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Molecular cloning and functional analysis of a salt-induced gene encoding an RNA-binding protein in alfalfa

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Abstract Based on the sequence of an expressed sequence tag of alfalfa (*Medicago sativa* L. cv. Zhongmu-1), a full-length sequence of 1,551 nucleotides was isolated using RACE-PCR techniques. This gene (*MsRBP*) was predicted to encode a protein of 409 amino acids, which contained three RNA recognition motifs comprising highly conserved RNA-binding domains at the N-terminus. Sequence analysis indicated that the C-terminus of MsRBP was rich in proline and glutamine residues. Subcellular location analysis suggested that MsRBP preferentially localized in the nucleus. Real-time fluorescent quantitative PCR analysis showed that the transcript level of *MsRBP* was upregulated after treating with 20 % polyethylene glycol (PEG6000), 100 μM abscisic acid, or 300 mM NaCl. Compared with the wild-type plant, transgenic *Arabidopsis* plants overexpressing *MsRBP* displayed retarded germination and root growth under salt stress. The results suggested that *MsRBP* may play a negative role in salt stress regulation of alfalfa.

Keywords Alfalfa · *Arabidopsis* · RRM domain · Salt stress · Subcellular localization · Overexpression

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Introduction

Post-transcriptional control of gene expression plays a vital role in the normal growth and development of eukaryotes. This level of regulation includes pre-mRNA splicing, capping, polyadenylation, mRNA transport, stability, and translation (Burd and Dreyfuss 1994; Dreyfuss et al. 2002). The regulation is accomplished either directly by RNA-binding proteins (RBPs) or indirectly via the interaction between RBPs and other regulatory factors. The superfamily of plant RBPs is extensive compared with other eukaryotes (Peal et al. 2011). In fact, RBPs are characterized by conserved domains in the protein sequence. These domains include RNA recognition motif (RRM, also known as the consensus-sequence RNA-binding domain (cs-RBD)) or ribonucleo-protein motif (RNP domain), K homology motif, zinc finger domain,

DEAD/DEAH box, RGG box, and serine-arginine-rich motif, among others. RBPs containing RRMs are the most common among eukaryotes. Structurally, RRM is comprised of 80–90 amino acids and occurs in one or more copies in various RBPs. The RRM consists of a highly conserved octamer or RNP-1 amino acid sequence and a less well-conserved hexamer or RNP-2 amino acid sequence. The RRMs are conserved structurally rather than sequentially (Lorkovic and Barta 2002).

Due to the lack of suitable plant-derived systems in vitro for studying post-transcriptional events, RBPs have received much less attention in plants than in other organisms (Lorkovic 2009), despite the extensive superfamily of RBPs. In plants, the only RBP database available is POGs/PlantRBP (Walker et al. 2007), which contains predicted plant RBPs that are identified by sequence similarity. The genome of *Arabidopsis* contains 279 RBPs (Anantharaman et al. 2002) compared with 257 RBP genes identified in *Oryza sativa* L. (Morris et al. 2011), 100 in *Caenorhabditis elegans* (Crittenden et al. 2002), and 117 in *Drosophila* (Lasko 2000). However, the functions of only a few plant RBPs have been reported thus far. Interestingly, plant RBPs are emerging with increasing frequency as the regulatory factors in floral transition, floral patterning, abscisic acid (ABA) signaling, stress responses, circadian rhythms, and chromatin modification (Lorkovic 2009; Lu and Fedoroff 2000; Staiger 2001).

Indeed, recent identification and analyses of plant RBPs have elucidated their important role in regulating diverse developmental processes and environmental stresses (Lorkovic 2009). For example, the expression of *GR-RBP4*, one of the eight GR-RBP family members in *Arabidopsis thaliana*, increased markedly with cold stress, while dehydration and salt stress strongly increased *GR-RBP1* transcription (Kwak et al. 2005). Three *UBA2* genes encoding hnRNP-type nuclear RNA-binding proteins were shown to be involved in a novel wound signal transduction pathway (Bove et al. 2008). Recent work found that *SaRBP1*, an RNA-binding protein from a salt-treated halophyte *Suaeda asparagoides*, enhanced tolerance to osmotic, freezing, and heat shock stresses when overexpressed in yeast (Ayarpadikannan et al. 2012). *HYL1* encoding a nuclear dsRNA-binding protein in the Arabidopsis was associated with ABA-regulation at the transcriptional or post-transcriptional level (Lu and Fedoroff 2000). Similarly, *ABH1* *encodes* the Arabidopsis homolog of a nuclear mRNA-cap-binding protein, and modulated ABA signaling (Hugouvieux et al. 2001). *atRZ-1a* has a negative impact on seed germination and seedling growth of *Arabidopsis thaliana* under salt or drought stress conditions (Kim et al. 2007). These reports support the role of RBPs in stress response.

