Characterization of transgenic Arabidopsis plants overexpressing GR-RBP4 under high salinity, dehydration, or cold stress

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Abstract

A glycine-rich RNA-binding protein4 (GR-RBP4), one of the eight GR-RBP family members in Arabidopsis thaliana, was investigated for its stress-related expression, nuclear acid-binding property, and functional roles in plants subjected to various stresses including cold, high salinity, and dehydration. Real-time RT-PCR and GUS expression analyses showed that GR-RBP4 was abundantly expressed in young plants, root tips, and flowers, but weakly in mature leaves and stems, implying that GR-RBP4 is highly expressed in actively proliferating organs. The transcript level of GR-RBP4 increased markedly with cold stress, decreased significantly with salt stress, and decreased slightly with dehydration stress. In vitro nucleic acid-binding assays revealed that GR-RBP4 protein binds sequence non-specifically to RNAs and DNAs. Characterization of the transgenic Arabidopsis plants overexpressing GR-RBP4 under the control of the 35S promoter revealed that 35S::GR-RBP4 lines displayed retarded germination compared with the wild type under salt or dehydration stress. Despite the marked up-regulation of GR-RBP4 expression by cold stress, the 35S::GR-RBP4 lines did not show any noticeable changes in cold or freezing tolerance compared with wild-type plants. These results indicate that GR-RBP4 contributes differentially to altered germination and seedling growth of Arabidopsis plants under various stress conditions.

Key words: Glycine-rich RNA-binding protein, RNA-binding protein, stress, transgenic Arabidopsis thaliana plants.

Introduction

The regulation of gene expression in living organisms occurs at the post-transcriptional level as well as at the transcriptional level. Post-transcriptional gene regulation includes pre-mRNA splicing, capping, polyadenylation, mRNA transport, stability, and translation (Higgins, 1991; Simpson and Filipowicz, 1996). In these processes, regulation is mainly achieved either directly by RNA-binding proteins (RBPs) or indirectly, whereby RBPs modulate the function of other regulatory factors. How a protein recognizes a specific RNA site and promotes a specific RNA function are central problems in understanding many cellular processes. In recent years, considerable progress has been made in the discovery of RBPs that contain one or more RNA-recognition motifs (RRMs) at the N-terminus and a variety of auxiliary motifs at the C-terminus, such as glycine-rich, arginine-rich, acidic, SR-repeats, and RD-repeats (Kenan et al., 1991; Fukami-Kobayashi et al., 1993; Burd and Dreyfuss, 1994; Albà and Pagès, 1998). In contrast to the well-defined function of the RRM, functional roles of these auxiliary domains have only been established in some cases, and suggested to be involved in binding to nucleic acids and other protein factors (Kenan et al., 1991; Fukami-Kobayashi et al., 1993).

Proteins that contain RRMs at the N-terminus and a glycine-rich region at the C-terminus (glycine-rich RNA-binding proteins, GR-RBPs) have been described in plants, and their involvement in plant stress response has been indicated by several expression analyses. Since the first gene encoding GR-RBP was identified in maize (Gómez et al., 1988), cDNAs encoding homologous proteins have been found in various other plant species, including Arabidopsis thaliana (van Nocker and Vierstra,
Materials and methods

Plant materials and stress treatments

*A. thaliana* ecotype Col-0 was used throughout the study. Seeds were sown on a 2:1:1 by vol. mixture of vermiculite, peat moss, and perlite.

The pots were placed at 4 °C for 4 d in the dark and transferred to normal growth conditions. Plants were grown at 23 °C under long day conditions (16/8 h light/dark cycle). For cold treatment, the plants in the pots were placed at 4 °C for 1–6 d under dim light. For the dehydration treatment, the 4-week-old plants were placed on a filter paper for 1 h at room temperature to remove residual water completely, and then placed in a growth chamber at 23 °C. For salt or ABA treatment, the plants were placed in Petri dishes with their roots submerged into the solutions containing 250 mM NaCl or 100 µM ABA. The samples were collected at the time intervals indicated, frozen immediately into liquid nitrogen, and were used for RNA extraction and subsequent analysis. The entire experiments were repeated at least three times.

