Structural determinants crucial to the RNA chaperone activity of glycine-rich RNA-binding proteins 4 and 7 in Arabidopsis thaliana during the cold adaptation process

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Abstract

Although glycine-rich RNA-binding proteins (GRPs) have been determined to function as RNA chaperones during the cold adaptation process, the structural features relevant to this RNA chaperone activity remain largely unknown. To uncover which structural determinants are necessary for RNA chaperone activity of GRPs, the importance of the N-terminal RNA recognition motif (RRM) and the C-terminal glycine-rich domains of two Arabidopsis thaliana GRPs (AtGRP4 harbouring no RNA chaperone activity and AtGRP7 harbouring RNA chaperone activity) was assessed via domain swapping and mutation analyses. The results of domain swapping and deletion experiments showed that the domain sequences encompassing the N-terminal RRM of GRPs were found to be crucial to the ability to complement cold-sensitive Escherichia coli mutant cells under cold stress, RNA melting ability, and freezing tolerance ability in the grp7 loss-of-function Arabidopsis mutant. In particular, the N-terminal 24 amino acid extension of AtGRP4 impedes the RNA chaperone activity. Collectively, these results reveal that domain sequences and overall folding of GRPs governed by a specific modular arrangement of RRM and glycine-rich sequences are critical to the RNA chaperone activity of GRPs during the cold adaptation process in cells.

Key words: Arabidopsis thaliana, cold stress, glycine-rich RNA-binding protein, RNA-binding protein, RNA chaperone.

Introduction

It is increasingly evident that RNA chaperones play an important role during cold adaptation process in both prokaryotes and eukaryotes. It has been determined that the cold shock proteins (CSPs) in prokaryotes, harbouring only the cold shock domain (CSD) comprised of ~65–75 amino acid residues, are induced at high levels during the cold acclimation phase (Goldstein et al., 1990), and function as RNA chaperones which facilitate translation at low temperatures via the blockade of secondary structure formation in mRNAs (Jiang et al., 1997; Graumann and Marahiel, 1998). Plant cold shock domain proteins (CSDPs) differ from those detected in prokaryotes, as they harbour additional glycine-rich regions interspersed with CCHC-type zinc fingers in the C-terminal half. Plant CSDPs have been suggested to play similar roles to CSPs in prokaryotes during the cold acclimation process (Kingsley and Palis, 1994; Karlson et al., 2002; Karlson and Imai, 2003), and it has recently been determined that specific types of CSDPs function as RNA chaperones (Nakaminami et al., 2006; JS Kim et al., 2007), and confer cold tolerance in Arabidopsis thaliana (Kim et al., 2009; Park et al., 2009).

With the increasing numbers of reports demonstrating the role of CSDPs as RNA chaperones during the cold adaptation process, it is interesting to note that cyanobacteria lack CSPs, instead harbouring cold-induced glycine-rich RNA-binding proteins (GRPs) that contain one or more RNA recognition motifs (RRMs) at their N-terminus and a glycine-rich region at the C-terminal region. It was
thus hypothesized that the function of GRPs may substitute for the function of CSPs in cyanobacteria (Graumann and Marahiel, 1998; Maruyama et al., 1999). Plants harbour different types of RNA-binding proteins, and the roles of plant RNA-binding proteins in development, stress response, and genome organization have recently been reviewed (Lorković, 2009). A variety of GRPs have been identified in different plant species (Albà and Pagès, 1998; Sachetto-Martins et al., 2000), and it has been suggested that GRPs may perform a function during cold acclimation, as they were profoundly induced by cold temperatures (Sachetto-Martins et al., 2000, and references therein). AtGRP2 and AtGRP7, the two GRPs of eight GRP family members in *A. thaliana* (Lorković and Barta, 2002), have been implicated to perform functions in cold stress responses (Carpenter et al., 1994; Vermel et al., 2002). Therefore, it appears likely that plant GRPs perform a function as RNA chaperones, in a fashion similar to that of bacterial CSPs and/or plant CSDPs during cold stress. However, the functions and importance of GRPs in the cold stress response, and, in particular, their functions as RNA chaperones during the cold adaptation process largely remain to be verified.

