Structural and Biochemical Analysis of the *Hordeum vulgare* L. *HvGR-RBP1* Protein, a Glycine-Rich RNA-Binding Protein Involved in the Regulation of Barley Plant Development and Stress Response

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**Supporting Information**

**ABSTRACT:** The timing of whole-plant senescence influences important agricultural traits such as yield and grain protein content. Post-transcriptional regulation by plant RNA-binding proteins is essential for proper control of gene expression, development, and stress responses. Here, we report the three-dimensional solution NMR structure and nucleic acid-binding properties of the barley glycine-rich RNA-binding protein *HvGR-RBP1*, whose transcript has been identified as being 45-fold up-regulated in early—as compared to late—senescing near-isogenic barley germplasm. NMR analysis reveals that *HvGR-RBP1* is a multidomain protein comprising a well-folded N-terminal RNA Recognition Motif (RRM) and a structurally disordered C-terminal glycine-rich domain. Chemical shift differences observed in 2D 1H−15N correlation (HSQC) NMR spectra of full-length *HvGR-RBP1* and N-*HvGR-RBP1* (RRM domain only) suggest that the two domains can interact both in-trans and intramolecularly, similar to what is observed in the tobacco *NtGR-RBP1* protein. Further, we show that the RRM domain of *HvGR-RBP1* binds single-stranded DNA nucleotide fragments containing the consensus nucleotide sequence 5′-TCTGGX-3′ with low micromolar affinity *in vitro*. We also demonstrate that the C-terminal glycine-rich (*HvGR*) domain of *HvGR-RBP1* can interact nonspecifically with ssRNA *in vitro*. Structural similarities with other plant glycine-rich RNA-binding proteins suggest that *HvGR-RBP1* may be multifunctional. Based on gene expression analysis following cold stress in barley and *E. coli* growth studies following cold shock treatment, we conclude that *HvGR-RBP1* functions in a manner similar to cold-shock proteins and harbors RNA chaperone activity. *HvGR-RBP1* is therefore not only involved in the regulation of barley development including senescence, but also functions in plant responses to environmental stress.

**S**enescence is the final stage in a monocarpic plant’s life cycle and is critical for plant reproduction, crop yield, and the grain protein content of its seeds. This highly coordinated and active molecular process involves the dismantling of leaf cellular components, degradation of cell constituents, and remobilization of nutrients, in particular, nitrogen, to developing seeds in annual plants, or stems and roots in perennial species.†‡

The timing of whole-plant and leaf senescence is genetically controlled. However, senescence can also be induced by a variety of environmental factors such as light intensity and duration, temperature, nutrient availability, pathogens, and other environmental stresses, indicating that cross-talk exists between biochemical signaling pathways modulating abiotic and biotic stress responses and those controlling developmentally regulated plant senescence. Despite the biochemical complexity of plant senescence, significant advances have been made in our understanding of critical components of signaling pathways that regulate leaf senescence. This knowledge has arisen by characterizing biochemical traits of plant lines and varieties varying in their senescence behavior and the function of senescence-associated genes. Despite these advances, it is clear, however, that more complete molecular characterizations of specific regulatory factors associated with the process of plant senescence are needed.

*Hordeum vulgare* L. (barley) is one of the most economically important cereal crops grown worldwide. Barley seeds are used for food, as a principal feed for livestock, or for malting for alcoholic beverage production. The timing of senescence strongly impacts barley yield and the grain protein content (GPC) of its seeds. Early senescing barley plants generate seeds that are high in GPC and more ideally suitable as feed, whereas late-senescent barley plants produce seeds with a lower GPC and higher carbohydrate content, characteristics that are preferred for malting. The characterization of senescence-associated gene products in cereals such as barley is critical to help us understand how senescence signaling pathways modulate GPC and affect crop yield and crop quality. Ultimately, data generated by fundamental biochemical studies...
of molecular processes critical to barley senescence will be useful to develop new barley varieties that have been modified with respect to senescence initiation and timing for optimal and specific agro-economic purposes.