**Table 1. Gene-specific primer pairs used in the RT-PCR experiments**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>GR-RBP1</td>
<td>Forward: 5′-ATGCGTCCAAAGAGAGTGTATTGG-3′ Reverse: 5′-TCATTGCTCTCCAGCTCTCC-3′</td>
</tr>
<tr>
<td>GR-RBP2</td>
<td>Forward: 5′-GGAACTTCTTCTCTGAAATGCTTCTTG-3′ Reverse: 5′-GTCTCTTCTTACGACATCTTC-3′</td>
</tr>
<tr>
<td>GR-RBP3</td>
<td>Forward: 5′-GATGGCTTATAGCATGATGAGG-3′ Reverse: 5′-TCACCTCCTTTGGAACATCTTC-3′</td>
</tr>
<tr>
<td>GR-RBP4</td>
<td>Forward: 5′-GAATGCGTCTTCTGGAACACACG-3′ Reverse: 5′-TTCTGCTGCTTCAACCCACCGCC-3′</td>
</tr>
<tr>
<td>GR-RBP5</td>
<td>Forward: 5′-TCAAGCTGAAAGGTTGTAATCCTGCC-3′ Reverse: 5′-GAAACTCACATGCCACATCGTGT-3′</td>
</tr>
<tr>
<td>GR-RBP6</td>
<td>Forward: 5′-GGGCAATCTTTCATTAACTGAC-3′ Reverse: 5′-CCCTTGAATAAACCACCTTCTTACGGTC-3′</td>
</tr>
<tr>
<td>GR-RBP7</td>
<td>Forward: 5′-GAATTTGACATGCTTACGATC-3′ Reverse: 5′-AGGAAATTTACCCTCCTCACCAC-3′</td>
</tr>
<tr>
<td>GR-RBP8</td>
<td>Forward: 5′-AAATGCTCTGAGGGTGTGAGATCCTCAGTG-3′ Reverse: 5′-CAGACGACAAACACCTCATT-3′</td>
</tr>
<tr>
<td>RD29A</td>
<td>Forward: 5′-CGGAGGATATTCCATCATCGG GCCG-3′ Reverse: 5′-ACGAAGTATGGCGGCGATGTG-3′</td>
</tr>
<tr>
<td>RD29B</td>
<td>Forward: 5′-TCTCTGAGGCTTTCTTCCACCACAC-3′ Reverse: 5′-TGCGTTCCTCCAGCTCAGCC-3′</td>
</tr>
<tr>
<td>ACTIN</td>
<td>Forward: 5′-CAGCAGACCAGGGAATTTGAGAGA-3′ Reverse: 5′-TTCTTCTACGGTGTGCAACAGAC-3′</td>
</tr>
</tbody>
</table>
was monitored by a melting curve analysis of the PCR products as suggested by the manufacturer (Corbett Research, Australia). The PCR primer sets used for real-time RT-PCR are shown in Table 1. The real-time quantification of RNA target was performed in the Rotor-Gene 2000 real-time thermal cycling system by Corbett Research) using the QuantiTect SYBR Green RT-PCR kit (Qiagen) as previously described by Kim et al. (2003). The reaction mixture (25 µl) contained 200 ng of total RNA, 0.5 mM of each primer, and appropriate amounts of enzymes and fluorescent dyes as recommended by the manufacturer (Qiagen). For a control reaction, no RNA was added to the reaction mixture, resulting in no detectable fluorescence signal from the reaction. To determine the copy number of the GR-RBP4 gene present in the total RNA samples, the pGEM-T easy plasmid containing GR-RBP4 was used as a reference matrix. A range of five dilutions of the DNA was tested in the same conditions as the RNA samples. The number of the GR-RBP4 gene present in this dilution range was determined based on the size and the mass of the DNA, which can be used as a reference for the calculation of the copy number of the GR-RBP4 gene present in the total RNA sample.