Alfalfa (*Medicago sativa* L.) is a perennial forage legume. It is widely distributed in irrigated arid and semi-arid regions worldwide. Water deficit and salinity severely restrict alfalfa growth. In our previous study, we constructed an alfalfa cDNA library induced by salt stress using suppression subtractive hybridization (SSH) technology (Jin et al. 2010). In this study, we cloned an RNA-binding protein gene (*MsRBP*) from alfalfa based on an EST sequence (GenBank Accession No. FE896907) in the SSH library. We also investigated the subcellular location of *MsRBP* and its expression patterns under different stresses by using real-time RT-PCR. Furthermore, we overexpressed *MsRBP* in *Arabidopsis* and examined the effect on seed germination and seedling growth of transgenic plants under salt stress.

Materials and methods

Plants and stress treatments

Seeds of alfalfa (*Medicago sativa* L. cv. Zhongmu-1, a salt-tolerance alfalfa cultivar) were sown on MS medium and germinated in the dark at 25 °C for 1 day, followed by growth under alternating 16-h light and 8-h darkness at 25 °C. We transferred 6-day-old seedlings into Hoagland's nutrient solution. The solution was changed every 5 days. We transferred 2-week-old seedlings into Hoagland's nutrient solution supplemented with 20 % PEG6000, 100 μM ABA, and 300 mM NaCl, respectively. PEG6000 was used to simulate drought stress. ABA was used to simulate endogenous ABA after salt stress. *Arabidopsis thaliana* ecotype Col-0 was used as a recipient host for plant transformation. *Arabidopsis* plants were grown in a greenhouse at a constant temperature of 22 °C with 16-h light and 8-h darkness.

Isolation of MsRBP gene from alfalfa

Based on one EST sequence (GenBank Accession No. FE896907) from an alfalfa salt-stress-induced SSH

cDNA library, two gene-specific primers (GSP) were designed to amplify the 3′- and 5′-ends of *MsRBP*. P1: 5′-AGTTTATTAGCCGTGCTGGTGCTGAGA-3′ was used to amplify the 3′-RACE end, and P2: 5′-AACAG-CAACATCAACAACAACCACCCT-3′ was used to amplify the 5′-RACE end with Advantage™ 2 PCR Enzyme kit (Clontech, USA). Total RNA was isolated from 2-week-old alfalfa seedlings by TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. Any contaminated genomic DNA was removed by incubating the total RNA with RNase-free Dnase I (Promega, USA) at 37 °C for 30 min. The total RNA was used to synthesize 3′-RACE-Ready-cDNA and 5′-RACE-Ready-cDNA according to the manufacturer's recommendation of SMART™ RACE cDNA amplification kit (Clontech, USA). Thermocycling was performed for 30 cycles at 95 °C for 3 min, 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min, and an additional polymerization step at 72 °C for 10 min. RACE products were separated by electrophoresis on 1 % agarose gel stained with ethidium bromide and extracted using a DNA gel extraction kit (Takara, Japan). The products were cloned into the pMD-19T vector (Takara, Japan) and then transformed into *Escherichia coli* DH5α. Recombinant plasmids were sequenced by Biomed Company (Beijing, China). By comparing and aligning the sequences of the known EST, the 5′- and 3′-end sequences, the full-length sequence of *MsRBP* was obtained.

Bioinformatics analysis

Overlapping and assembly of sequences were accomplished with DNAMAN software (http://www.lynnon.com/). Homology searches were performed with BLAST algorithms (http://blast.ncbi.nlm.nih.gov/) to confirm sequence identity. The Open Reading Frame Finder (http://blast.ncbi.nlm.nih.gov/) was used to search for ORF of *MsRBP*. The theoretical isoelectric point, molecular weight, and hydrophobic structure of the putative protein were predicted on ExPASy website (http://us.expasy.org). Secondary structure of the putative protein was predicted in PBIL LYON-GERLAND database (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). The subcellular localization of MsRBP was analyzed by ProtComp version 9.0 software (http://linux1.softberry.com/berry.phtml). Amino acid sequence alignment was carried out by ClustalX 2.0 (http://www.clustal.org/), and the phylogenetic tree of MsRBP and RNA-binding proteins with triple RNA recognition motifs of some other plants was constructed by MEGA 5.0 software with the neighbor-joining method (Saitou and Nei 1987).