Previously, RNA transcripts extracted from the topmost leaves (i.e., leaves growing directly below the barley ears) of a late-senescing barley variety, referred to as "Karl", were compared to those of a near-isogenic early senescing barley line, referred to as "10_11", at 14 and 21 days past anthesis (dpa) (i.e., past flowering). The two barley varieties "Karl" and "10_11" vary genetically in the alliecal state of a particular locus on chromosome 6H, which was shown to influence leaf senescence and grain protein content of these two barley lines.5−7 As anticipated, the earlier senescent "10_11" barley line displayed increased abundance of gene transcripts related to Senescence Associated Genes (SAGs) which code for proteins with putative or established roles in senescence, such as transcription factors, kinases, phosphatases, lipases, proteases, and nucleases. Interestingly, a transcript coding for a putative RNA-binding protein termed HvGR-RBP1 (GenBank JX126694) was identified as one of the most up-regulated gene transcripts (i.e., >45-fold) in the early senescing "10_11" barley line, when compared to its transcript level in the late senescing "Karl" variety.5

Examination of its amino acid sequence indicated that HvGR-RBP1 shares 65% protein sequence identity with the Arabidopsis thaliana RNA-binding protein AtGRP7 which has been implicated in the regulation of flowering, and has been shown to be up-regulated during Arabidopsis senescence.8,9 Specifically, Atgrp7 T-DNA mutant and RNAi interference Arabidopsis lines with reduced levels of AtGRP7 flowered late when compared to wild-type plants under both long- and short-day conditions, but this late-flowering phenotype was canceled by vernalization.8 Similarly, the late-senescing barley variety "Karl" with very low Hvgr-rbp1 expression flowered later than "10_11" (with high Hvgr-rbp1), and as in Arabidopsis, these differences disappeared when plants were vernalized.10,11 These observations thus suggest that strong functional similarities exist between HvGR-RBP1 and AtGRP7.

Further inspection of the amino acid sequence of HvGR-RBP1 suggests that the protein is composed of a highly conserved N-terminal RNA Recognition Motif (RRM) spanning residues 1−92, and a C-terminal glycine-rich region containing a series of (GGR or RGG) amino acid repeats, spanning residues 93−162 of full-length HvGR-RBP1. This protein thus appears to be a member of a large family of less well-characterized plant glycine-rich RNA-binding proteins (referred to as GR-RBPs), which are implicated in the regulation of diverse cellular processes including plant development, responses to environmental stress, and post-transcriptional gene regulation.12−19

While the structural architectures of several eukaryotic RRM-containing proteins have been reported,20−22 our structural and functional understanding of plant RRM and plant glycine-rich RNA-binding proteins is less well developed. To this date, the three-dimensional (3D) structures of only two plant-derived GR-RBPs have been reported,23,24 and none from barley. The present work is the first structural report of a barley GR-RBP protein.

In this work, we report structural and biochemical findings regarding barley HvGR-RBP1, including a detailed characterization of the 3D structure of its N-terminal RRM domain, and the nucleic acid binding properties of full-length HvGR-RBP1 and of its associated RRM and glycine-rich (GR) domains. Our structural studies indicate that HvGR-RBP1 is a bipartite protein, comprised of a well-folded RNA Recognition Motif (RRM) at its N-terminus, and a largely unstructured glycine-rich (GR) C-terminal domain.

Moreover, protein−protein interactions were detected using NMR chemical shift perturbation experiments between the RRM and GR domains of HvGR-RBP1, suggesting that at least in vitro, these two domains self-associate, results that are consistent with what has been observed for the Nicotiana tabacum NiGR-RBP1 protein.24 Nucleic acid interaction assays indicated that the GR domain of HvGR-RBP1 can bind to single-stranded RNA (ssRNA) in the absence of the RRM domain. As anticipated based on the HvGR-RBP1 sequence similarity to the Nicotiana tabacum NiGR-RBP1 protein and a recent report by Khan et al.,24 1H/15N chemical shift perturbation experiments established that the N-terminal RRM domain of HvGR-RBP1 can bind, albeit weakly, to a 6-nucleotide single-stranded DNA fragment containing the consensus sequence S′-TTCTG-3′, a DNA equivalent of the S′-UUCUGA-3′ RNA sequence previously identified as a key nucleotide binding motif for the Arabidopsis protein AtGRP7.25

Taken together, these results suggest a model of nucleotide binding for HvGR-RBP1 involving cooperative binding of single-stranded nucleic acid targets and the RRM and GR domains of HvGR-RBP1. In addition, our observations that expression levels of the barley Hvgr-rbp1 transcript are increased under cold stress, and that the protein enhances E. coli growth following cold-shock treatment, strongly suggest that HvGR-RBP1 harbors RNA chaperone activity in addition to its ability to bind single-stranded nucleic acids.

