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Overexpression of a novel salt stress-induced glycine-rich protein gene from alfalfa causes salt and ABA sensitivity in Arabidopsis

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Abstract

Key message **We cloned a novel salt stress-induced glycine-rich protein gene (*MsGRP*) from alfalfa. Its overexpression retards seed germination and seedling growth of transgenic *Arabidopsis* after salt and ABA treatments.**

Abstract Since soil salinity is one of the most significant abiotic stresses, salt tolerance is required to overcome salinity-induced reductions in crop productivity. Many glycine-rich proteins (GRPs) have been implicated in plant responses to environmental stresses, but the function and importance of some GRPs in stress responses remain largely unknown. Here, we report on a novel salt stress-induced GRP gene (*MsGRP*) that we isolated from alfalfa. Compared with some glycine-rich RNA-binding proteins, *MsGRP* contains no RNA recognition motifs and localizes in the cell membrane or cell wall according to the sub-cellular localization result. *MsGRP* mRNA is induced by salt, abscisic acid (ABA), and drought stresses in alfalfa seedlings, and its overexpression driven by a constitutive cauliflower mosaic virus-35S promoter in *Arabidopsis* plants confers salinity and ABA sensitivity compared with WT plants. *MsGRP* retards seed germination and seedling growth of transgenic *Arabidopsis* plants after salt and ABA treatments, which implies that *MsGRP* may affect germination and growth through an ABA-dependent regulation pathway. These results provide indirect evidence that *MsGRP* plays important roles in seed germination and seedling growth of alfalfa under some abiotic stress conditions.

Keywords Alfalfa · Glycine-rich protein · Abiotic stress · Overexpression · Sensitivity

Introduction

Salinity stress is one of the primary causes of crop losses worldwide, but plants have evolved sophisticated mechanisms to adapt to salinity and other environmental stresses (Zhu 2002). Soil salinity is one of the most significant abiotic stresses for crop plants including legumes (Duzan et al. 2004; Golldack et al. 2011; Zahran 1999). Legumes are very important both ecologically and agriculturally since their roots are able to interact symbiotically with soil microorganisms to form nodules that fix atmospheric nitrogen. Several legumes, such as *Medicago sativa* (alfalfa) and the model *Medicago truncatula*, have a large diversity of cultivars that have adapted to soil salinity. Many *M. sativa* cultivars show high salinity tolerance (Munns and Tester 2008), especially the cultivar of *Medicago sativa* L. cv. Zhongmu-1 that we cultivated. Abiotic stresses, such as drought, salinity, and cold, regulate the expression of thousands of genes in plants at both the transcriptional and the posttranscriptional levels. The molecular mechanism of plant salt tolerance is very complex (Munns and Tester 2008; Zhu 2002). To date, little is

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known about the molecular links between morphological adaptations to salt stresses and endogenous signals. Suppressive subtractive hybridizations (SSH), array technologies, transcriptome high-throughput sequencing, and proteome difference analysis have become powerful and useful tools for identifying and analyzing the gene expression profiles of plants that are exposed to abiotic stresses such as drought, cold, and high salinity. We previously reported that an alfalfa cDNA library induced by salt stress was constructed using the SSH technique, which resulted in the identification of 82 expressed sequence tags (ESTs) induced by salt stress (Jin et al. 2010). In this study, we reported cloning and characterizing a novel glycine-rich protein (GRP) gene (*MsGRP*) using one of the EST sequences (GenBank accession No. FE896860) (Jin et al. 2010) that was previously identified from alfalfa.

Plant GRPs are a group of proteins characterized by their high content and repetitive sequences of glycine residues. A number of GRP-coding genes have been isolated from various plant species and can be divided into three main classes according to their amino acid sequences. The first GRP class consists of cell wall component proteins that contain signal peptide sequences that indicate protein export into the apoplastic space (Ringli et al. 2001). The second GRP class contains RNA-binding proteins that contain RNA recognition motifs (RRMs) at the N-terminal and a glycine-rich region at the C-terminal (Burd and Dreyfuss 1994). The third GRP class contains dehydrins, which are homologous to stress and/or developmentally regulated proteins and are thought to protect plasma membranes during stresses that involve increased osmotic pressure. Some structural cell wall GRPs have glycine contents that comprise up to 60 or 70 % of all amino acid residues (Ringli et al. 2001). Their role has yet to be elucidated. Bean GRP1.8 is a structural cell wall GRP evidenced to locate in the cell wall (Keller et al. 1988). Glycine-rich RNA-binding proteins have been described in plants including maize (Gomez et al. 1988), *Arabidopsis thaliana* (Carpenter et al. 1994; van Nocker and Vierstra 1993), barley (Dunn et al. 1996; Molina et al. 1997), and alfalfa (Ferullo et al. 1997). Some of these proteins are suggested to play a role in stress responses, as their mRNA levels increased following exposure to a variety of environmental stresses including cold, injury, water stress, and heavy metals (Sachetto-Martins et al. 2000). At least one GRP has been found in each higher plant species that has been examined. This finding suggests that these proteins are involved in essential plant functions, but not study to date has provided clear evidence of their functional roles. This leads us to hypothesize the possible involvement of these proteins in plant responses to changing environmental conditions.

The functions and importance of GRP in stress responses are speculative since the in vivo functional data collected so far remains limited. *MsGRP* is predicted to be the ortholog of one GRP gene (AT4G21620) in *Arabidopsis thaliana* due to their high amino acid sequence similarity. The function of AT4G21620 is currently unknown. In this paper, we demonstrate the subcellular location of *MsGRP* in the onion epidermis as well as its expression patterns under salt, ABA, and drought stresses. We also demonstrate its in vivo function in salt and ABA sensitivity by analyzing *MsGRP*-overexpressing transgenic *Arabidopsis* plants (35S::*MsGRP*). Our results suggest that *MsGRP* can negatively affect seed germination and seedling growth of transgenic *Arabidopsis* plants after salt and ABA treatments.