Subcellular localization

To observe the subcellular localization of MsRBP, a MsRBP-GFP fusion was constructed and transiently expressed in onion epidermal cells. Two primers P3 and P4 were designed to amplify the coding sequence of *MsRBP*, one with *Xho* I restriction site (P3: 5′-TACTCGAGATGATGCAACCAGGAGGACCAG-3′) and another with *Spe* I restriction site (P4: 5′-GCACTAGTCTATATCCTAATTGCTGTTGCT-3′). The PCR product was digested with *Xho* I and *Spe* I and ligated with the *pA7-GFP* vector (Supplementary Figure 1), which was digested with the same restriction enzymes. The *pA7-GFP* vector was used as the control. The *pA7-MsRBP-GFP* vector was then transformed into onion epidermal cells using a particle gun (Bio-Rad, USA) and visualized under laser scanning confocal microscopy (Nikon, Japan).

Transcriptional analysis by real-time PCR

To investigate the expression patterns of *MsRBP*, 2-week-old alfalfa seedlings were transferred into Hoagland's solution with NaCl (300 mM), ABA (100 μM), and PEG 6000 (20 %), respectively. Plants were sampled at 0, 2, 4, 10, and 24 h after starting each treatment. The 0-h seedlings were used as control. The roots and shoots were harvested separately. The total RNA was reverse transcribed, and the synthesized cDNA was used as template in real-time PCR. P5: 5′-CCGATGAAGTCAAAACTCTCTGGAT-3′ and P6: 5′-GCACGGCTAATAAACTCAAGAAACC-3′ were used for the amplification of *MsRBP*. Actin gene was used as a housekeeping gene to normalize the target gene quantities. P7: 5′-TGGGCTGCCACAGAACATTTGA-3′ and P8: 5′-GCTGTGGTTGCTTTTTTGGTGTCTC-3′ were used for the amplification of actin gene. The real-time PCR was performed following the instructions of SYBR *Premix Ex Taq*™ (Takara, Japan) using the fluorescent quantitative PCR amplification instrument (ABI7500, USA). The relative expression level of *MsRBP* was calculated by –ΔΔCt method.

Transformation and selection of transgenic Arabidopsis lines

Based on the cDNA sequence, the following two gene-specific primers were designed to amplify the coding sequence, with *Xba* I and *Sma* I restriction sites: P9: 5′-TATCTAGAATGATGCAACCAGGAGGACCAG-3′ and P10: 5′-ATCCCGGGCTATATCCTAATTGCTG TTGCT-3′, P9 and P10 were used to amplify the coding sequence of *MsRBP* without stop codon and fuse with the MsRBP-GUS for overexpression. The amplification product was subcloned into *pBI121* vector (Supplementary Figure 1). The pBI121-*MsRBP* vector was transformed into *Arabidopsis* with a floral-dipping method by *Agrobacterium* GV3101 and expressed under the control of CaMV 35S promoter. The transgenic *Arabidopsis* plants were selected on MS medium supplemented with kanamycin (50 mg/L) and verified by PCR, RT-PCR. The tissue expression pattern of *MsRBP* was analyzed by GUS staining.

Salt stress regulation in transgenic Arabidopsis

Wild-type seeds and T_3 generation transgenic *Arabidopsis* seeds were sterilized by 0.1 % $HgCl_2$ for 8 min and then rinsed with sterile deionized water. Germination assays were carried out with 40–50 seeds in each MS medium agar plates. The plates were placed at 4 °C for 3 days in the dark and then transferred to normal growth conditions. Each plate was divided into two parts, one each for wild-type and the T_3 transgenic *Arabidopsis* seeds. To determine the effect of salt stress on germination and seedling growth, the medium was supplemented with 0, 75, 125, or 175 mM NaCl. Germinating seeds were identified by radicle protrusion from seed coat. To determine the effect of treatment on seedling growth, we placed the plates vertically in a growth chamber and measured root length every day.