■ MATERIALS AND METHODS

Cloning, Protein Expression, and Purification. Plasmid constructs encoding amino acid residues 1−162 (FL-HvGR-RBP1), 1−137 (137-HvGR-RBP1), 1−112 (112-HvGR-RBP1), 93−162 (HvGR), and 1−92 (N-HvGR-RBP1) of HvGR-RBP1 were prepared similarly to that described in ref 26. The vector encoding the FL-HvGR-RBP1(F54A) protein variant was generated using the QuickChange Lightening (Agilent Technologies) site directed mutagenesis kit using pET46-FL-HvGR-RBP1 vector as the starting template. For pET32-C-HvGR-RBP1, DNA coding for amino acid residues 93−162 of HvGR-RBP1 was amplified using PCR from pET46-FL-HvGR-RBP1 and ligated into pET-32 Xa/LIC (Novagen) plasmid. The proteins expressed using the pET46 expression vectors resulted in protein constructs containing a 6-histidine tag for purification and added the following 14 residues MAHHHHHHHHDDDKK to the native sequences, as previously reported.26

Recombinant protein expression was carried out using Escherichia coli BL21(DE3) (Invitrogen) cells transformed with plasmids containing Hvgr-rbp1 constructs and grown at 37 °C in LB supplemented with 50 μg/mL ampicillin for 6 h, followed by induction of protein synthesis via addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) to cell cultures at an OD600 of 0.6. For production of uniformly 15N or 15N/13C labeled HvGR-RBP1 proteins, transformed BL21 cells were grown in M9 minimal media containing 15NH4Cl (1 g/L) and [13C6]-glucose (2g/L) (Cambridge Isotope Lab) as the sole sources of nitrogen and carbon, respectively. Following 10 h of cell growth post IPTG induction, bacterial cells were harvested by centrifugation, resuspended in 250 mM Na2HPO4, 1 M...
NaCl, 10 mM imidazole, 0.1 mM PMSF, pH 8 buffer, and lysed using an M-110L microfluidizer (Microfluidics). Cell debris were subsequently removed by centrifugation at 24 000 g for 20 min, and resulting supernatant applied to a nickel affinity column containing 3–5 mL bed volume of His Pur Ni-NTA resin (ThermoScientific), followed by protein elution using standard nickel affinity column wash and purification procedures.26 Protein-containing fractions were pooled and dialyzed twice against 1 L buffer solution containing 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 0.1% PMSF, at pH 6.8, and resulting HsGr-RBP1 protein was further purified by gel filtration using a Superdex 75 size exclusion column (1 × 30 cm, GE Life Sciences) pre-equilibrated in the same buffer. Final protein containing fractions were pooled and concentrated to 1 mM protein concentration as determined by Bradford (Bio-Rad) assay and OD280 nm absorbance readings. Similar purification protocols were used for all HsGr-RBP1 protein constructs of interest. For NMR analysis of 15N-labeled FL-HsGr-RBP1 which displayed significant aggregation properties at 1 mM, 2D 1H−15N correlation (HHSQC) NMR experiments were also performed on samples at different pH conditions (pH 6.2, 6.8, and 7.2), temperature (298 and 303 K), and more dilute sample concentrations (0.25 mM). Resulting spectra for FL-HsGr-RBP1 were compared to the 2D 1H−15N correlation NMR spectra acquired for 15N-labeled N-HsGr-RBP1 under the same conditions.

**NMR Spectroscopy.** Multidimensional NMR spectra of N-HsGr-RBP1 were acquired at 298 K (25 °C) in 50 mM NaH2PO4/Na2HPO4, 300 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.01% NaN3 pH 6.8, containing 10% (v/v) D2O or 100% D2O (for acquisition of 3D 13C-edited 1H−D2O or 100% D2O) for (acquisition of 3D 13C-edited 1H−1H-TOCSY, 1H−1H-NOESY, and 2D 1H−13C-CT-HSQC spectra) buffer using a 5 mm triple axis (1H, 15N, 13C) TCI cryogenically cooled probe (cryoprobe) and a Bruker AVANCE III 600-MHz (1H Larmor frequency) NMR spectrometer. NMR spectra were processed using NMRPipe28 and analyzed using the software program Sparky.28 Chemical shift assignments of backbone and side-chain atoms (1H, 13C, 15N) were obtained using the following double and triple resonance solution NMR experiments: 1H−13N-HSQC, 1H−13C-CT-HSQC, HNCA, HNCCACB, CBCA(CO)NH, CC(CO)NH, HBHA(CO)NH, H(CCOC)NH, HCHC(TOCSY), (HB)CB(CGGDCD)HE, and (HB)CB(CGGCD)HD, as reported in ref 26. 1H, 13C, and 15N chemical shifts were indirectly referenced to DSS. Dihedral angle (φ and ψ) restraints were obtained from chemical shift data using the program TALOS+.29 Interproton distance restraints were obtained from 3D 13N-edited and 13C-edited NOESY spectra using a combination of automated and manual assignment methods. Hydrogen-bond restraints were determined using the algorithm VADAR30 in combination with H2O/D2O solvent exchange experiments that were performed by first lyophilizing 500 μL of a 1 mM 15N-labeled N-HsGr-RBP1 protein sample, followed by resuspension of the lyophilized protein in the same volume of 100% D2O, and acquisition of a series of 2D 1H−15N-HSQC correlation spectra at subsequent time points. Additionally, a 2D-CLEANEX-PM NMR experiment31 was performed to detect amide protons participating in fast chemical exchange with solvent.