Over the last several years, the functional roles of GRPs in *Arabidopsis* and rice (*Oryza sativa*) have been evaluated under stress conditions. *Arabidopsis* and rice GRPs which harbour an N-terminal RRM and C-terminal glycine-rich domains interspersed with CCHC-type zinc fingers were determined to perform a function as an RNA chaperone in the enhancement of cold and freezing tolerance in *Arabidopsis* plants (Kim et al., 2005; Kim and Kang, 2006; Kim et al., 2010a). In addition, GRPs from *Arabidopsis* and rice were shown to exert an impact on the seed germination, seedling growth, and stress tolerance of *Arabidopsis* plants under cold or freezing stress conditions (J. Y. Kim et al., 2007, 2010b). In particular, it was demonstrated that AtGRP7, but not AtGRP4, from *Arabidopsis* has an RNA chaperone activity during the cold adaptation process in *Escherichia coli* (JS Kim et al., 2007), and that GRP7 confers freezing tolerance in *Arabidopsis* (Kim et al., 2008).

As it has become increasingly clear that GRPs may function as RNA chaperones during the cold adaptation process in plants, it is important to understand what structural features are critical to their RNA chaperone activities. Considering that AtGRP7 has an RNA chaperone activity, but AtGRP4 has no RNA chaperone activity (J. S. Kim et al., 2007), it is necessary to understand whether or not the N-terminal or C-terminal regions are crucial to the RNA chaperone activity of GRPs. In an effort to answer these questions, domain-swapping experiments between the N- and C-terminal domains of GRPs were conducted. The recombinant proteins were then evaluated with regard to their complementation abilities in the cold-sensitive *E. coli* mutant BX04 under cold stress and their RNA melting activities. In this study, new evidence is provided that the domain sequences encompassing the N-terminal RRM of GRPs are crucial to the RNA chaperone activity in cells.

### Materials and methods

**Domain swapping, cold shock test, and transcription anti-termination assay in *E. coli***

To construct the chimeric version of the expression vector for AtGRP4 and AtGRP7, the cDNA harbouring the N-terminal region (from amino acid 1 to 122 of AtGRP4 and from amino acid 1 to 87 of AtGRP7) and the C-terminal region (from amino acid 123 to 136 of AtGRP4 and from amino acid 88 to 177 of AtGRP7) were cloned into pGEM-T vector (Promega). The chimeric construct 7R2+4R1 was constructed by replacing the ribonucleoprotein 1 (RNP1) of AtGRP7 (from amino acid 42 to 87) with the RNP1 of AtGRP4 (from amino acid 68 to 122), and the chimeric construct 4R2+7R1 was constructed by replacing the RNP2 of AtGRP7 (from amino acid 1 to 41) with the RNP2 of AtGRP4 (from amino acid 1 to 67). The pNIIII expression vectors harbouring either the native or chimeric GRPs were utilized in the cold shock test. *Escherichia coli* BX04 mutant cells (Xia et al., 2001) that lacked four CSPs and were confirmed to be highly sensitive to cold stress were obtained from Dr M. Inouye. The experimental procedures were conducted as described previously (J. S. Kim et al., 2007). In brief, the BX04 mutant cells transformed with each vector were grown in Luria–Bertani (LB) medium and, when the optical density at 600 nm reached ~1.0, the cell cultures were subjected to serial dilution, spotted in LB-agar plates containing 0.2 mM isopropyl-D-thiogalactopyranoside (IPTG), and were then incubated at low temperatures. For the transcription anti-termination assay, *E. coli* RL211 cells obtained from Dr R. Landick (Landick et al., 1990) were transformed with each construct, and the cell cultures were spotted on LB-carbenicillin plates with or without chloramphenicol. The growth of the cells was inspected daily.

**Recombinant protein purification and RNase cleavage assay***

The native and chimeric constructs were subcloned into the EcoRI/Xbal site of the pGEX-4T-3 vector (Amersham Pharmacia Biosciences). For the expression and purification of recombinant glutathione S-transferase (GST)–GRP fusion proteins in *E. coli*, the constructs were transformed into BL21 DE3 competent cells (Promega), and the recombinant proteins were purified with glutathione-Sepharose 4B resin. To prepare the RNA substrate, the parent unmodified pET-22(+) plasmid was cut with *Bam*HI and transcribed using T7 RNA polymerase, which results in a 175 nucleotide long RNA. The 32P-labelled RNA substrates were incubated with the purified GST–GRP fusion proteins in binding buffer [20 mM TRIS-HCl, pH 7.5, 2 mM EDTA, 4 mM KCl, 5% glycerol, 50 μg ml−1 bovine serum albumin (BSA)] for 15 min on ice. The reaction mixture was loaded onto an 8% acrylamide gel. All experimental conditions were maintained as previously described (Jiang et al., 1997; J. S. Kim et al., 2007).