**Vector construction and plant transformation**

To generate GR-RBP4 overexpression constructs, the coding region of GR-RBP4 cDNA was obtained by PCR, and was ligated into the pGEM-T easy vector (Promega). The vector was then digested using an XbaI/BamHI double digestion, and the resulting DNA was subcloned into the pBluescript KS(−) vector linearized by a double digestion with the same restriction enzymes. All DNA manipulations were according to standard procedures (Sambrook et al., 1989), and the GR-RBP4 coding region and the junction sequences were confirmed by DNA sequencing. Transformation of *Arabidopsis* was according to the vacuum infiltration method (Bechtold and Pelletier, 1998) using *Agrobacterium tumefaciens* GV3101. Seeds were harvested and plated on the selection medium containing kanamycin (50 µg ml−1) and carbenicillin (250 µg ml−1) to identify transgenic plants. After further selection of transgenic lines with a 3:1 segregation ratio, T0 or T1 homozygous lines were used for the phenotypic investigation.

**Germination and seedling growth under stress conditions**

Seeds harvested from individual plants grown in identical environmental conditions were used for the germination assays. Germination assays were carried out with three replicates of 40–50 seeds. Seeds were sown on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, and the plates were placed at 4 °C for 3 d in the dark and then transferred to normal growth conditions. To determine the effect of salt on germination, the medium was supplemented with 75, 100, 125, or 150 mM NaCl. To determine the effect of dehydration on germination, the medium was supplemented with 200 or 300 mM mannitol. To determine the effect of cold on germination, the seeds on MS plates were placed in an incubator maintained at 12 °C under white light. A seed was regarded as germinated when the radicle protruded through the seed coat. To determine the effect of salt or dehydration on seedling growth, seeds were fully germinated on normal MS medium, and the 4-d-old seedlings were transferred to medium supplemented with 75, 100, or 125 mM NaCl, which was supplemented with 200 or 300 mM mannitol, and seedling growth was monitored for 14 d. To test root growth under stress condition, the plates were placed vertically in a growth chamber and the root length was measured each day.

**Freezing tolerance and electrolyte leakage test**

Two-week-old seedlings of the wild-type and 35S::GR-RBP4 plants grown on MS agar plates were used for the freezing tolerance test. The plates were first placed at 4 °C for 1 d and then subjected to a series of temperature treatments; −1 °C for 1 d, −5 °C for 1 d, and −10 °C for 2–5 h under continuous light. After freezing shock, the plates were immediately placed at 4 °C for 1 d in the dark, and then into a growth chamber under normal conditions. The plants were examined for damage at the times indicated. The percentage of electrolyte leakage was measured to evaluate the degree of freezing injury in *A. thaliana*. Two leaves were cut from each plant and carefully placed in polypropylene centrifuge tubes. After incubation at various temperatures ranging from −5 to −10 °C for different time periods, 1.5 ml of distilled water was added to the tubes. The tubes were agitated gently for 3 h at room temperature, and the electrolyte content was measured by using a conductivity meter (Cole-Parmer Instrument Co.). The solutions and leaves were then autoclaved, and the electrolyte content was measured again. The ratio of electrolyte content before and after autoclaving was used as an indicator for membrane damage after the freezing treatment.

**Construction of GR-RBP4PRO::GUS expression vector**

For the isolation of the promoter for GR-RBP4 gene, two primers (forward; 5′-ATCGAGGGATGATTCGAGATG-3′ and reverse; 5′-GTCCTACACAAAAACTAAATC-3′) were synthesized that were designed to obtain a DNA fragment of 1.4 kb in size ranging from the initiation ATG codon to the upstream region that corresponds to the putative promoter for the GR-RBP4 gene. PCR was performed by using the Col-0 genomic DNA as a template. The amplified fragment was gel-purified and cloned into the pGEM-T easy vector. The vector was then digested with the HindIII/BamHI restriction enzymes and the fragment was ligated into the HindIII/BamHI digested pBluescript KS(−) vector (Clontech) to create the GR-RBP4PRO::GUS. The entire promoter sequence was confirmed by DNA sequencing.