Materials and methods

Plant material and treatments

Medicago sativa L. cultivated variety Zhongmu-1 was gown in hydroponic culture of Hoagland's solution in a growth chamber at 25 °C under a 16 h light/8 h dark photoperiod and 75 % relative humidity. The solution was changed every 5 days. To investigate the expression pattern of *MsGRP* under different stresses, 30-day-old Zhongmu-1 seedlings were transferred into Hoagland's solution of 300 mM NaCl, 0.1 mM ABA, and 20 % PEG-6000. Shoot and root of the seedlings were harvested separately at different time points (0, 2, 4, 8, 12, and 24 h) and frozen in liquid nitrogen for further analysis. To investigate the expression pattern of *MsGRP* in different tissues, the leaf, stem, and root of 30 days old Zhongmu-1 seedlings grown in normal Hoagland's solution were harvested separately for further analysis.

Arabidopsis thaliana ecotype Col-0 was used in transgene experiment. *Arabidopsis thaliana* plants were grown at 22 °C under long-day conditions (16 h light/8 h dark). Seeds harvested from individual plants grown under identical environmental conditions were used for germination and seedling growth assays. Germination assays were carried out on three replicates of 80–100 seeds. Seeds were sown on MS medium supplemented with 3 % sucrose, and the plates were placed at 4 °C for 2 days in the dark prior to germination. To determine the effect of salt stress on germination, the medium was supplemented with 100, 200 mM NaCl. The effect of ABA on germination was measured on medium supplemented with 5 and 10 μM ABA. To determine the effect of salt stress on seedling growth, 10-day-old seedlings germinated in normal medium were transferred to medium supplemented with 200 mM NaCl, and the chlorophyll *a* and *b* content of the seedlings was measured after treating for 10 days. To test the effect of ABA stress on seedling growth, the seeds were

allowed to germinate under normal growth conditions and then transferred to medium supplemented with 10 μM ABA. And the length of the roots was measured at the indicated times after transfer to medium with ABA for 10 days.

RNA isolation and reverse transcription

Total RNA was extracted using Trizol (Invitrogen) and then dissolved in ddH$_2$O that was treated with diethylpyrocarbonate. Prior to the reverse transcription process, the total RNA was treated with RNase-free DNase I (MBI Fermentas) for 30 min at 37 °C. The cDNA was synthesized using PrimeScript reverse transcriptase (Takara). The harvested complementary cDNA was stored at −20 °C until use.

Rapid amplification of cDNA ends (RACE) of the *MsGRP* 3′ and 5′ ends

A SSH cDNA library of Zhongmu-1 alfalfa induced by salt was previously constructed and screened. An EST (GenBank accession No. FE896860) of a novel gene was isolated. RACE was performed to amplify its unknown 3′ and 5′ ends. Total RNA was used to synthesize 5′-RACE-Ready-cDNA and 3′-RACE-Ready-cDNA according to the manufacturer's recommendation of BD SMARTTM RACE cDNA amplification kit (Clontech). Based on the EST sequence already obtained, we designed gene-specific primers P1 (5′-CGGAACGGCTGAATGAGGAGAAA-CACT-3′) to amplify the 5′-cDNA end, and P2 (5′-CAAATGGAGGACACTCTAAAAATGGCA-3′) to amplify the 3′-cDNA end. The polymerase chain reaction (PCR) conditions were as follows: pre-denaturalization at 94 °C for 5 min, 30 cycles of thermocycling at 94 °C for 30 s, 68 °C for 1 min, and an additional polymerization step at 72 °C for 10 min. The PCR products were detected using electrophoresis on a 1 % agarose gel stained with ethidium bromide and then purified using the DNA gel extraction kit (Takara). The products were cloned into the pMD-19T vector (Takara) and then transformed into *E. coli* DH5α. And recombinant plasmids were sequenced by Beijing Genome Institute.

We obtained the full-length cDNA sequence of *MsGRP* by comparing and aligning the sequences of the known EST, 5′RACE, and 3′RACE sequences. *MsGRP* and its putative encoding protein was subsequently analyzed for molecular characterization purposes such as the conserved motifs, sequence homology, secondary structure, hydropathicity analysis, phylogenetic analysis, subcellular localization, and expression patterns under different environmental stresses.

Bioinformatics analysis

The secondary structure of the putative protein was analyzed using the HNN SECONDARY STRUCTURE PREDICTION METHOD (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). The specifically functional sites of the putative protein were analyzed using the SMART (http://smart.embl-heidelberg.de/). Sequences homology analysis was performed against the nucleotide and protein database of GenBank using the BLAST (NCBI) program. The phylogenetic relationship between the amino acid sequences was analyzed using the ClustalX 2.0 and MEGA 5.0 programs using the neighbor joining method. A potential signal peptide cleavage site was identified using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). The pI/MW of the putative protein was predicted using the ExPaSy website (http://us.expasy.org/tools/pi-tool.htm).

Subcellular localization of MsGRP

To observe the subcellular localization of MsGRP, an MsGRP–GFP fusion was constructed. Primers P3 (5′-ctcgagATGAAAACTATGACCACCTTTCT-3′) and P4 (5′-gtcgacACAATAGGCACTGCACTTCTT-3′) were designed to amplify the *MsGRP*-coding sequence. The PCR product was cloned into a pMD19T vector. The recombinant vector pMD19T-*MsGRP* and transient expression vector pA7-*GFP* were digested by *Xho*I and *Sal*I restriction endonucleases. The digested pA7-*GFP* fragment and the *MsGRP* gene fragment were linked with T4-ligase to obtain the fusion construct pA7-*MsGRP-GFP*. *MsGRP-GFP* was driven by a 35S promoter. The pA7-*MsGRP-GFP* vector was transformed into onion inner epidermis using a particle gun (Bio-Rad) and visualized under laser scanning confocal microscopy (Nikon).