**Seed germination and seedling growth assays under cold stress and freezing tolerance assay***

To generate transgenic *Arabidopsis* plants expressing each construct in the *grp7* mutant background, the chimeric constructs were cloned into the pCambia1301 vector to express each gene under the control of the cauliflower mosaic virus 35S promoter. The transformation of *Arabidopsis* was conducted via vacuum infiltration using *Agrobacterium tumefaciens* GV3101. Phenotypic investigation was carried out in the T1 transgenic lines in which the expression of each chimeric GRP in *grp7* mutant plants was analysed via reverse transcription-PCR (RT-PCR) using the genespecific primers. Seeds of the wild-type, *grp7* mutant, and GRP-expressing *grp7* plants were sown on Murashige and Skoog (MS) medium supplemented with 1.5% sucrose, and the plates were incubated for 3 d at 4°C in darkness. To determine the effects of cold stress on seed germination, the MS plates were placed in an
incubator maintained at 10 °C under white light. To assess the effects of cold stress on seedling growth, the seeds were first allowed to germinate under normal growth conditions, and then transferred to an incubator maintained at 10 °C under a 16 h light/8 h dark photocyte. The plates were then placed vertically in a growth chamber and the length of the roots was measured under stress conditions. For the freezing tolerance test, the 10-day-old wild-type, grp7 mutant plants, and GRP-expressing grp7 plants grown in MS medium were subjected to freezing shock at −5 °C for 1 h, immediately placed at 4 °C for 1 d in the dark, and then placed in a growth chamber under normal conditions. The plants were examined for damage at the indicated times.

Poly(A) mRNA in situ localization assay
Poly(A) mRNA in situ hybridization was conducted essentially as described (Gong et al., 2005; Kim et al., 2008). Briefly, the leaves of 2-week-old Arabidopsis plants were fixed in a fixation buffer [120 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4, 2.7 mM KCl, 0.1% Tween-20, 80 mM EGTA, 5% formaldehyde, 10% dimethyl sulphoxide (DMSO), and 50% heptane], and the leaf samples were hybridized in perfect Hyb Plus hybridization buffer (Sigma-Aldrich) containing 5 pmol of 45-mer oligo(dT) labelled with fluorescein at the 5′ end. After washing the samples, the leaves were immediately observed under an Olympus IX71 FV500 confocal laser-scanning microscope (Olympus America Inc.). All samples were observed under the same conditions, including the same laser strength and the same ×20 objective. Each experiment was repeated at least three times, and similar results were obtained.

Results
Complementation ability of the native and chimeric GRPs in a cold-sensitive E. coli mutant during cold shock
AtGRP7 has been implicated to function as an RNA chaperone, and confer cold tolerance to E. coli (J. S. Kim et al., 2007). To understand the importance of the structural motifs necessary for RNA chaperone activity during the cold adaptation process in E. coli, the native and chimeric GRPs were evaluated for their ability to complement defects in the growth of BX04 cells that lack four CSPs and are highly sensitive to cold stress (Xia et al., 2001). The chimeric construct 7N4C was constructed by joining the N-terminal amino acids (from 1 to 87) of AtGRP7 with the C-terminal amino acids (from 123 to 136) of AtGRP4, and the chimeric construct 4N7C was constructed by joining the N-terminal amino acids (from 1 to 122) of AtGRP4 with the C-terminal amino acids (from 88 to 177) of AtGRP7, as shown in Fig. 1A. The native and chimeric constructs, in addition to the E. coli CspA gene as a positive control, were inserted into a pInIII vector, and the colony-forming abilities of the BX04 cells transformed with these clones were assessed on LB plates at 17 °C in the presence of IPTG. The expression of each gene in BX04 cells was confirmed by RT-PCR analysis (data not shown) and western analysis (Supplementary Fig. S1 available at JXB online). When the BX04 cells harbouring each construct were incubated at 37 °C, all cells grew well with no noticeable differences (data not shown). CspA, which was utilized as a positive control, successfully complemented the cold-sensitive phenotype of BX04, as was observed in a previous report (Xia et al., 2001). AtGRP7 was capable of complementing the cold-sensitive phenotype of BX04, whereas the BX04 cells expressing AtGRP4 did not grow well when incubated at 17 °C (Fig. 1A; J. S. Kim et al., 2007). It was also observed that the 7N4C of GRP successfully complemented the cold-sensitive phenotype of BX04, whereas the 4N7C of GRP did not have this ability (Fig. 1A). These results demonstrate that the N-terminal domain of AtGRP4 and AtGRP7 determine the growth-stimulatory abilities.