**NMR Structure Calculations.** 3D structure determination of N-HsGr-RBP1 was achieved using standard simulated annealing protocols as implemented in the program CYANA,32,33 and NMR chemical shifts, dihedral angles, and NOE-derived interproton distance restraints as experimental input constraints. From a set of 200 structures generated, 40 conformers with the lowest residual target function values were selected and subjected to further restraints minimization with AMBER93 using the AMBER 2003 force field, a generalized Born model, and a 10 Å TIP3P water box of the AMPS-NMR web portal.35 From these calculations, the 20 lowest conformational energy conformers most consistent with the experimental NMR data were chosen as representative conformers of the ensemble of N-HsGr-RBP1 protein structures. The stereochemistry and quality of these structures were analyzed using PROCHECK-NMR,36 MolProbity,37 Verify3D,38 and the protein structure validation suite (PSVS) software.39 Statistics resulting from the structural calculations and assessment of the structural quality of the calculated protein models are presented in Table 1. An ensemble of the 20 lowest energy conformers of N-HsGr-RBP1 has been deposited in the Protein Data Bank as PDB entry 2M PU, and BMRB code 18776 for the NMR structure validation.

**Table 1. Structural Statistics for the Ensemble of N-HsGr-RBP1 NMR Structures**

| Constraints for final structure calculations |  |
|---------------------------------------------|---|
| Total NOE distance restraints               | 683 |
| Intraresidue                                | 352 |
| Sequential (i−j−i−j)                        | 165 |
| Medium range (i−i−i−j)                      | 46 |
| Long range (i−i−j−j)                        | 120 |
| Dihedral angle restraints•                  |  |
| Φ angles                                    | 83 |
| Ψ angles                                    | 83 |
| Hydrogen bond distance restraints•          | 74 |
| Structure statistics (20 conformers)        |  |
| CYANA target function (Å²)                  | 0.58 |
| Residual distance violations                |  |
| Number >0.5 Å                               | 0 |
| Number >0.3 Å                               | 0 |
| Residual dihedral angle violations          |  |
| Number >5°                                  | 0 |
| AMBER mean restraint violation energy (kcal/mol) | 6.11 |
| Ramachandran plot statistics (%)•          |  |
| Residues in most favored regions            | 85.3 |
| Residues in additionally allowed regions    | 13.6 |
| Residues in generously allowed regions      | 1.2 |
| Residues in disallowed regions              | 0 |
| Average RMSD to mean structure (Å)•         |  |
| Protein backbone                            | 0.66 ± 0.14 |
| Protein heavy atoms                         | 1.36 ± 0.18 |

•Φ and Ψ angles were derived from the program TALOS+.29 Interproton distance restraints were obtained from 3D 13N-edited and 13C-edited NOESY spectra using a combination of automated and manual assignment methods. Hydrogen-bond restraints were determined using the algorithm VADAR30 in combination with H2O/D2O solvent exchange experiments that were performed by first lyophilizing 500 μL of a 1 mM 15N-labeled N-HsGr-RBP1 protein sample, followed by resuspension of the lyophilized protein in the same volume of 100% D2O, and acquisition of a series of 2D 1H−15N-HSQC correlation spectra at subsequent time points. Additionally, a 2D-CLEANEX-PM NMR experiment31 was performed to detect amide protons participating in fast chemical exchange with solvent.