Real-time fluorescent quantitative PCR

The 30-day-old alfalfa seedlings were transferred into Hoagland's solution with salt (300 mM), ABA (0.1 mM), and PEG-6000 (20 %) for 0, 2, 4, 10, and 24 h. Seedlings grown on normal Hoagland's solution were used as controls. The roots and shoots of the seedlings were harvested. Total RNA of each was reverse transcribed, and the synthesized cDNA was used as a template in real-time PCR. The real-time fluorescent quantitative PCR was analyzed by ABI-7500 (ABI). Primers P5 (5′-CAAATGGAGGA-CACTCTAAAAATGGC-3′) and P6 (5′-GGAACGGCT-GAATGAGGAGAAACA-3′) were used for amplification of *MsGRP*. β-actin gene was used as housekeeping gene to normalize the target gene quantities. The amplified fragment

of *MsGRP* was 122 bp. P7 (5′-TGGGCTGCCACAGAA-CATTTGA-3′) and P8 (5′-GCTGTGGTTGCTTTTTTGG TGTCTC-3′) were used to amplify the actin gene. The amplified fragment of actin gene was 117 bp. The PCR annealing temperature of all primers was 60 °C.

Overexpression of *MsGRP* in *Arabidopsis*

The full-length coding region of *MsGRP* was cloned into the *Xba*I and *Bam*HI site of the pBI121 vector to create *pBI121-MsGRP* that over-expresses *MsGRP* and *GUS* (*uidA*) reporter gene under control of the *CaMV 35S* promoter (*35S::MsGRP*). Primers P9 (5′-tctagaATGAAAAC TATGACCACCTTTCT-3′) and P10 (5′-ggatccACAA-TAGGCACTGCACTTCTT-3′) were designed to amplify the coding sequence of *MsGRP*. The construction process of the overexpression vector *pBI121-MsGRP* was performed using the construction method of the subcellular localization vector above. The *pBI121-MsGRP* vector was transformed into *A. tumefaciens* strain GV3101. Transformation of *Arabidopsis* was performed by the vacuum infiltration method (Bechtold and Pelletier 1998) using *A. tumefaciens* GV3101. The seeds were harvested and plated on kanamycin (50 μg/mL) selection medium for identification of the transgenic plants. After further selection of the transgenic lines using a 3:1 segregation ratio, T_3 homozygous lines were used for the phenotypic investigation. *MsGRP* overexpression in the transgenic plants was verified using RT-PCR analysis. *GUS* activity was visualized in the leaf tissue as described previously (Jefferson et al. 1987). Chlorophyll *a* and *b* were measured after extraction in 80 % acetone (Lichtenthaler 1987).

Results

Cloning of the full-length *MsGRP* cDNA

In the 5′ RACE PCR reaction, a 486-bp fragment was obtained using primers P1 and UPM (contained in the BD SMARTTM RACE cDNA amplification kit). A 344-bp fragment was isolated using the 3′ RACE primers P2 and UPM. A full-length 631-bp sequence was obtained by assembling the known EST sequence with the 3′ and 5′ end sequences. The full length contained a 492-bp open reading frame (ORF) that encoded a protein containing 163 amino acids (26 % glycine, 10 % serine) (Fig. 1). The molecular weight of the putative protein was approximately 16.02 kDa, while the *pI* was 9.67. It was tentatively named *MsGRP* for its high glycine content. (Its nucleotide accession number is JQ340083 and the coded protein accession number is AFA45120.)


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1    TATGGCCGGGGATCATCTATCTTCACCTTCTTCTCACATCTCATAAGTATATTTTCCCTA
61   AACCAAAAAAAATGAAAACTATGACCACCTTTCTCTTGCTTACTACTCTATTGCTCATAG
1                  M  K  T  M  T  T  F  L  L  L  T  T  L  L  L  I
121  CTTCATCATCTTTGGCAACCCGACCCGTTTCATCCGGATCTTCTCCTTCTACTCAAGTCA
17    A  S  S  S  L  A  T  R  P  V  S  S  G  S  S  P  S  T  Q  V
181  AACACCCTAAGGACAAAAACAACAACCAAGGTGGTGGTATTCCAGCTGGTGGTGGTAGTA
37    K  H  P  K  D  K  N  N  Q  G  G  G  I  P  A  G  G  G  S
241  TTCCAGGTGTTGGTGGAATTCCAGGTGGCTATTTTGGACCTGGAGGAGGGTTTAACATTC
57    I  P  G  V  G  G  I  P  G  G  Y  F  G  P  G  G  G  F  N  I
301  CAGGATTTGGAAACGGGTTTGGTAATGGAATTGGAGGTGGATATGGATCCGGTTATGGAG
77    P  G  F  G  N  G  F  G  N  G  I  G  G  G  Y  G  S  G  Y  G
361  GTCCAAATGGAGGACACTCTAAAAATGGCATAGTAAGACCTACTGTTGTGTGTGCAAAGATA
97    G  P  N  G  G  H  S  K  N  G  I  V  R  P  T  V  V  C  K  D
421  AAGGTCCATGTTACCAGAAAAAGTTAACATGTCCAGCTAAGTGTTTCTCCTCATTCAGCC
117   K  G  P  C  Y  Q  K  K  L  T  C  P  A  K  C  F  S  S  F  S
481  GTTCCGGTAAGGGCTATGGCGGTGGTGGCGGTGGTGGTGGCTGTACCATTGATTGCAAAA
137   R  S  G  K  G  Y  G  G  G  G  G  G  G  C  T  I  D  C  K
541  AGAAGTGCAGTGCCTATTGTTAAATGAAGTGCATGCATTAGTCATAAATATATGGTAACA
157   K  K  C  S  A  Y  C  *
601  GTTGTTGTTGTTTAAAAAAAAAAAAAAAAAAAA
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Fig. 1 Nucleotide and putative encoding amino acid sequence of *MsGRP*. The *underlined* amino acids indicate the glycine-rich region. The *italic amino acids* indicate the putative SCOP domain (d1a0tp) in some membrane and cell surface proteins and peptides (porins)

Analysis result of the putative MsGRP protein

The secondary structure of the MsGRP protein was predicted to contain 11.6 % alpha helix, 6.13 % extended strand, and 82.21 % random coil using the hierarchical neural network secondary structure prediction method. According to the prediction results obtained by SMART, there was a Structural Classification of Proteins (SCOP) domain of *d1a0tp* from Lys^{40} to Ser^{103}, which acts as a transmembrane domain in the membrane and in cell surface proteins and peptides. There was a glycine-rich region from Gly^{46} to Gly^{150} (Fig. 1). A signal peptide was predicted to be present in MsGRP by SignalP 4.0. Based on the amino acid sequence, a phylogenetic tree was constructed by using the neighbor joining method. The results showed that *MsGRP* was predicted to be ortholog of some GRPs in some other plants, such as NP_001190788 (AT4G21620) of *A. thaliana* (Fig. 2).