RNA melting and RNA chaperone activities of the native and chimeric GRPs
To determine whether the chimeric 7N4C has RNA chaperone activity in vivo, the transcription anti-termination assay system developed by Landick et al. (1990) was
employed. An *E. coli* RL211 strain harbours a chloramphenicol resistance gene downstream from the trpL terminator, and functions as an efficient system by which the transcription anti-termination activity can be assessed (Bae *et al.*, 2000; Phadtare *et al.*, 2002; Nakaminami *et al.*, 2006). The same pINIII constructs employed for the low temperature complementation assay were transformed into RL211 cells, and the *in vivo* transcription anti-termination activity was evaluated. It was observed that RL211 cells expressing 7N4C as well as AtGRP7 grew on the growth media containing chloramphenicol, whereas RL211 cells harbouring 4N7C or AtGRP4 did not exhibit growth on chloramphenicol plates (Fig. 1B). CspA, which was employed as a positive control, evidenced transcription anti-termination activity, whereas the RL211 cells harbouring the pINIII vector did not grow on chloramphenicol plates. These results show that the chimeric 7N4C of GRP has an activity that is capable of melting the secondary structure of RNA *in vivo*, which further verifies that the N-terminal domain of GRPs is critical to the RNA chaperone activity.

As the chimeric 7N4C of GRP successfully complemented the cold-sensitive phenotype of the Csp-deficient mutant and also had RNA melting activity, it was speculated that the 7N4C of GRP may function as an RNA chaperone, as does AtGRP7 (J. S. Kim *et al.*, 2007). Whether the 7N4C of GRP facilitates the susceptibility of RNA to RNase T1 was further assessed via an RNase cleavage assay. The recombinant proteins of native and chimeric GRPs were purified from *E. coli* cultures (Supplementary Fig. S2 at *JXB* online), and the same RNA substrate utilized in a previous study was prepared via the transcription of the pET-22b(+) plasmid (J. S. Kim *et al.*, 2007). When RNase T1 was added to the RNA substrate and the mixture was incubated on ice for 15 min, several RNase T1-resistant bands appeared at lower positions, as indicated by arrows. However, when the GST–7N4C, in addition to the native GST–GRP7, was added prior to the addition of RNase T1, the RNase-resistant bands disappeared (Fig. 2). In contrast, no novel RNA substrate cleavage products appeared as the result of the addition of GST–4N7C or GST–GRP4 that do not show the ability to suppress the cold sensitivity of the BX04 cells (Fig. 2). These results suggest that the chimeric 7N4C destabilizes the secondary structures of the RNA molecules in order to allow RNase T1 to digest the RNAs further into smaller fragments, as does AtGRP7 (J. S. Kim *et al.*, 2007), further supporting the notion that the chimeric 7N4C operates as an RNA chaperone during the cold adaptation process.

**The importance of the N-terminal domain of GRPs for RNA chaperone activity**

Because it is evident that the N-terminal domain harbouring the RRM is critical to the RNA chaperone activity of GRPs, analysis the importance of domain sequences encompassing RNP1 and RNP2 at the N-terminal region of AtGRP4 and AtGRP7 was probed further by sequence motif swapping. When the RNP1 of AtGRP7 (from amino acid 42 to 87) was replaced by the RNP1 of AtGRP4 (from amino acid 68 to 122), the swapped construct (7R2+4R1) maintained growth-stimulatory activity at low temperatures, whereas the swapped construct harbouring the RNP2 of AtGRP4 (from amino acid 1 to 67) and RNP1 of AtGRP7 (from amino acid 42 to 87) (4R2+7R1) lost the ability to complement cold-sensitive BX04 cells (Fig. 3A). It was also observed that the 7R2+4R1 construct had transcription anti-termination activity, whereas the 4R2+7R1 construct had no transcription anti-termination activity (Fig. 3D). These results clearly show that the domain sequences encompassing the RNP1 and RNP2 of AtGRP4 and AtGRP7 are important for RNA chaperone activity.

