. 3C and D).

3.6. The effect of constitutively expressing MsMYB2L on the transcription of known abiotic stress-responsive genes

The transcriptional response of known stress-responsive genes was compared between OE and WT seedlings grown in the absence of any stress. The experiment revealed that all of *AtP5CS1*, *AtRD22*, *AtADH1*, *AtRD29A* and *AtCOR15A* were more abundantly transcribed in the OE plants than in the WT ones (Fig. 4).

4. Discussion

R2R3 MYB TFs have been shown to participate in the stress response of a number of plant species. To date, the only such TF encoded by alfalfa is MYB4, which is involved in the plant's defense against soil salinity (Dong et al., 2018). This is now joined by a second MYB member, denoted here as MsMYB2L. TFs, which can be either up- or down-regulated in response to abiotic stress, tend to respond transcriptionally rather rapidly (He et al., 2012). For example, the abundance of transcript generated from the soybean gene *GmMYB84* is raised within 1 h of the plants' exposure to either salinity or exogenously supplied ABA (Wang et al., 2017a,b). Similarly, *MsMYB2L* was very rapidly induced by each of the three stressors investigated here. In each case, the abundance of its transcript remained higher in the more stress tolerant of the two cultivars tested, which suggested that this TF likely plays some part in stress tolerance.

The accumulation of compatible osmolytes supports cellular homeostasis in plants experiencing stress (Rosa et al., 2009). Proline has repeatedly been implicated as an osmoprotectant (Szekely et al., 2008). Both salinity- and drought-hypersensitive *A. thaliana* mutants have been shown to build up their content of proline to a high level (Rosa et al., 2009), and similarly, the proline (as well as the soluble sugar) content of the leaves of the *MsMYB2L* transgenic plants was higher than that of WT leaves. The activity of the enzyme pyrroline 5-carboxylate synthase (P5CS) represents a rate-limiting step in proline synthesis: its loss-of-function is typically associated with a reduced capacity to tolerate salinity (Liu and Zhu, 1997), while its over-expression has the opposite effect (Kishor et al., 1995). The up-regulation of *AtP5CS1* in the transgenic plants constitutively expressing *MsMYB2L* may therefore make a contribution towards the observed increased salinity tolerance of these plants.

Plants respond to a number of stress factors by raising the endogenous level of ABA. The response to both drought and salinity stress can be manifested via both ABA-independent and ABA-dependent pathways (Yoshida et al., 2014). The *MsMYB2L* transgenic plants exhibited a varied degree of ABA sensitivity during their germination and seedling development which are in line to the salt tolerance MYB genes such as *MtMYBS1* and *AtMYB2* (Dong et al., 2017; Abe et al., 2003). The ABA-dependent genes *AtRD22*, *AtADH1*, *AtRD29A* and *AtCOR15A* were all transcribed more strongly in the MsMYB2L transgenic plants than in WT plants. The dependency of *AtRD22* is thought be largely due to co-expression activation of AtMYB2/AtMYC2 (Abe et al., 2003), while the up-regulation of *AtADH1* only requires the activation of *AtMYB2* to act in a protective protein (Hoeren et al., 1998). Both *AtRD29A* and *AtCOR15A* are known to exist in DRE or related motifs promoter regions which are induced by a number of abiotic stress factors (Narusaka et al., 2003; Yang et al., 2011). The likelihood is that the benefit gained by the heterologous expression of *MsMYB2L* operates through an ABA-dependent pathway, with enhanced water retention abilities against osmotic stress condition by promoting the content of proline and soluble sugar, although the possibility also exists that ABA regulates the growth of the transgenic plants' roots.