**In Vitro Transcription and Generation of Single Stranded RNA (ssRNA).** Using standard Qiagen cloning methods, the Hsgr-rbp1 (GenBank id: JX126694) gene was cloned into a pDrive cloning vector and used as template for in
*in vitro* transcription and production of single-stranded RNA (ssRNA). More specifically to generate ssRNA, 2.5 μg of plasmid DNA in 20 μL was linearized by restriction digest with SalI (*Pronega*) for 2 h at 37 °C and heated at 65 °C for 20 min. The resulting sample was treated with 40 units of RNase inhibitor (New England Biolabs), followed by addition of T7 RNA polymerase (50 units plus nucleotide containing buffer) and the reaction incubated for 2 h at 37 °C. To remove the DNA template, the sample was subsequently treated with DNase I (New England Biolabs), and the ssRNA isolated using QIAGEN RNeasy spin columns (*Qiagen*).

**Generation of Single Stranded DNA Templates (ssDNA).** DNA coding for HvGR-RBP1 was amplified by PCR using a pET46-FL-HvGR-RBP1 vector as template and biotin-5′-biotin-5′ primer (3′ microcentrifuge tube) and rotated for 1 h. The beads were then washed with 2 × 2 mL aqueous ethanol and 2 × 1 mL DNA fragments were dissolved in nucleic acid binding NMR buffer (25 mM sodium phosphate, 100 mM potassium chloride, 95% H2O/5% D2O, pH 6.8) and slow-tilt-rotated for 10 min. Following centrifugation, the supernatant was removed and 40 mM HCl added to bring the solution pH back to 7.0. The concentration and purity of the ssDNA was determined by UV spectroscopy and agarose gel electrophoresis.

**Gel Electrophoretic Mobility Shift Assay (EMSA).** Purified protein samples (FL-HvGR-RBP1, N-HvGR-RBP1; 137-HvGR-RBP1) were dialyzed against nucleic acid binding buffer (25 mM Tris-HCl, 10 mM HEPES, 1 mM EDTA; 50 mM NaCl, pH 7.8) and diluted to 4.4 μM with binding buffer supplemented with 40 units of RNase inhibitor (New England Biolabs) and 50 μg/mL bovine serum albumin. In a 10 μL reaction volume, 1 μL of ssRNA, ssDNA, or dsDNA (stock concentrations of 125 ng/μL, 0.042 μM, 900 bp) was mixed with increasing concentrations of protein (from 0 μM to 1.0 μM) and incubated at 25 °C for 30 min. Loading buffer (2.5 μL of 20 mM Tris-HCl, 10 mM EDTA, 50% glycerol, pH 7.8) was added, and the total sample mixture analyzed on a 1.2% agarose gel with 0.5X TBE running buffer run at 60 V for 45 min, and visualized under UV light following SybrGold (Invitrogen) staining.

**Preparation of ssRNA-Bound Affinity Chromatography Columns.** Covalent coupling of ssRNA (25 μg) to 200 mg of cyanogen-bromide (CNBr)-activated Sepharose beads was carried out as described in refs 47 and 48 to assess whether expression of HvGR-RBP1 enables *E. coli* to grow better following cold shock treatment. These experiments are analogous to the ones conducted with *Arabidopsis thaliana* glycine-rich RNA-binding proteins (AtGRPs) that demonstrated the functional role of AtGRPs in cold adaptation. In these experiments, *E. coli* BL21 (DE3) cells transformed with vectors, pET46-FL-HvGR-RBP1 (encoding full-length HvGR-RBP1), pET46-N-HvGR-RBP1 (encoding the N-terminal RRM domain of HvGR-RBP1), pET32-C-HvGR-RBP1 (encoding the C-terminal glycine rich domain of HvGR-RBP1), pET46-FL-HvGR-RBP1(F54A) (encoding a full-length version of HvGR-RBP1 containing a phenylalanine to alanine substitution at residue position 54), and pET46-IsdBN1 (encoding an unrelated protein used as control), were grown in LB medium containing 50 μg/mL ampicillin to an optical density at 600 nm (OD600) of 0.6. This *E. coli* strain is not deficient in cold-shock proteins, and thus not the BX04 strain that is more sensitive to cold shock and used by Kim et al.77,48 Thus, any observed effects with the *E. coli* BL21 (DE3) cells are in addition to these cells’ native cold-adaptation process.