Subcellular localization of MsGRP

The online ProtComp version 9.0 program (http://www.softberry.com) was used to predict the subcellular localization. The result predicted by the integral prediction method showed that MsGRP is most likely localized in the extracellular space. (The prediction score was approximately 8.68.) To observe the subcellular localization of MsGRP, the MsGRP-GFP fusion was transformed into onion inner epidermis using a particle gun. In cells where the *pA7-GFP* vector was introduced alone (Fig. 3), fluorescence was visualized throughout those cells with no clear preference for localization. However, in cells expressing the MsGRP-GFP fusion, fluorescence was found to be clearly localized near the plasmalemma or cell wall (Fig. 3).
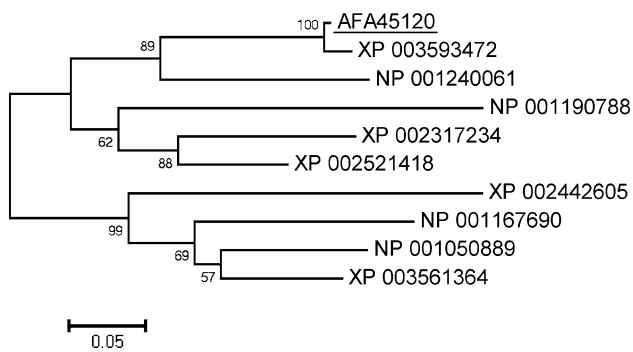
Fig. 2 Phylogenetic tree analysis of MsGRP and other proteins. The phylogenetic tree was constructed using the neighbor-joining method and employed amino acid sequences. The amino acid sequences were obtained from NCBI with the following accession numbers: AFA45120 from *Medicago sativa* (*MsGRP*), XP_003593472 from *Medicago truncatula*, NP_001167690 from *Zea mays*, NP_001190788 from *Arabidopsis thaliana*, NP_001050889 from *Oryza sativa*, XP_002317234 from *Populus trichocarpa*, NP_001240061 from *Glycine max*, XP_002521418 from *Ricinus communis*, XP_002442605 from *Sorghum bicolor*, and XP_003561364 from *Brachypodium distachyon*. Tree reliability was verified by bootstrap analysis with 1,000 replicates

Expression of *MsGRP* in different environmental stresses

A real-time PCR analysis was carried out to investigate *MsGRP* gene expression in alfalfa under different environmental stresses. The alfalfa seedlings were treated with 300 mM NaCl, 0.1 mM ABA, and 20 % PEG-6000 for 2, 4, 10, and 24 h. Seedlings grown on Hoagland's solution without treatment were used as controls. The relative expression of *MsGRP* was calculated as follows: ratio $= 2^{-\Delta\Delta C_t} = 2^{-[C_{t,t}-C_{t,r}]}$ (C_t cycle threshold, $C_{t,t}$ C_t of the target gene, $C_{t,r}$ C_t of the control gene β-actin). The

expression levels of *MsGRP* in different alfalfa tissues were also detected using real-time PCR.

The expression level of *MsGRP* in different time periods after 300 mM NaCl treatment is showed in Fig. 4c. Compared with control (0 h), the transcripts of *MsGRP* in the roots and shoots increased significantly after 4-h treatment, while the *MsGRP* expression level in the roots increased more significantly than that in the shoots (Fig. 4c). After treatment with 0.1 mM ABA, the relative expression level of *MsGRP* in the roots began to increase significantly after 4-h treatment, while the *MsGRP* mRNA level in the shoots increased obviously after 2-h treatment (Fig. 4b). After 2-h treatment with 20 % PEG, the relative expression level of *MsGRP* mRNA increased significantly. At 10 h, the expression level of *MsGRP* in the roots reached its maximum, while the expression level of *MsGRP* in the shoots reached its maximum at 4 h (Fig. 4a). Of the three detected tissues (root, stem, and leaf) without any stress, the transcripts of *MsGRP* in the leaf were the highest (Fig. 4d).

Analysis of the transgenic *Arabidopsis* plants

To investigate the functional role of the *MsGRP* gene, a complete ORF (Fig. 1) was overexpressed in *Arabidopsis* plants using *Agrobacterium*-mediated transformation. Preliminary screening of kanamycin-resistant putative *Arabidopsis* transformants was performed using a GUS assay (Fig. 5). The presence of *MsGRP* was confirmed in the GUS-positive plants by PCR using primers P9 and P10 (data not shown). Sixteen independently transformed T_0 transgenic plants were selected and grown to maturity to obtain homozygous lines. Two T_3 homozygous lines, *G1* and *G5*, were randomly selected and used throughout the study.
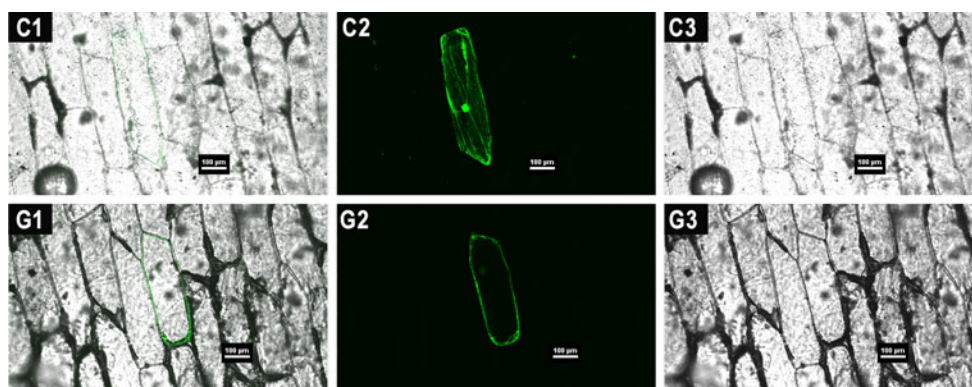