**GUS staining in transgenic plants**

Seeds were sown on MS medium with kanamycin, and 4 d after germination, kanamycin-resistant seedlings were either stained for GUS expression or transferred to soil for further growth. Plant tissues were directly submerged into a solution containing 50 mM Na2HPO4, 30 mM NaH2PO4, 0.4 mM K3[Fe(CN)6], 0.4 mM K4[Fe(CN)6], 10 mM Na-EDTA, 0.1% Triton X-100, 5 ml MeOH, and 2 mM 5-bromo-4-chloro-3-indol-glucuronide cyclohexylamine salt. After incubation overnight at 37 °C, plant samples were washed by several changes of 70% ethanol and photographed by a digital camera under the microscope.

**In vitro nucleic acid binding assay**

The proteins used for the *in vitro* nucleic acid binding assay were synthesized by *in vitro* transcription and translation. The cDNA encoding GR-RBP4 was subcloned into the pET-22b (+) vector (Novagen). The *in vitro* transcription/translation/reaction performance was performed using the TnT® Quick Coupled Transcription/Translation System with T7 RNA polymerase (Promega). One microgram of DNA was mixed with the reaction mixture containing 40 µl TnT® Quick Master Mix, 2 µl [35S]methionine, and 6 µl nuclelease-free water. The reaction mixture was incubated at 30 °C for 90 min. Five microliters of the *in vitro*-synthesized protein was mixed with 5 µl of ribonucleopolymer-agarose beads or DNA-cellulose beads at a concentration of 1 mg ml−1 in 20 µl of binding buffer (10 mM TRIS-HCl, pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100, and 125–1000 mM NaCl) with 1 mg ml−1 heparin. The mixture was incubated on ice for 30 min, and the beads were washed three to four times to remove the unbound-proteins with the binding buffer containing 125–1000 mM NaCl (no heparin). After the last wash, the samples were dried, and resuspended by boiling in 30 µl of SDS loading buffer. The released proteins were separated by SDS-12% PAGE, and the relative intensities of the protein bands were quantified by a PhosphorImager (Fuji, Japan).
Results

Organ-specific expression of GR-RBP4

To examine the organ-specific expression of GR-RBP4 in *A. thaliana*, the DNA fragment of 1.4 kb in size including the initiation ATG codon to the upstream region that corresponds to the putative promoter for the GR-RBP4 gene was isolated and fused to the GUS reporter, and transgenic Arabidopsis plants expressing the GR-RBP4::GUS were generated. Histochemical analysis of the transgenic Arabidopsis plants (Fig. 1) showed that GUS activity varied depending on the developmental stage of the plants in an organ-specific manner. In 14-d-old seedlings, strong GUS activities were detected in the entire region of the plant. Strong GUS activities were also observed in the root tips. In 5-week-old mature plants, strong GUS activities were detected in flowers, flower buds, pedicels, and both ends of siliques. By contrast, only weak GUS expression was observed in mature leaves and stems (Fig. 1). To test the organ-specific expression of GR-RBP4 in *A. thaliana* further, the transcript levels of GR-RBP4 in different organs including stems, roots, leaves, flowers, and siliques were analysed by quantitative real-time RT-PCR. The transcript level of GR-RBP4 in different organs of Arabidopsis plants ranged from 200–1500 copies ng\(^{-1}\) total RNA, and it was most highly expressed in flowers compared with other organs (data not shown). These results indicate that GR-RBP4 is highly expressed in actively proliferating organs, yet the importance of these expression patterns needs to be verified by further analysis.