Protein expression was induced using 1 mM IPTG and resulting cell cultures were allowed to grow for 2 h at 30 °C, prior to being subjected to cold shock at 10 °C for 24 h. Following a 24 h period of cold shock treatment, the cells were returned to growth conditions at 37 °C, and cell growth was monitored by recording OD600 readings. HvGR-RBP1 protein levels were assessed by SDS-PAGE using 200 μL aliquots of cell cultures obtained from the last recorded time point of growth.

**Hvgr-rbp1 Transcript Expression Following Cold Treatment.** To analyze the effect of cold stress on *Hvgr-rbp1* expression, transcript levels were compared before and after chilling treatment of barley plants. Gene expression data for this analysis were obtained from the BarleyBase/PLEXdb database (http://www.plexdb.org/plex.php?database=Barley),49 experiment BB81 (entitled “Low temperature stress in cv. Dicktoo”), deposited by L. Tommasini (University of California, Riverside). These published data were generated by growing barley plants at 20 °C for 7 days and then chilled.
Temperature was decreased from 20 to 4 °C at a rate of 1.3 °C per hour, and plants were then kept at 4 °C (day)/2 °C (night) for 5 days. Leaves were harvested and analyzed using the Affymetrix Barley1 GeneChip. RMA-normalized data for contig17116_at (representing Hvgr-rbp1 transcript; three replicates) were downloaded from BarleyBase, and analyzed for significance using a Student’s t test (two-sided; two independent samples assuming equal variance) using Microsoft Excel for Windows (Microsoft Corporation, Redmond, WA).

RESULTS

Three Dimensional Structure of HvGR-RBP1. Full-length HvGR-RBP1 is composed of 162 amino acids. Sequence analysis indicated that the N-terminal region of HvGR-RBP1 (residues 1–92) shares sequence homology with proteins containing RNA-recognition motifs (RRMs), while the C-terminal region (residues 93–162) is enriched in glycine residues and contains a series of (GGR/RGG) repeats that are commonly found in glycine-rich RNA-binding proteins from plants. Full-length HvGR-RBP1 is very similar in sequence (65% sequence identity) to the Arabidopsis thaliana glycine-rich RNA-binding protein AtGRP7, which has been found to regulate numerous developmental and environmental adaptation processes in Arabidopsis, and to the Nicotiana tabacum NtGR-RBP1 protein (71% sequence identity) (Figure 1), thus is in all likelihood an ortholog of these proteins.

Initial in vitro studies of full-length HvGR-RBP1 (FL-HvGR-RBP1) indicated that the C-terminal glycine-rich region is easily degraded following purification from cell extracts, whereas the N-terminal RRM domain (residues 1–92) is more resistant to protease degradation (data not shown). Further and as previously reported, full-length HvGR-RBP1 is prone to aggregation and precipitation at the millimolar concentrations and buffer conditions required for multidimensional heteronuclear (1H, 15N, 13C) NMR analysis. At lower protein concentrations, the 2D-1H − 15N HSQC spectra of 15N-labeled FL-HvGR-RBP1 exhibited significantly broadened 1H/15N amide resonances positioned within the central "random coil" chemical shift region (i.e., 1H ~ 8 ppm; 15N ~ 120 ppm) of the HSQC spectrum, and broader NMR resonances for signals outside of this random coil region than is expected for a protein of ~18 kDa (Figure 2A). These data would indicate that under these conditions the protein aggregates and nonspecific intermolecular association takes place. Interestingly, this resonance line-broadening phenomenon appears to be dependent on pH and temperature. Raising the temperature from 298 to 303 K resulted in 2D-1H − 15N HSQC spectra with narrower resonance line-widths and improved resolution of 1H/15N amide signals (Figure 2B).
Comparing the 2D $^1$H−$^{15}$N correlation (HSQC) spectra of $^{15}$N-labeled FL-HvGR-RBP1 and N-HvGR-RBP1 (RRM domain only) recorded under identical NMR conditions at pH 6.2 and temperature of 303 K (Figure 2C and D) revealed small but significant differences in the chemical shift positions of several resonances originating from the RRM domain of HvGR-RBP1.