Results

Cloning of a cDNA encoding RBP

An 1,127-bp fragment was isolated by using 3′-RACE PCR, and a 424-bp fragment was obtained by using 5′-RACE PCR. A full-length of 1,551 bp cDNA sequence was assembled by the 1,127 bp 3′-end sequence, 424 bp 5′-end sequence, and the known EST sequence (Supplementary Figure 2). A 1,230-bp open reading frame (ORF) encoding a protein of 409 amino acids was predicted by ORF Finder. Sequence analysis indicated that the theoretical *pI* of the putative protein was 6.31 and its Mw was 45.6 kDa. Secondary structure analysis of the putative protein indicated α-helix, extended strand, and random coil. The functional site prediction showed three RRMs and a Glu-Pro-rich domain at the C-terminal ends of the protein. It was tentatively named *MsRBP* (GenBank Accession No. JN986878). A phylogenetic tree was constructed using neighbor-joining method. It was based on the amino acid sequence of MsRBP (GenBank Accession No. AEW68341) as well as RNA-binding proteins containing triple RNA recognition motifs. The result revealed that MsRBP was homologous with RNA-binding protein in other plants, such as XP_003610417 (*Medicago truncatula*) and XP_004507695 (*Cicer arietinum*) (Supplementary Figure 3).

Subcellular localization

The online ProtComp version 9.0 program was used to predict the subcellular localization. Based on the integral prediction method, the MsRBP was most likely localized in the nucleus. A strong fluorescence signal was observed in the nucleus of onion epidermal cells transformed with *pA7-MsRBP-GFP* vector under a confocal laser scanning microscope (Fig. 1a–c). In contrast, the fluorescence signal was distributed throughout the onion epidermal cells transformed with *pA7-GFP* control vector (Fig. 1d–f). The results indicated that MsRBP was a nuclear-localized protein.

Expression analysis of MsRBP mRNA

In order to determine the expression pattern of *MsRBP*, real-time RT-PCR was carried out to examine the transcriptional levels of *MsRBP* in alfalfa seedlings under various stresses including high salinity, drought, or abscisic acid (ABA) treatments. The seedlings treated at 0 h were used as control. As shown in Fig. 2, the expression of *MsRBP* was affected differently depending on stress conditions. The transcriptional level of *MsRBP* in roots increased up to 5–6-fold after salt stress for 10 h and then decreased marginally afterward, while the *MsRBP* transcript level in shoots was slightly upregulated by NaCl (Fig. 2a). The transcriptional level of *MsRBP*