Regulation of GR-RBPs expression by various abiotic stress treatments

To test the effect of various abiotic stresses including cold, dehydration, and high salinity on the transcript level of GR-RBP family members, the Arabidopsis plants were subjected to the indicated stress, and the expression levels of GR-RBPs were investigated for a period of time. Since the expression of some of the GR-RBPs is influenced by circadian rhythm as well as stress, the expression level of GR-RBPs in the non-stressed control plants immersed in water was carefully measured at each time point. Figure 2 shows the histograms for the mean values and standard errors of triplicate experiments conducted with different RNA preparations. Among the abiotic stresses tested, cold treatment greatly increased the expression of most of the

![Fig. 1. Expression pattern of GR-RBP4 in different organs of A. thaliana. GUS expression in the transgenic Arabidopsis plants expressing the GR-RBP4::GUS was observed in (A) 14-d-old seedling, (B) roots, (C) root tip, (D) mature plant, (E) mature leaf, and (F) siliques.](https://academic.oup.com/jxb/article-abstract/56/421/3007/593491)
GR-RBP family members. Except GR-RBP5 and GR-RBP6 that showed about a 2-fold increase of expression by cold treatment, the transcript levels of other GR-RBPs increased 5–7-fold by cold treatment. The transcript level of GR-RBP4 investigated in this study increased up to 5-fold at 2 d after cold treatment. To test whether the elevated transcript level of GR-RBP4 upon cold treatment resulted from enhanced gene transcription or increased transcript stability, the GUS expression was measured in the GR-RBP4PRO::GUS plants subjected to cold stress. Results showed that much stronger GUS activity was observed in the plants receiving cold treatment (data not shown), indicating that the elevated transcript level of GR-RBP4 under cold stress resulted from the enhanced gene transcription.

Contrary to the large increase in the expression of most of the GR-RBPs by cold stress, the expression patterns of GR-RBPs by dehydration or salt stress differed from gene to gene. As shown in Fig. 2, only GR-RBP1 was up-regulated by either dehydration or salt stress. The expression of GR-RBPs 3, 5, 6, and 8 was not altered significantly by dehydration stress, and the transcript levels of GR-RBPs 2, 4, and 7 decreased marginally by dehydration stress. High salinity down-regulated the expression of GR-RBPs 3, 4, 6, and 7. High salinity or dehydration stress decreased the expression of GR-RBP4 down to a quarter or a third, respectively, of the non-stressed control (Fig. 2). No significant changes in the transcript level of Actin were observed, and the expressions of stress response markers RD29A and RD29B were markedly increased by cold, dehydration, or high salt stress, indicating that the experimental conditions and real-time RT-PCR analysis used in this study were valid to follow the changes in transcript levels in stress-treated samples.

In vitro nucleic acid-binding property of GR-RBP4
The bindings between the [35S]methionine-labelled GR-RBP4 protein and single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), or homoribopolymers (poly(A), poly(C), poly(G), and poly(U)) were tested at different NaCl concentrations. As shown in Fig. 3, the GR-RBP4 bound strongly to all DNAs and RNAs tested in the presence of 250 mM NaCl. These bindings were also observed even at high salt concentrations of 1.0 M NaCl, suggesting that the binding capability of GR-RBP4 to nucleic acids is strong. It is interesting to note that GR-RBP4 has high affinity to ssDNA and dsDNA as well as RNAs. To verify the specificity of this binding assay further, GR-RBP2 and GR-RBP7 as other members of the GR-RBP family, and luciferase as a negative control, were tested. GR-RBP2 bound most strongly to poly(U) as observed by Vermel et al. (2002), and GR-RBP7 showed higher affinity to poly(G), poly(U), and ssDNA as observed in many other GR-RBPs (Ludevid et al., 1992; Hirose et al., 1994). No binding was detected for the luciferase that contains neither RRM nor a glycine-rich motif. These observations support the reliability of the binding assay, and indicate that GR-RBP4 binds sequence non-specifically to RNAs and DNAs.