With the observation that the chimeric construct harbouring the RNP2 of AtGRP4 and RNP1 of AtGRP7 lost the ability to complement cold-sensitive BX04 cells, it is of interest to determine which structural features of the domain sequences encompassing the RNP2 of AtGRP4 are relevant to the inhibitory effect of RNA chaperone activity. Because AtGRP4 harbours an additional 24 amino acids at the N-terminal region compared with AtGRP7 (Fig. 3B), it was reasoned that these additional amino acids of AtGRP4...
might impede RNA chaperone activity. As a step to address this, deletion analysis was performed. When the N-terminal 24 amino acids were deleted from AtGRP4, the mutant construct (GRP4 ΔN) gained the complementation ability (Fig. 3C) and transcription anti-termination activity (Fig. 3D). In contrast, when the N-terminal 24 amino acids of AtGRP4 were fused to the N-terminus of AtGRP7, the mutant construct (GRP7 +N) lost these activities (Fig. 3C, D). These results clearly show that the N-terminal domain sequence of AtGRP4 impedes RNA chaperone activity.

The N-terminal domain of GRPs is important to confer freezing tolerance in Arabidopsis

Since the chimeric 7N4C but not 4N7C complemented the cold-sensitive phenotype of E. coli mutant cells and possessed RNA chaperone activity, it is of interest to determine whether 7N4C is capable of conferring cold or freezing tolerance in plants. Here, the Arabidopsis grp7 mutant plant which showed decreased survival rates under freezing stress was used (Kim et al., 2008). As AtGRP7 was determined to have RNA chaperone activity (J. S. Kim et al., 2007), it was reasoned that 7N4C harbouring RNA chaperone activity should complement the freezing-sensitive phenotype of grp7 mutant plants. Multiple transgenic Arabidopsis grp7 plants expressing either 7N4C or 4N7C were generated, and the expression of chimeric constructs in two representative transgenic plants is shown in Supplementary Fig. S3 at JXB online. The wild-type, grp7 mutant, and 7N4C- or 4N7C-expressing plants were tested for their germination rates, root growth, and fresh weight under low temperatures, and for freezing tolerance. The experiments were repeated three times with 16 plantlets each, and similar results were obtained. The response of the transgenic and mutant plants to low temperature was examined at 10 °C, the temperature which was used to evaluate the cold stress response in Arabidopsis grp7 mutant plants (Kim et al., 2010a, b). The expression of cold stress-responsive marker genes, such as COR6.6, COR15a, COR47, LT129, and RD29A, was markedly up-regulated at 10 °C (data not shown), indicating that cold stress was adequately applied to the plants. When the seeds of the wild-type, grp7 mutant, and 7N4C- or 4N7C-expressing grp7 plants were germinated at low temperatures (10 °C), no noticeable differences in germination rates were observed between the genotypes (Fig. 4A). The growth of 7N4C-expressing grp7 plants as measured by root length and fresh weight at low temperatures was marginally better than that of wild-type, grp7 mutant, and 4N7C-expressing grp7 plants (Fig. 4B, C). These results show that 7N4C, but not 4N7C, positively affects the seedling growth of the plants under cold stress conditions. Whether the chimeric GRPs contribute to enhance freezing tolerance in Arabidopsis was evaluated next. Ten-day-old seedlings of the wild-type, grp7 mutant, and 4N7C-expressing grp7 plants (Fig. 4B, C). The growth of 7N4C-expressing grp7 plants as measured by root length and fresh weight at low temperatures was marginally better than that of wild-type, grp7 mutant, and 4N7C-expressing grp7 plants (Fig. 4D). In contrast, the survival rates of 4N7C-expressing plants were similar to those of grp7 plants (Fig. 4D). This experiment was repeated at least three times with the plants grown either in MS medium or in soil, and similar results were observed, although the survival rates differed among independent experiments. All of these results show that 7N4C-expressing plants are more tolerant to cold and freezing stress compared with grp7 mutant and 4N7C-expressing plants.