Fig. 3 Subcellular localization of MsGRP in the onion inner epidermis. The pA7-*MsGRP-GFP* and pA7-GFP vectors were transformed into onion epidermal cells using a particle gun. The fluorescence signals were examined using confocal laser scanning microscopy. The GFP fluorescence from cells expressing MsGRP-GFP fusion protein was localized near the plasmalemma (G1–G3). The GFP fluorescence was distributed throughout the cells expressing pA7-GFP vector (C1–C3). The photographs were taken in superposition of bright and dark vision (G1 and C1), dark field vision (G2 and C2), and bright light vision (G3 and C3)

Fig. 4 Relative expression levels of *MsGRP* in Zhongmu-1 alfalfa roots and shoots under different stresses. **a–c** The relative expression levels of *MsGRP* in Zhongmu-1 alfalfa treated with 20 % PEG-6000, 0.1 mM abscisic acid, or 300 mM NaCl. The *y*-axis indicates the relative fold increased expression level compared to 0 h. **d** The expression level of *MsGRP* in Zhongmu-1 alfalfa roots, leaves, and stems under normal conditions. The *y*-axis indicates the relative fold increased expression level in the root
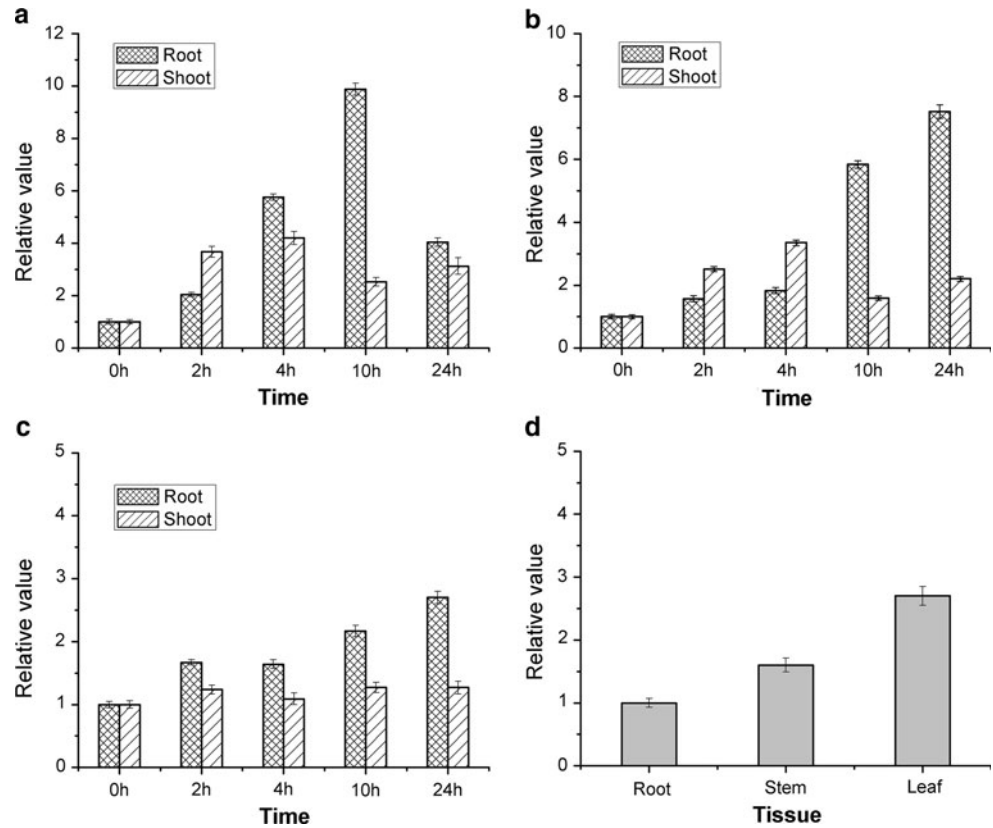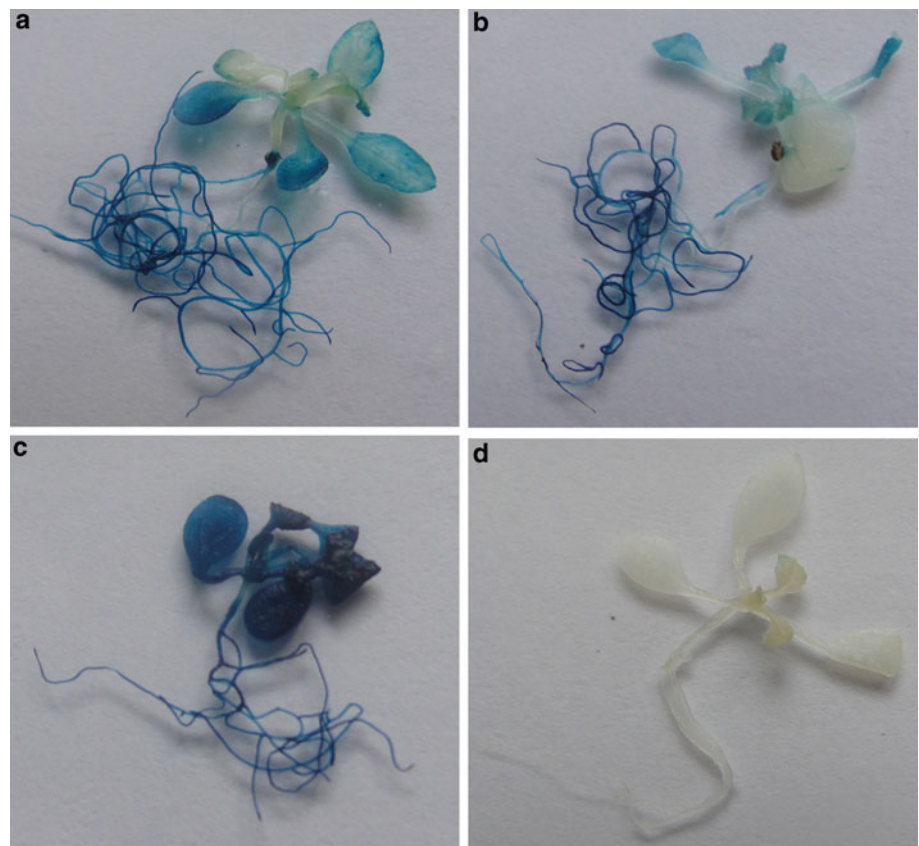