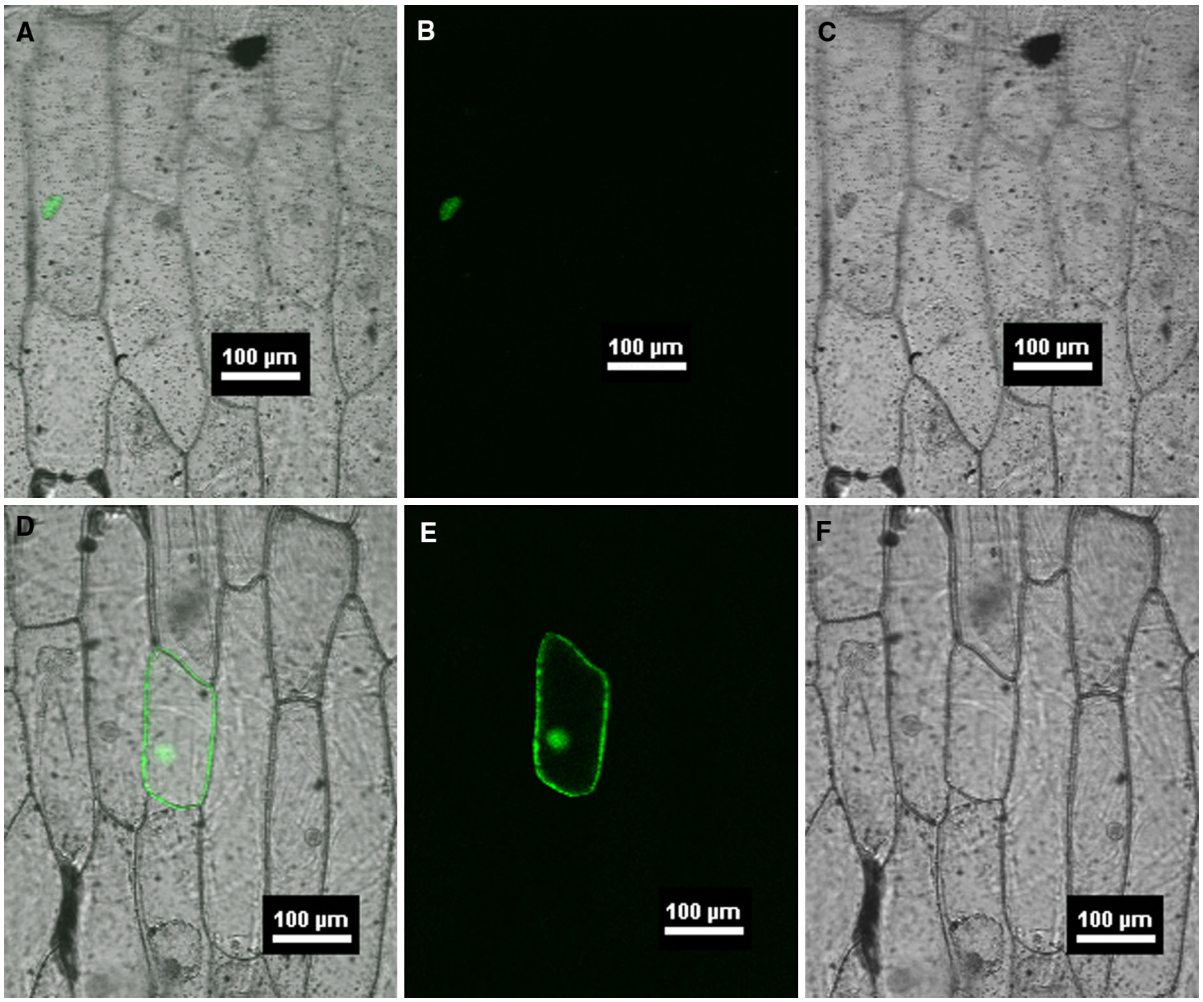

Fig. 1 Subcellular localization of *MsRBP* in onion. The pA7-*MsRBP*-GFP and *pA7-GFP* vectors were transformed into onion epidermal cells using a particle gun. The fluorescence signal was examined by a confocal laser scanning microscope. GFP fluorescence from cells expressing *MsRBP*-GFP fusion protein (**a**–**c**). GFP fluorescence from cells expressing GFP protein (**d**–**f**). **a**, **d** Superposition of the bright and dark vision. **b**, **e** Dark field vision. **c**, **f** Bright light vision

was increased sharply after the first 2 h of ABA treatment and then gradually decreased afterward, but still higher than control at 24 h (Fig. 2b). When treated by PEG6000, the relative transcriptional level of *MsRBP* reached the highest at 4 h in roots, but at 2 h in shoots (Fig. 2c).

Characterization of transgenic Arabidopsis plant

The *35S::MsRBP* transgenic plants of T_0 generation were screened using kanamycin (50 mg/L) and confirmed further by genomic PCR, RT-PCR. The presence of *MsRBP* in transgenic plants was confirmed by genomic PCR (Supplementary Figure 4). Thirty-seven independently transformed T_0 transgenic plants were selected and grown to maturity to obtain homozygous lines. Three T_3 homozygous lines, T31, T33, and T35, were randomly selected and used throughout the study. The *MsRBP* gene was amplified with cDNA of T_3 transgenic plants using P9/P10 primers. The results showed that *MsRBP* had integrated into transgenic *Arabidopsis* plants genome and expressed at the transcription level by RT-PCR (Supplementary Figure 4). GUS staining analysis result indicated that *MsRBP-GUS* fusion was expressed in almost all of the transgenic *Arabidopsis* (Fig. 3).