Salt response of 35S::GR-RBP4 transgenic Arabidopsis plants
Since the expression of GR-RBP4 was down-regulated by high salinity (Fig. 2), an investigation was made into the roles of GR-RBP4 on the germination and growth of A. thaliana under salt stress conditions. The transgenic Arabidopsis plants overexpressing GR-RBP4 under the control of the CaMV 35S promoter were generated and verified by RT-PCR analysis (Fig. 4A). Among the seven independent transgenic lines generated, the three transgenic lines, designated T-13, T-14, and T-20, were selected, and germination and seedling growth of 35S::GR-RBP4 plants were compared with those of the wild-type plants at various salt concentrations. No significant changes in germination rate and seedling growth were observed between wild-type and 35S::GR-RBP4 lines under normal growth conditions (Fig. 6A). No significant changes in germination rate were observed between wild-type and 35S::GR-RBP4 plants by abscisic acid treatment (data not shown). When the seeds of wild type and 35S::GR-RBP4 lines were germinated in the presence of 100 mM NaCl, about 85% of wild-type seeds germinated by the 3rd day, whereas only 50–70% of 35S::GR-RBP4 seeds had germinated by the 3rd day. All wild-type seeds had completed germination by the 5th day, whereas the germination rate of 35S::GR-RBP4 lines was only 80–85% by the 5th day (data not shown). This retardation of germination in 35S::GR-RBP4 lines was more severe at higher NaCl concentrations in that about 28% and 70% of wild-type seeds germinated by the 3rd and 6th days, respectively, whereas only 10–15% and 30–45%
of 35S::GR-RBP4 seeds germinated by the 3rd and 6th days, respectively, in the presence of 150 mM NaCl (Fig. 4B). These results indicate that GR-RBP4 affects in seed germination a negative way under high salinity stress.

The next aim was to test whether GR-RBP4 plays a role in the growth of Arabidopsis under high salt stress. Seeds of wild-type and 35S::GR-RBP4 lines were allowed to germinate fully in normal growth medium for 3 d, and the germinated seedlings were transferred to the medium supplemented with different NaCl concentrations. The survival rates of the seedlings on the MS medium supplemented with 200–300 mM NaCl were compared between the wild-type and 35S::GR-RBP4 lines. As shown in Fig. 4C, the survival rate of 35S::GR-RBP4 lines was lower than that of the wild type in the medium supplemented with 200 mM NaCl. At higher salt concentrations, similar patterns of survival rates were observed between wild-type and 35S::GR-RBP4 lines (data not shown). These results suggest that GR-RBP4 affects in a negative way the seedling growth under salt stress condition.

Dehydration response of 35S::GR-RBP4 transgenic Arabidopsis plants

With the observation that the expression of GR-RBP4 was down-regulated slightly by dehydration stress (Fig. 2), the next aim was to investigate the roles of GR-RBP4 on germination and growth of A. thaliana under osmotic stress condition. When the seeds of wild-type and 35S::GR-RBP4 lines were germinated in the presence of 200 mM mannitol, about 60% of wild-type seeds germinated by the 3rd day, whereas only 45–55% of 35S::GR-RBP4 seeds germinated by the 3rd day. About 90% of wild-type seeds germinated by the 6th day, but the germination rates of 35S::GR-RBP4 lines were 75–85% by the 6th day (Fig. 5A). This retardation of germination in 35S::GR-RBP4 lines was more...
Cold response of 35S::GR-RBP4 transgenic Arabidopsis plants

Since cold stress greatly increased the expression of GR-RBP4 (Fig. 2), it was expected that GR-RBP4 could contribute to the enhancement of germination or seedling growth of A. thaliana under cold stress. At the normal growth temperature of 23 °C, germination of wild-type and 35S::GR-RBP4 lines was initiated on the 1st day and was completed by the 2nd day (Fig. 6A). When the seeds of wild-type and 35S::GR-RBP4 lines were incubated at the low temperature of 12 °C, the wild-type and 35S::GR-RBP4 lines initiated germination on the 3rd day, and completed germination by the 5th day. However, no significant changes in germination rate and seedling growth were observed between the wild-type and 35S::GR-RBP4 lines at low temperature (Fig. 6A). The next test was to determine whether the overexpression of GR-RBP4 confers freezing tolerance in A. thaliana. When the 14-d-old seedlings of wild-type and 35S::GR-RBP4 lines were subjected to freezing shock at −5 °C to −10 °C for 2–7 h, the wild-type and 35S::GR-RBP4 plants either all survived or all died depending on the stress conditions, with no noticeable differences (data not shown). In the separate experiments with 4-week-old plants grown in the pots, all the wild-type and 35S::GR-RBP4 lines survived when subjected to freezing shock at −7 °C for 1 h, but all died when subjected to freezing shock at −7 °C for 2 h (data not shown).