Fig. 5 GUS assay of wild-type *Arabidopsis*, G1 and G5 *Arabidopsis* transformants. **a**, **b** GUS staining of transgenic line G1 and G5. **c** GUS staining of *35S::GUS* transformant performed as a positive control. **d** GUS staining of wild-type *Arabidopsis* plant performed as a negative control

The wild-type and *35S::MsGRP* plants were compared for their germination and seedling growth on medium with NaCl and ABA concentrations. The germination rates of the transgenic plant and wild-type seeds were not significantly different in normal MS medium (Fig. 6). And all the germination retarded by a few days in NaCl or ABA MS medium compared with that in normal MS medium. In NaCl or ABA medium, the germination rate of the transgenic plant seeds was lower than that of the wild-type (Figs. 7a, b, 8a, b). The inhibition of transgenic plant germination was more significant as the concentration of NaCl or ABA increased. In the MS medium with ABA, >90 % of the wild-type seeds germinated by day 10. The germination rates of G1 and G5 were approximately 70 % in 5 μM ABA and 50 % in 10 μM ABA (Fig. 6a). In the MS medium with 100 mM NaCl, the germination rates of the transgenic plants and wild-type seeds were >90 % at day 10, while the germination rate of the wild-type was a little higher than those in lines G1 and G5 (Fig. 6b). The germination rate of the wild-type was significantly higher than that of the transgenic plants in 200 mM NaCl stress from day 5 (Fig. 6b). These results clearly indicate that *MsGRP* retarded seed germination under salt and ABA conditions.

The growth influences of salt and ABA conditions were also tested for the wild-type plant and *35S::MsGRP* plants. Seeds of wild-type and *35S::MsGRP* plants were allowed to fully germinate on normal MS medium, and 10-day-old seedlings were transferred to MS medium containing 200 mM NaCl or 10 μM ABA. The two *35S::MsGRP* lines were observed to be more sensitive to NaCl and ABA than the wild-type plants (Figs. 7c, d, 8c, d). The seedlings grown in 10 μM ABA or 200 mM NaCl were monitored by measuring the content of chlorophyll (Fig. 9). The chlorophyll contents of the transgenic lines were similar with the content of the wild-type plant under normal conditions, but the chlorophyll contents of the transgenic lines were significantly lower than those of the wild-type plants under 10 μM ABA or 200 mM NaCl treatment. These results indicate that *MsGRP* overexpression retarded seed germination of *Arabidopsis* plants and retarded the seedling growth under salt or ABA treatments.

Discussion

We are interested in studying the molecular mechanism of salt stress on plant gene expression. To begin this work, we isolated a cDNA that is inducible in alfalfa (*M. sativa* L. cv. Zhongmu-1) tissues by salt stress. This cDNA was predicted to encode a glycine-rich protein (*MsGRP*). The subcellular localization analysis experiment showed that the MsGRP protein is localized near the membrane, and we had predicted
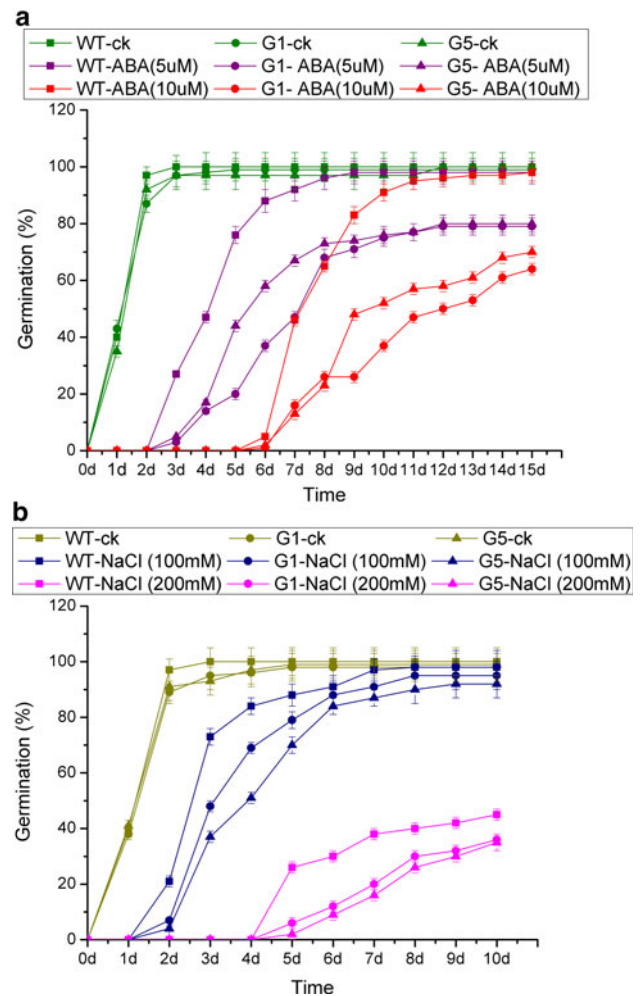