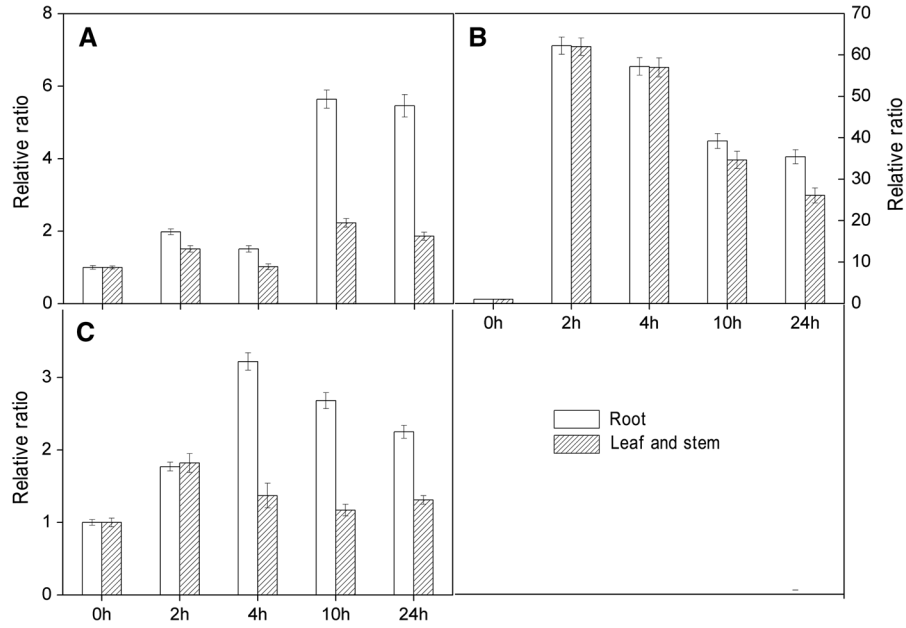

Fig. 2 Expression level of *MsRBP* in alfalfa after 300 mM NaCl treatment (**a**), 100 μM ABA treatment (**b**), and 20 % PEG treatment (**c**). The *y*-axis indicates the increased expression level of relative fold compared with control (0 h), and the expression level of control was set to 1. *Vertical bars* indicate the mean ± SE of three independent experiments

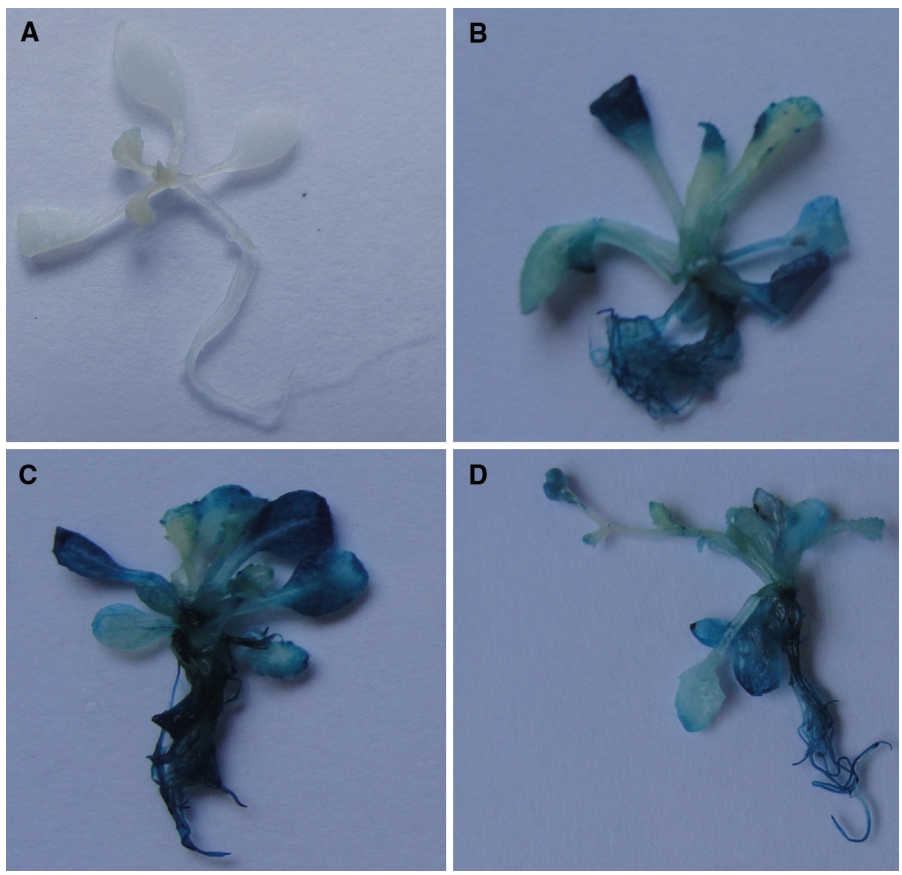

Fig. 3 GUS staining of wild-type *Arabidopsis thaliana* and *MsRBP*-overexpressing lines. **a**–**d** The GUS staining of wild-type *Arabidopsis thaliana*, and overexpression lines T31, T33, and T35