Freezing tolerance of 35S::GR-RBP4 transgenic plants

Since it has been shown that GR-RBP7 overexpression influenced the expression of GR-RBP8 encoding a closely related RNA-binding protein (Staiger et al., 2003), the aim was to test whether GR-RBP4 overexpression affects the expression of other GR-RBP family members in 35S::GR-RBP4 transgenic plants. The transcript level of each GR-RBP member was measured by RT-PCR analysis in wild-type and 35S::GR-RBP4 plants. The position of the gene-specific primer set was chosen in such a way that no primer shared more than 70% identity with any other genes in the whole Arabidopsis genome sequence (Table 1). RT-PCR was performed by 20–25 cycles of amplification depending on the expression levels of each GR-RBP in the Arabidopsis plants. As shown in Fig. 7, it was evident that the transcript levels of GR-RBP4 in the 35S::GR-RBP4 lines were much higher than that

Effect of low or freezing temperatures on germination and growth of 35S::GR-RBP4 transgenic plants.

(A) Seeds of wild type (Col-0) and GR-RBP4-overexpressing lines (T-13, T-14, and T-20) were germinated at 23 °C or 12 °C, and the germination was scored at the indicated days. (B) Electrolyte leakages of wild type and 35S::GR-RBP4 transgenic plants were measured at −10 °C for the indicated time periods.

Expression of other GR-RBP family members in 35S::GR-RBP4 transgenic plants

Since it has been shown that GR-RBP7 overexpression influenced the expression of GR-RBP8 encoding a closely related RNA-binding protein (Staiger et al., 2003), the aim was to test whether GR-RBP4 overexpression affects the expression of other GR-RBP family members and the observed phenotypes during stress conditions resulted from the alterations in the expression level of other GR-RBP family members in the 35S::GR-RBP4 lines. The transcript level of each GR-RBP member was measured by RT-PCR analysis in wild-type and 35S::GR-RBP4 plants. The position of the gene-specific primer set was chosen in such a way that no primer shared more than 70% identity with any other genes in the whole Arabidopsis genome sequence (Table 1). RT-PCR was performed by 20–25 cycles of amplification depending on the expression levels of each GR-RBP in the Arabidopsis plants. As shown in Fig. 7, it was evident that the transcript levels of GR-RBP4 in the 35S::GR-RBP4 lines were much higher than that
Cold shock proteins (CSPs) in bacteria have been implicated to function as RNA chaperones that disrupt metabolism of RNA in actively proliferating organs. The down-regulation of GR-RBP4 expression by high salinity or dehydration stress, and the retardation of germination of the 35S::GR-RBP4 transgenic lines under salt or dehydration stress clearly demonstrate that GR-RBP4 affects in a negative way the germination of Arabidopsis under high salt or dehydration stress. At present it is not known how GR-RBP4 affects germination and seedling growth under salt or dehydration stress conditions. In this RT-PCR analyses of the RNA transcripts for SOS1 (At2g01980), SOS2 (At5g35410), SOS3 (At5g24270), proline dehydrogenase (At3g30775), glutathione reductase (At3g24170), and steroid sulphotransferase (At2g03760) that are known to play roles in germination and growth under high salt condition (Werner and Finkelstein, 1995; Quesada et al., 2000; Zhu, 2000), no differences in the RNA levels of these genes were observed between wild-type and transgenic plants (data not shown). Although GR-RBP4 did not influence the transcript levels of these stress-related genes, it may be possible that GR-RBP4 exerts its role by binding to target RNAs and ultimately regulating RNA processing and/or translation of target genes. It is likely that the negative effect of GR-RBP4 on the germination of Arabidopsis under salt or dehydration stress arose from the binding of GR-RBP4 directly to the target genes involved in the germination process, thus regulating the processing, stability, or translation of the target RNAs. It is also likely that GR-RBP4 binds to other protein factors that modulate the transcription of these target genes. This hypothesis can be tested by identifying the target RNAs by yeast three-hybrid screening and/or by determining the modulation of their protein products using two-dimensional PAGE and peptide mapping analysis under stress conditions.