Fig. 6 Germination rates of G1, G5 and wild-type *Arabidopsis* seeds. **a** The seed germination rates in 5 and 10 μM abscisic acid MS medium. **b** The seed germination rates in 100 and 200 mM NaCl MS medium. The seed germination rates in normal MS medium as a control (ck)

it to be a porin on the membrane or an extracellular structural component of the cell wall. The GRP group members have been found in the cell walls of many higher plants and form a third group of structural protein components of the wall in addition to extensins and proline-rich proteins. Each cell wall GRP is localized mainly in the vascular tissue of the plant. For instance, the major cell wall GRP of the French bean is localized at the ultrastructural level in the modified primary cell wall of protoxylem. Immunological studies have shown that these proteins form a major part of these highly extensible and specialized cell walls (Ringli et al. 2001). Each GRP is characterized by its high glycine residue content. However, a GRP is not necessarily a structural protein, as RNA-binding proteins also have glycine-rich domains (Burd and Dreyfuss 1994; Kim et al. 2008; Sachetto-Martins et al. 2000). Protein structure and domain analysis results suggest that MsGRP contains a glycine-rich

Fig. 7 Germination and seedling growth of G1, G5, and wild-type *Arabidopsis* under NaCl stress. **a**, **b** The germination and seedling growth after sowing in 100 mM NaCl MS medium for 20 days. **c**, **d** Several 10-day-old seedlings germinated in normal MS medium were transferred to 200 mM NaCl medium for 10 days
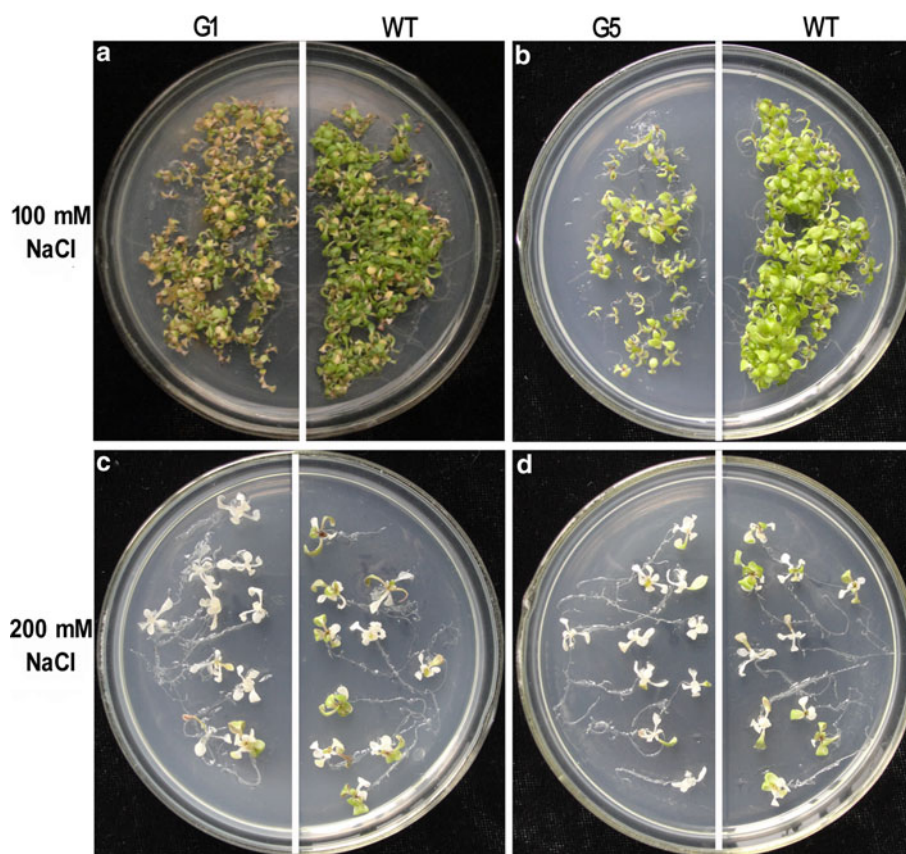
domain and a SCOP domain (SCOP is a basic domain of some membrane and cell surface porins) (Fig. 1) but does not contain any RNA-binding motifs. MsGRP also contains a $(Gly-X)_n$ repetitive motif of some extracellular GRP proteins in the cell wall (Fig. 1).

GRP expression has been shown to be developmentally regulated in some cases. For example, the *grp1.8* and *grp1.8*-like gene(s) are mainly expressed in young tissue, whereas the expression in older stages of development is strongly reduced (Condit 1993; Ye et al. 1991). In addition to developmental regulation, *grp* gene expression has been shown to be altered by external influences such as injury, hormone treatment, low temperatures, and water or ozone stress (Bergeron et al. 1994; Condit 1993; de Oliveira et al. 1990; Keller et al. 1988; Laberge et al. 1993; Lei and Wu 1991; No et al. 1997; Showalter et al. 1992). In fact, some *grp* genes were cloned in differential hybridization experiments performed with the goal of isolating genes induced by drought, water, or low-temperature stress (Chang et al. 1996; Laberge et al. 1993; No et al. 1997). Thus, the GRP proteins are not only required in certain stages of development but are often also stress-induced. Transcriptional expression experiments using real-time PCR showed that *MsGRP* gene expression is not tissue-specific as shown by

the accumulation of its transcript in roots, leaves, and stems. It also showed that *MsGRP* mRNA was induced by NaCl and ABA as well as significantly by PEG 6000 (Fig. 4). These results suggest that *MsGRP* gene expression is dependent upon the water status of plants and that ABA may act as a signal in this response.

We have shown that *MsGRP* affects seed germination and seedling growth of transgenic *Arabidopsis* plants under salt stress and ABA. The germination rate of *35S::MsGRP Arabidopsis* seeds was restrained more obviously in salt and ABA stress conditions. The growth of *35S::MsGRP Arabidopsis* seedlings was more sensitive to NaCl and ABA stresses. Although the molecular mechanisms underlying the roles of *MsGRP* under stress conditions are not clearly understood, the induction by salt and ABA as well as the overexpressing transgenic *Arabidopsis* showing more sensitivity to ABA and salt suggest that *MsGRP* may play a critical role in salt stress regulation and may cause an anaphylactic response in plants after salt and ABA treatments. It is interesting that, despite being induced by salt, ABA and PEG, *MsGRP* plays a negative role in salt and ABA stresses since our results showed that the *35S::MsGRP Arabidopsis* became more sensitive than the wild-type under those stresses.