These data indicate that under these conditions, the GR region of HvGR-RBP1 interacts with the RRM domain of the protein. Interestingly, further increases in pH (from 6.2 to 7.2) decreased these chemical shift differences, resulting in 2D $^{15}$N−$^1$H HSQC spectra that look very similar to the one of RRM-only N-HvGR-RBP1 with a corresponding disappearance of $^1$H/$^{15}$N signals originating from the HvGR region (see Supporting Information Figure S4). Similarly, truncation of the 25 and 50 most C-terminal residues of HvGR-RBP1 glycine-rich domain (137-HvGR-RBP1 and 112-HvGR-RBP1, respectively) eliminated $^1$H/$^{15}$N chemical shift differences for NMR signals corresponding to the RRM domain of HvGR-RBP1 (Supporting Information Figure S5). These data suggest that the interactions between the RRM and GR domains of HvGR-RBP1 either require the complete HvGR sequence, or that these interdomain interactions are specifically mediated by the 25 most C-terminal residues of HvGR.

Figure 2. Comparison of various 2D $^1$H−$^{15}$N-HSQC spectra showing interactions between the RRM and GR domains of HvGR-RBP1. (A,B) Effect of temperature on FL-HvGR-RBP1: (A) 2D $^1$H−$^{15}$N-HSQC spectra recorded at 298 K, pH 6.2, and 250 μM protein concentration, displaying severely broadened resonances and resulting in the observation of two resonances for the indole $^1$H/$^{15}$N signal of Trp18. Allogether, these observations are indicative of intermolecular association. (B) 2D $^1$H−$^{15}$N-HSQC spectra recorded at 303 K, pH 6.2, displaying sharp, well-resolved resonances with line widths consistent with an ~18 kDa protein. (C) Overlays of FL-HvGR-RBP1 (black) and N-HvGR-RBP1 (red) recorded at pH 6.2 and temperature of 303 K showing shifted NMR signal in the presence or absence of the glycine-rich (GR) GR region in HvGR-RBP1. (D) Expanded view of a spectral region of overlaid 2D $^1$H−$^{15}$N HSQC spectra of FL-HvGR-RBP1 (black) and N-HvGR-RBP1 (red), indicating notable chemical shift changes of the $^1$H/$^{15}$N NMR signal arising from HvGR-RBP1’s RRM domain. (E) 2D $^1$H−$^{15}$N-HSQC spectrum of the glycine-rich domain (HvGR) of HvGR-RBP1. $^{15}$N-labeled HvGR (residues 93−162) was produced using a pET46 expression vector, and the resulting 2D $^1$H−$^{15}$N NMR spectrum was recorded at pH 6.2 and temperature of 298 K. (F) 2D $^1$H−$^{15}$N-HSQC spectrum of $^{15}$N-labeled HvGR upon addition of unlabeled N-HvGR-RBP1.
A 2D $^1$H−$^{15}$N HSQC spectrum recorded at a pH of 6.8 and temperature of 298 K of an $^{15}$N-labeled glycine-rich (HvGR) protein sample (Figure 2E) reveals that the glycine-rich domain of HvGR-RBP1 is unstructured in solution, displaying only broad resonances in the central “random coil” region of the spectrum similar to that shown in Figure 2A. Titration of unlabeled N-HvGR-RBP1 into an $^{15}$N-labeled glycine-rich (HvGR) protein does perturb the spectral characteristics of the NMR resonances arising from $^{15}$N-labeled HvGR (Figure 2F). Spectral changes taking place upon addition of unlabeled N-HvGR-RBP1 to $^{15}$N-HvGR suggest that the two protein domains can interact in vitro in trans, although the biological significance of these interactions is unclear. Taken together, these data indicate that the glycine-rich GR region of HvGR-RBP1 can participate in intra- and intermolecular interactions with the protein RRM domain, although these protein–protein interactions appear to be weak. In contrast, N-HvGR-RBP1 composed solely of the protein RRM domain and spanning residues 1−92 is well-folded and amenable to detailed structural determination by NMR.

Solution Structure of N-HvGR-RBP1. The 3D solution structure of N-HvGR-RBP1 was solved using well-established, heteronuclear ($^1$H, $^{15}$N, $^{13}$C), multidimensional solution NMR experiments and standard computational torsion angle simulated annealing protocols. Resonance assignments of backbone and side-chain atoms were nearly complete (>97%) with only the $^1$H/$^{15}$N backbone amide resonances of V55 unobservable. The final 3D structure of N-HvGR-RBP1 was computed using 683 interproton ($^1$H−$^1$H) NOE, 83 dihedral angle, and 74 hydrogen bond distance restraints, as shown in Table 1 which summarizes the NMR experimental constraints used and resulting structural statistics. An overlay of the Cα backbone traces of the final set of 20 low energy conformers depicting the ensemble of N-HvGR-RBP1 structures is shown in Figure 3. These conformers exhibit excellent bond and stereochemical properties, with no NOE-derived distance violations greater than 0.5 Å or dihedral angle violations greater than 5°. Dihedral angle analysis indicates that all residues within the structurally ordered region (residues 10−84) of N-HvGR-RBP1 have $\phi$ and $\psi$ dihedral angle values that are within the allowed ranges of the Ramachandran plot. Residues 10−84 of N-HvGR-RBP1 are well ordered and converge into a well-folded structure with a pairwise backbone heavy atom RMSD of 0.66 Å (Table 1). In contrast, the N- and C-termini of the protein (residues 1−9 and 85−92) are less well-defined.