The importance of the N-terminal domain of GRPs for mRNA export during cold stress

It has been demonstrated that the mRNA export from the nucleus to the cytoplasm is impaired in grp7 mutant plants,
and one of the proposed roles of AtGRP7 under cold stress is to regulate mRNA export from the nucleus to the cytoplasm (Kim et al., 2008). To determine further whether the chimeric 7N4C GRP harbouring RNA chaperone activity can complement the defect in mRNA export of the grp7 mutant during cold stress, a poly(A) mRNA in situ hybridization assay was conducted. The results showed that the nuclei of the cells of the grp7 mutant plants grown under cold stress conditions exhibited strong fluorescence signals, indicating that mRNA export was impaired in the mutant cells (Fig. 5). In contrast, the grp7 mutant plants expressing 7N4C did not show any noticeable fluorescence signals (Fig. 5), indicating that mRNAs were efficiently exported to the cytoplasm. In comparison, the grp7 mutant plants expressing 4N7C exhibited strong fluorescence signals in the nuclei, indicating that mRNA export was impaired in the cells (Fig. 5). The strong fluorescent signals in the nuclei of grp7 mutant and 4N7C-expressing plant cells were detected only when the plants were subjected to cold stress, and no fluorescent signals were detected in the nuclei of the cells when the plants were grown under normal growth temperatures (data not shown). These results show that 7N4C but not 4N7C complements the defect in mRNA export from the nucleus to the cytoplasm in the grp7 mutant during cold stress.

**Discussion**

The potential roles of GRPs as RNA chaperones have been suggested owing to the fact that GRPs harbour structural features similar to those of the RRM proteins found in cyanobacteria, in which RRM proteins may substitute for the function of CSD proteins (Graumann and Marahiel, 1998; Maruyama et al., 1999). A previous report showed that AtGRP7, but not AtGRP4, successfully complements the cold-sensitive phenotype of the Csp-deficient mutant, has RNA melting activity, enhances RNase cleavage activity, and confers cold and freezing tolerance in Arabidopsis, thereby suggesting that AtGRP7 performs an RNA chaperone function (Kwak et al., 2005; J. S. Kim et al., 2007, 2008; J. Y. Kim et al., 2007). Although these works provide clear evidence that AtGRP7 but not AtGRP4 has RNA chaperone activity in E. coli and plants, the structural features of AtGRP7 relevant to RNA chaperone activity have not been determined. Because AtGRP4 harbours a glycine-rich region shorter than those of AtGRP7, it was initially thought that the size of the glycine-rich region in the C-terminus is crucial for the RNA chaperone activity of GRPs. However, the present findings clearly show that the domain sequences of the N-terminal region of AtGRP4 and AtGRP7 are critical to the RNA chaperone activity. It was
amino acid sequence adopts a highly disordered structure (Kim et al., 2007), and rice OsGRP 1, 4, and 6 harbour the glycine-rich regions of AtGRP4 and AtGRP7 adopt highly disordered structures (Supplementary Fig. 6), which makes it difficult to determine their precise three-dimensional structures. Although the structures between AtGRP4 and AtGRP7 cannot be compared directly with each other with this limited information, it is proposed that the N-terminal extension of AtGRP4 affects the overall structure of AtGRP4 and/or the additional peptide at the N-terminus of AtGRP4 interacts physically with the other parts of AtGRP4, and thereby impedes RNA chaperone activity. This hypothesis needs to be proved by more mutational and structural analyses.

In conclusion, the present study provides evidence that the domain sequences encompassing the RRM and the overall structure of GRPs are critical to the RNA chaperone activity of GRPs during the cold adaptation process in cells. Considering the importance of each domain and their arrangement in the RNA chaperone activity of GRPs, it is of critical interest to determine and compare the three-dimensional structures of the native and chimeric proteins. The currently limited information regarding the molecular mechanisms underlying GRP-mediated gene regulation demonstrated via domain swapping analysis that the domain sequences encompassing RNP2 in the N-terminal region of GRPs are crucial for its activity (Fig. 3). The amino acid sequences of the N-terminal region of AtGRP4 and AtGRP7 share high sequence homology with each other, and AtGRP4 contains additional amino acid residues in front of the domain sequence encompassing RNP2 compared with AtGRP7. To make sure that the N-terminal extension of AtGRP4 is naturally occurring and is not an alternatively spliced transcript, the sequence of the RNA transcripts corresponding to the N-terminal region of AtGRP4 was extensively analysed. Sequencing analysis of the RNA transcripts for AtGRP4 in Arabidopsis under both normal and cold stress conditions confirmed that every RNA transcript for AtGRP4 contains the N-terminal extension (data not shown). The findings that the recombinant AtGRP4 construct in which this additional region is deleted successfully complemented the cold sensitivity of the Csp-deficient mutant and had transcription anti-termination ability, whereas the recombinant AtGRP7 construct in which this additional region of AtGRP4 is inserted at the N-terminal end had no complementation and transcription anti-termination abilities (Fig. 3), clearly demonstrate that the amino acid sequences adjacent to RNP2 at the RRM are crucial to its activity. Although the current findings clearly show the inhibitory effect of the N-terminal extension of AtGRP4 on RNA chaperone activity, it should be noted that other GRP family members harbouring a similar N-terminal extension do exhibit different RNA chaperone activity. Arabidopsis AtGRP2 also contains the N-terminal extension but exhibits RNA chaperone activity (J. S. Kim et al., 2007), and rice OsGRP 1, 4, and 6 harbour the N-terminal extension but have RNA chaperone activity (Kim et al., 2010b). This suggests that the inhibitory effect of the N-terminal extension on RNA chaperone activity is not a common feature of all GRP family members.