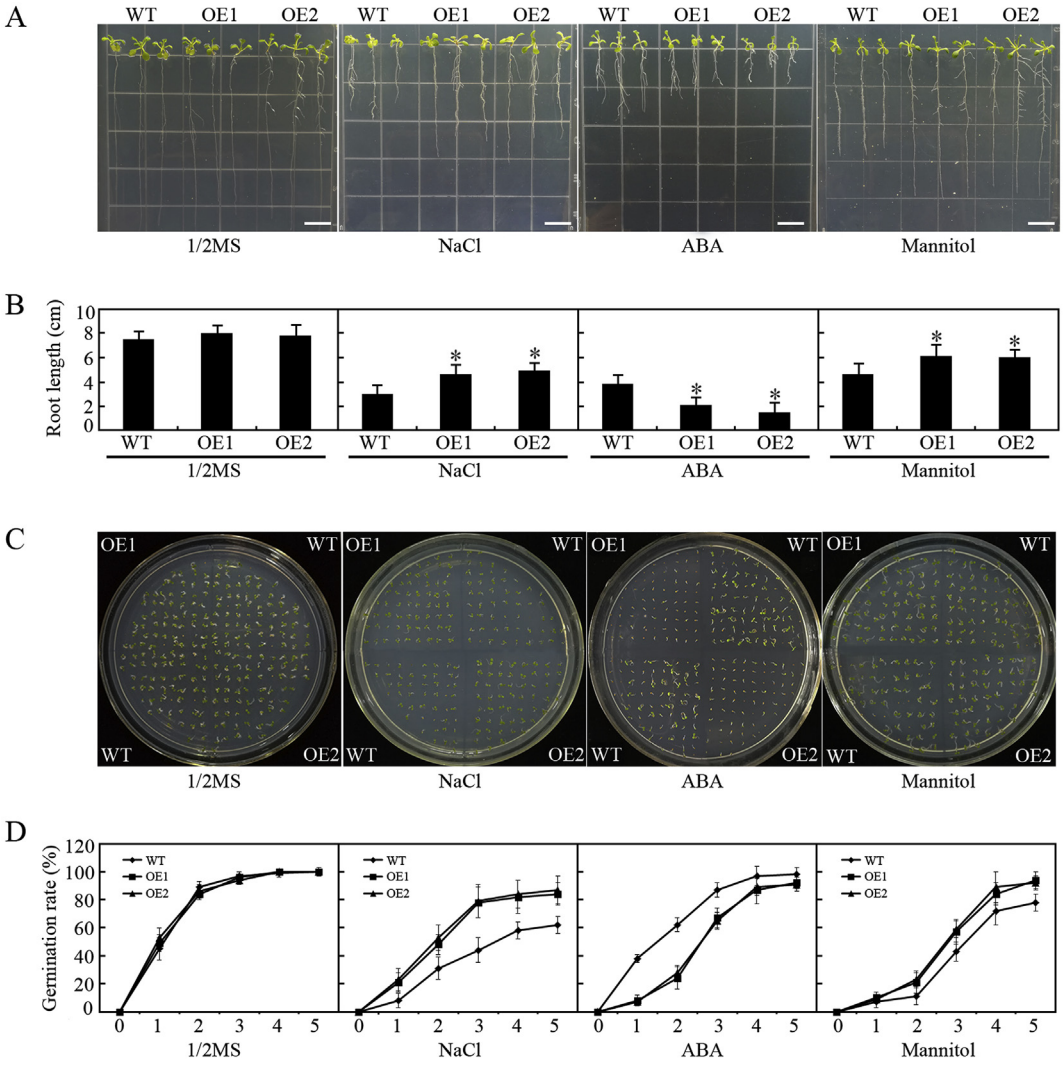

Fig. 3. Analysis of WT and *MsMYB2L* transgenic (OE) plants grown in the presence of salinity, mannnitol and ABA. (A) Seedlings exposed for ten days to either 125 mM NaCl, 10 μM ABA or 150 mM mannnitol. Bar = 1.5 cm (B) The root length of the seedlings shown in (A). (C) The germination of WT and OE line seeds in the presence/absence of 0.25 μM ABA, 150 mM NaCl or 200 mM mannitol. (D) The germination rate of the seedlings shown in panel (C). Values shown in the form mean ± SE (n = 3). Asterisks indicate a significant difference ($P < 0.05$) between the performace of OE line and WT seed.

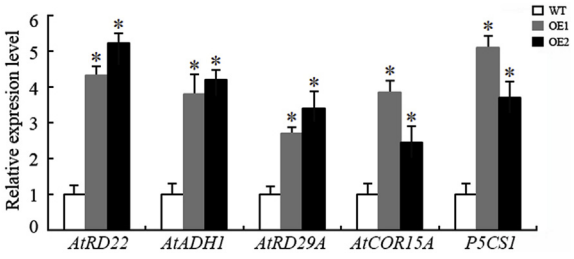

Fig. 4. The transcriptional behavior in two week old transgenic MsMYB2L (OE) seedlings of genes associated with the plant stress response. Data presented in the form mean ± SE (n = 3). Columns marked with an asterisk indicate mean abundances which differed significantly ($P < 0.05$) from those in WT plants exposed to the same treatment.

Genes which respond positively to salinity and drought stress in a manner which supports plant growth and development are an important resource in the context of breeding for crop resilience. *MsMYB2L* may represent such a gene, since it was inducible by multiple abiotic stress factors and, when constitutively expressed in *A. thaliana*, had a positive effect on the performance of plants exposed to stress. The assumption is – although this needs to be experimentally verified - that the same will be true when this gene is over-expressed in alfalfa.

Author contribution statement

YG Song and W Dong designed the research. W Dong, J Lv, NW Qiu, WT Bai and N Yang conducted the experiment and performed data analysis. W Dong wrote the paper.

Declarations of interest

None.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2019.06.007.

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