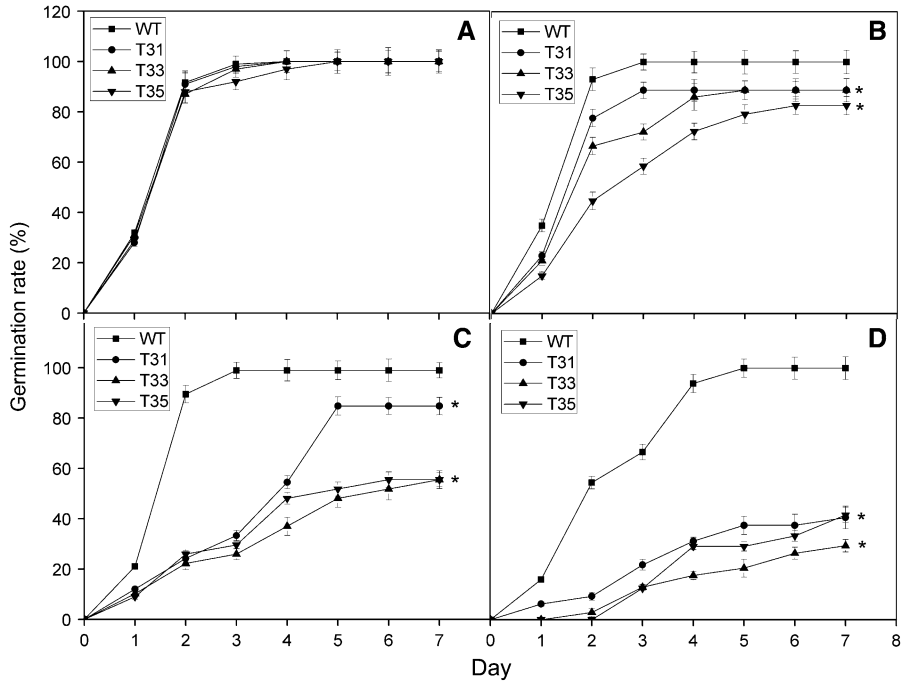

Fig. 4 Effect of salt stress on germination of WT (wild type) and *MsRBP*-overexpressing lines (T31, T33, and T35). Seeds were germinated in MS medium supplemented with 0 mM NaCl (**a**), 75 mM NaCl (**b**), 125 mM NaCl (**c**), and 175 mM NaCl (**d**). *Vertical bars* indicate the mean ± SE of three independent duplicate experiments. *Asterisk* indicates significant difference of germination rates on day 7 ($P < 0.05$, Student's *t* test)

We examined the effect of *MsRBP* in germinating seed and seedling growth of *Arabidopsis* under salt stress. Under normal growth condition, no significant differences in seed germination and seedling growth were observed between the wild-type and overexpressed lines (Fig. 4a). However, a large variation in germination rate and root growth was observed under high-salinity stresses. Under enriched NaCl conditions, increased inhibition of germination was seen. In the presence of 175 mM NaCl, the wild-type seeds germinated significantly earlier than transgenic seeds (Fig. 4). These results suggested that *MsRBP* negatively affected seed germination under salt stress. We tested the effect of *MsRBP* gene on seedling growth under salt stress, by measuring the root length of plants grown in 125 mM NaCl MS medium (the root length data in 75 mM and 175 mM NaCl treatments were not shown. In 75 mM NaCl, the difference of root length between transgenic plants and wild-type plants was not significant. In 175 mM NaCl, transgenic plants and wild-type plants can only germinate but not grow). Results indicated that transgenic seeds displayed retarded root growth, compared with wild type (Fig. 5). These results demonstrated that *MsRBP* played a negative role in seed germination and root growth of *Arabidopsis* under salt stress.

Discussion

RNA-binding proteins (RBPs) are important mediators of gene expression and involved in all aspects of mRNA existence. RBPs have been cloned from many plant species (Ayarpadikannan et al. 2012; Churin et al. 1999; Kwak et al. 2005; Morris et al. 2011; Wang et al. 2011). However, their regulatory importance or molecular mechanism of action is not clearly established. Evidence suggests that RNA-binding proteins also regulate plant response to environmental constraints (Ambrosone et al. 2012). RBP transcription is regulated by environmental variables, including dehydration, temperature, light, ABA, high salinity, wound, and low-oxygen stresses (Ayarpadikannan et al. 2012; Kwak et al. 2005; Ambrosone et al. 2012; Fusaro et al. 2007; Park et al. 2009; Sachetto-Martins et al. 2000; Sahi et al. 2007). However, the cloning and molecular characterization of *RBP* from alfalfa was not previously reported. In this report, we obtained a salt-stress -induced gene *MsRBP* from alfalfa on the basis of 321 bp EST in a SSH cDNA library. MsRBP contained three RRMs, which are commonly found in RBPs and sufficient for RNA binding.