The in vitro nucleic acid-binding assay revealed that GR-RBP4 interacts with ssDNA and dsDNA as well as RNA homopolymers with no sequence specificity (Fig. 3), which is in contrast to other reports showing that GR-RBPs had higher affinities to poly(G), poly(U), and ssDNA than poly(C), poly(A), and dsDNA (Ludevid et al., 1992; Hirose et al., 1994; Vermel et al., 2002). This discrepancy may result from the structural difference between GR-RBP4 and other GR-RBPs in that the C-terminus of GR-RBP4 is relatively short and contains only 12 glycine residues, whereas other GR-RBPs contain a much longer C-terminus rich in glycine residues. This glycine-rich domain has been suggested to be involved in binding to nucleic acids and other protein factors (Kenan et al., 1991; Fukami-Kobayashi et al., 1993), which contributes to the specificity of binding to target nucleic acids. Therefore, it is tempting to speculate that the lack of binding specificity of GR-RBP4 to RNAs and DNAs resulted from the absence of the long C-terminal region rich in glycine residues. However, the binding specificity of GR-RBP4 in the living cells may be different from that observed in vitro as the RNA-binding specificities of MA16 in maize depend on phosphorylation and interaction with other proteins (Freire and Pagès, 1995). Finding RNA targets in Arabidopsis plants and/or comparative analysis of protein patterns between wild-type and transgenic plants by two-dimensional PAGE and peptide mapping analysis would be a valuable way to identify any target genes and their protein products modulated by GR-RBP4, which could provide clues for better understanding the binding specificity and the functional roles of GR-RBP4 on germination and seedling growth under stress conditions.

Cold shock proteins (CSPs) in bacteria have been implicated to function as RNA chaperones that disrupt...
the over-stabilized secondary structures in mRNAs for an efficient translation at low temperatures (Jiang 
 et al., 1997; Graumann and Marahiel, 1998; Phadtare et al., 1999; Xia et al., 2001). Since cyanobacteria do 
not have CSPs found in other bacteria but contain RRM-type RBPs instead, it was hypothesized that 
RRM proteins may substitute the function of CSPs in cyanobacteria (Graumann and Marahiel, 1998, 
Maruyama et al., 2002). The functional role of plant GR-RBPs in cold acclimation processes has been 
suggested by the cold-induced expression patterns and the structural similarity to cyanobacterial RRM-type 
RBPs. Although experimentally not proven, it has been hypothesized (Vermel et al., 2002) that GR-RBP2 
could play roles as RNA chaperones similar to the bacterial CSPs. The present analysis of the 35S::GR-RBP4 
transgenic Arabidopsis plants revealed that GR-RBP4 does not contribute to enhance germination under 
cold stress or freezing tolerance in Arabidopsis plants (Fig. 6). This lack of a functional role of GR-RBP4 in 
the cold or freezing adaptation process may result from the unique structural features of GR-RBP4 
that, contrary to other GR-RBPs, contains relatively short (12 residues) C-terminus glycine residues that have 
been suggested to be involved in binding to nucleic acids and other protein factors (Kenan et al., 1991; Fukami-Kobayashi 
et al., 1993). At the time of writing is is not known whether other GR-RBP family members, that contain much longer 
C-terminus glycine-rich domains, have functional roles in the cold adaptation process. Analysis of other GR-RBP 
members would be important to understand better the roles of GR-RBPs under cold stress.

In conclusion, the present work has provided novel information to increase our knowledge about the involvement 
of GR-RBPs in response to environmental stresses. The complexity in the proposed function of GR-RBPs and the 
limited information on the biological roles of plant GR-RBPs are challenging aspects for further investigation. 
More analyses of the transgenic plants or knockout mutants of other GR-RBP family members would warrant better 
understanding of the functional roles of GR-RBPs in plants under various stress conditions.

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References