Although there is no direct evidence to understand how the additional amino acid sequence at the N-terminal region of AtGRP4 hampers RNA chaperone activity, it is noteworthy that the disorder prediction program DISOPRED2 (http://bioinf.cs.ucl.ac.uk/disopred/) predicts that this amino acid sequence adopts a highly disordered structure (Supplementary Fig. S4 at JXB online). The structural disorder of RNA chaperones is considered to be important for RNA chaperone function (Tompa and Csermely, 2004; Rajkowitsch et al., 2007). Considering that the additional peptide in the N-terminal region of AtGRP4 has a cysteine residue at the fourth position (Fig. 3B), it was initially thought that this cysteine residue may form a disulphide bond with the cysteine residue at the 85th position within the RRM domain of AtGRP4, which results in a decrease in overall disorderness of the protein and concomitant lack of RNA chaperone activity. This hypothesis was tested by mutating the fourth cysteine amino acid to an alanine residue and measuring the complementation ability of the mutant protein in E. coli. However, the complementation ability of AtGRP4 was not recovered by this point mutation (data not shown), suggesting that the presence or absence of a potential disulphide bond in AtGRP4 is not important for RNA chaperone activity. Determination and comparison of the three-dimensional structures of RNA chaperones should provide a deeper insight into the importance of structural features crucial to RNA chaperone activity. However, due to the lack of stable three-dimensional folds for the proteins harbouring RNA chaperone activity, no high-resolution structures of most of the RNA chaperones are available at present. Given the limited feasibility of determining the three-dimensional structures of AtGRP4 and AtGRP7 due to the disordered nature of the glycine-rich regions of the proteins, an attempt was made, via a molecular modelling approach, to obtain some clues on the importance of additional N-terminal extension of AtGRP4 in determining RNA chaperone activity. A web-based structure prediction program CPHmodels 3.0 (http://www.cbs.dtu.dk/services/CPHmodels/) predicted that both AtGRP4 and AtGRP7 share highly similar structures at the RRM domain (Supplementar Fig. S5 at JXB online). In comparison, the N-terminal extension of AtGRP4 and the C-terminal glycine-rich regions of AtGRP4 and AtGRP7 adopt highly disordered structures (Supplementary Fig. S6), which makes it difficult to determine their precise three-dimensional structures. Although the structures between AtGRP4 and AtGRP7 are not a common feature of all GRP family members.

In conclusion, the present study provides evidence that the domain sequences encompassing the RRM and the overall structure of GRPs are critical to the RNA chaperone activity of GRPs during the cold adaptation process in cells. Considering the importance of each domain and their arrangement in the RNA chaperone activity of GRPs, it is of critical interest to determine and compare the three-dimensional structures of the native and chimeric proteins. The currently limited information regarding the molecular mechanisms underlying GRP-mediated gene regulation.
under cold stress should prompt further studies aimed at the identification of RNA targets and any protein factors interacting with GRPs during the cold adaptation process in plants as well as in bacteria.

Supplementary data
Supplementary data are available at JXB online.

Figure S1. Confirmation of the expression of native and chimeric GRPs in BX04 E. coli cells.

Figure S2. Purification of the recombinant proteins of the native and chimeric constructs.

Figure S3. Confirmation of 7N4C- or 4N7C-expressing grp7 mutant plants.

Figure S4. Structural disorder plot of AtGRP4 and AtGRP7.

Figure S5. Structural modelling of the conserved RRM domain of AtGRP4 and AtGRP7.

Figure S6. Structural modelling of the full-length AtGRP4 and AtGRP7.

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