Fig. 8 Germination and seedling growth of G1, G5, and wild-type *Arabidopsis* in abscisic acid (ABA) stress. **a, b** The germination and seedling growth after sowing in 5 μM ABA medium for 20 days. **c, d** Several 10-day-old seedlings germinated in normal MS medium were transferred to 10 μM ABA medium for 10 days
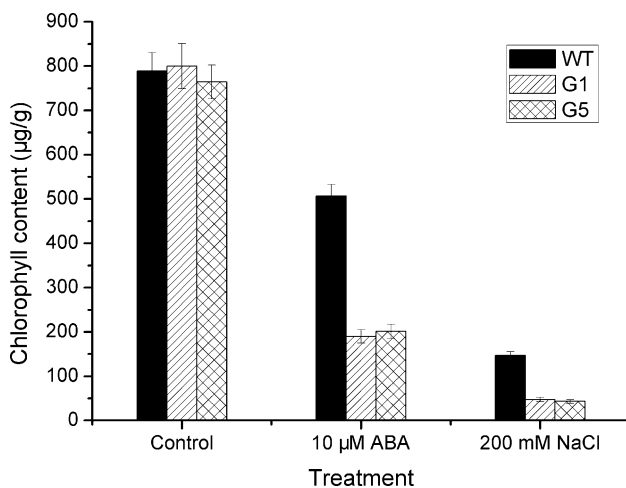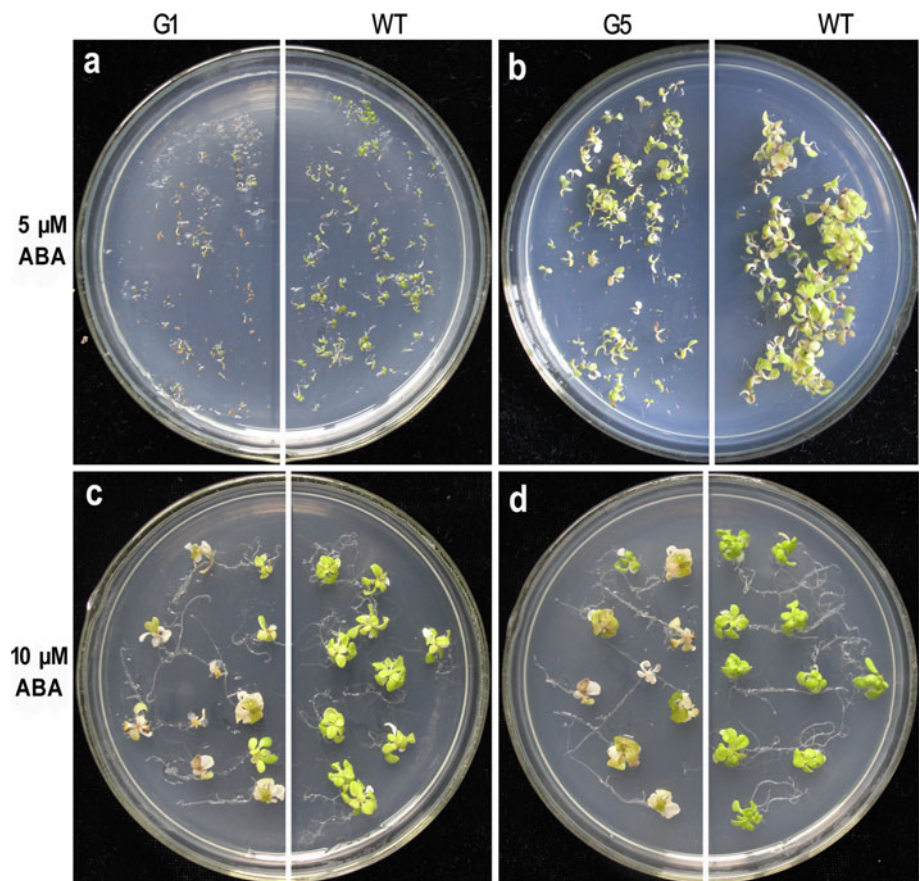

Fig. 9 Chlorophyll content of the leaves of wild-type, G1, and G5 plants. Several 10-day-old transgenic G1, G5, and wild-type plants were transferred to MS medium with 10 μM abscisic acid (ABA) or 200 mM NaCl, and the chlorophyll contents of these plants were measured after 10-day treatment. The *different letters* in *each column* indicate significant differences ($p < 0.05$) (Student's *t* test)

The function of *MsGRP* is similar to that of *Arabidopsis GRP7* reported previously (Kim et al. 2008). However, these two genes have many differences. *Arabidopsis GRP7*

is an RNA-binding protein in the nucleus, but MsGRP does not contain an RRM domain and probably localizes in the plasmalemma or cell wall. Based on the results of subcellular localization and bioinformatics analysis (Fig. 3), we predict that MsGRP probably plays a role in the porins on the membrane and participates in iron or water transport. Further studies are needed to investigate the molecular mechanism of MsGRP in the plant salt stress response. The knockout of *MsGRP* in alfalfa will be another way to investigate its function in future studies.

In conclusion, the present work provides novel information that increases our current knowledge of the functional roles of *GRP* in response to environmental stresses. The phenotypic analysis of *MsGRP*-overexpressing transgenic plants implies that *MsGRP* plays an important role on germination and seedling growth under salt and ABA conditions. The proposition that *MsGRP* negatively affects plant tolerance under salt and ABA stresses and has a negative impact on seed germination and seedling growth under salt and ABA stresses encourages further investigations into the cellular functions of *MsGRP* in stress responses. Since our knowledge regarding the cellular functions of the GRP family in stress responses remains far from sufficient, further studies might help us discover more of their functional roles in stress conditions.

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