Previous results showed that the expression of *GRRBP4* was downregulated by either dehydration or

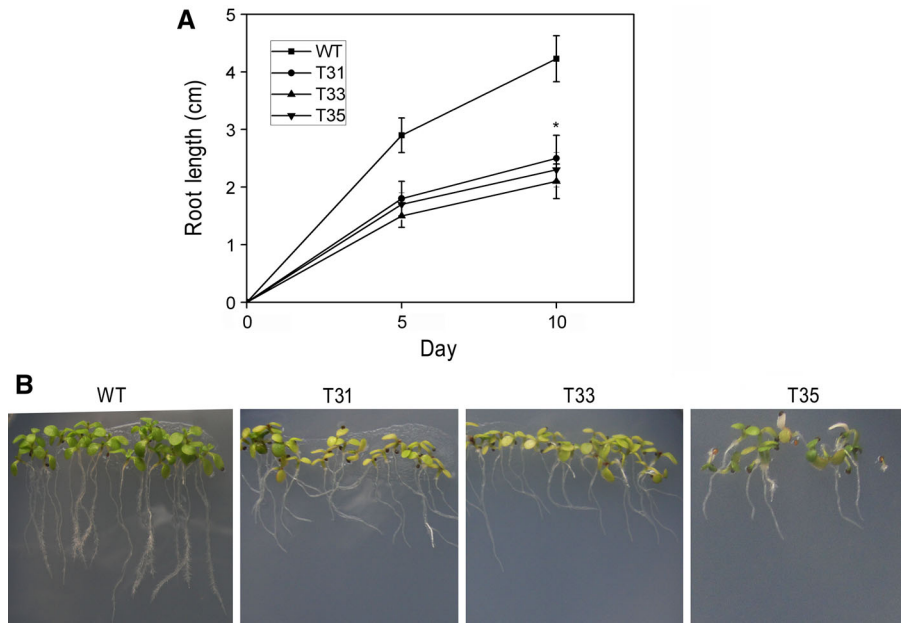

Fig. 5 Effect of salt stress on root length of WT (wild type) and *MsRBP*-overexpressing lines (T31, T33, and T35). Root length (mean ± SE) of plants treated with 125 mM NaCl was observed by incubating the seedlings on MS plates in the vertical orientation (**a**), and the photographs were taken 10 days after germination (**b**). Data were obtained from three independent duplicate experiments. *Asterisk* indicates significant difference of root length on day 10 ($P < 0.05$, Student's *t* test)

salt stress. The overexpressed *GR-RBP4* transgenic lines displayed retarded seed germination of *Arabidopsis* plant under salt or dehydration stress (Kwak et al. 2005). The transcription of *atRZ-1a* was marginally downregulated by drought stress and was not altered by salt stress (Kim et al. 2005). However, transgenic Arabidopsis plants overexpressing *atRZ-1a* displayed retarded germination and seedling growth (Kim et al. 2007). While the transcription of *GR-RBP2* decreased marginally under dehydration and slightly increased during high salinity, over-expressing *GR-RBP2* enhanced seed germination, but not seedling growth under salt stress. *GR-RBP2* played no role in seed germination or seedling growth of *Arabidopsis* plants under drought stress (Kim et al. 2007; Kwak et al. 2005). Therefore, we examined the expression levels of *MsRBP* in alfalfa by real-time quantitative RT-PCR. *MsRBP* was induced in alfalfa by NaCl, ABA, or PEG6000, and the expression of *MsRBP* was affected differently depending on stress conditions. These results also suggested that *MsRBP* might participate in adaptation to environment conditions in alfalfa. However, the importance of its expression pattern required additional analysis (e.g., Western blot). We constructed plant overexpression vector and transferred *MsRBP* gene into *Arabidopsis* successfully. We used *35S::MsRBP* seeds of transgenic *Arabidopsis* plant (T_3 generation) and wild type to analyze the germination and seedling growth under salt stress. The results suggested retardation in seed germination rate and root growth of T_3 generation compared with wild type in response to NaCl, indicating that *MsRBP* negatively affected seed germination and root growth under salt stress. These studies illustrate the myriad functional roles of plant RBPs during normal plant development and in response to environmental perturbations, mediated by the structural differences between MsRBP and other RBPs.

Currently, the role of *MsRBP* in germination and root growth under salt stress is unknown. It has been hypothesized that *GR-RBP2* and *atRZ-1a* may play a role as an RNA chaperone similar to bacterial CSPs (Kim et al. 2005). Probably, *MsRBP* influences the transcriptional levels of other stress-related genes, or *MsRBP* binds to the target RNA to regulate the processing, stability, and/or translation of target gene involved in germination and root growth. Our present work provides novel data related to the functional role of RBPs in response to environmental stresses. The

discovery of *MsRBP* gene may contribute to the study of mechanisms of salt tolerance in *Medicago sativa* L. It also provides an additional tool for the genetic improvement of stress tolerance in crop plants.

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