The solution structure of N-HvGR-RBP1 adopts a canonical RRM structural fold21,22 consisting of a four-stranded antiparallel $\beta$-sheet and two $\alpha$-helices arranged in a $\beta_1\alpha_1\beta_2\alpha_2$ secondary structural topology (Figure 4). The two $\alpha$-helices ($\alpha_1$ and $\alpha_2$) are positioned on the same side of the protein almost at a right angle to each other, as has been seen in other RRM structural folds.22 The three loops connecting the various $\beta$-strands and $\alpha$-helices all appear to be well-defined, with two loops (loops 3 and 5) incorporating...
two additional short β-strands to create two β-hairpin structures (β2/β2' and β4/β4') spanning the structural elements β2 and β3 and α2 and β4, respectively. Such hairpin structures are not typically observed in other RRM structural folds.20–22,56

The hydrophobic core of the protein comprises several conserved hydrophobic residues (highlighted in yellow in Figure 1), which are located primarily within the βαββ region of the protein. This region also includes the two conserved RNA-binding consensus sequences RNP2 and RNP122 that project the aromatic side chains of Phe12, Phe52, and Phe54 from the plane formed by the two central β-strands β1 and β2, respectively, priming these residues for base stacking interactions with potential RNA targets.56 Interestingly, the hydrophobic packing of the side chain of Leu16 into the hydrophobic core results from a sharp curvature of the polypeptide backbone chain formed by a β-bulge created by Ser15 and Leu16 within the β1-loop1 region (Figure 4). RRM-containing proteins with a β-bulge have been observed previously, but typically, the bulge is located within the β2 strand of the proteins (see, for example, the RNA-binding domains, RBD1 and RDB2, of U1A, Sex lethal, and hnRNP1 D057).

Lastly, the N- and C-termini (i.e., residues 1–9 and 85–92, respectively) of N-HvGR-RBP1 are positioned on the same side in the 3D structure of the protein. The N-terminal segment is largely disordered and most likely highly flexible, as judged by the intensity of the NMR signals, the presence of random coil 1H, 15N, and 13C chemical shifts, the lack of observable amides protected from 1H/2H solvent exchange, and the scarcity of interproton NOE-based distance restraints for residues located within this region. Residues in the C-terminal end of N-HvGR-RBP1 exhibit similar characteristics, except for the formation of a short α-helical turn (α3) within residue stretch 86–89 (Figure 4).

### Structural Comparison of N-HvGR-RBP1 with Other RRM-Containing Proteins

The DALI31 and SSM58 databases were utilized to search for structural homologues of the RRM domain of HvGR-RBP1. Homology search results reveal that RRM of N-HvGR-RBP1 is most structurally similar to other eukaryotic RRM domains, including the RRM2 domain of the T-cell restricted intracellular antigen-1 (TIA-1) protein (PDB ID = 3BS9, Z-score = 12.7, RMSD = 1.5 Å, 43% sequence identity),59 and the RRM1 domain of the polyadenylate-binding protein 1 (PABP1) (PDB ID = 4F02, Z-score = 12.2, RMSD = 2.4 Å, 24% sequence identity),60 both of which are involved in post-transcriptional regulation of gene expression including pre-mRNA splicing in the nucleus and mRNA translation in the cytoplasm (see Supporting Information Figures S1 and S2).

Restricting our DALI structural homology search to other RRM-containing plant proteins yielded the closest structural homology to the RRM domain of the Nicotiana tabacum glycine-rich RNA-binding protein 1, NtGR-RBP1 (PDB ID = 4C7Q, Z-score = 11.1, RMSD = 2.6 Å, 72% sequence identity), suggested to function as an RNA chaperone,24 and the RRM domain of the poly(A) binding protein 1 from Citrus sinensis, CsPABP1 (PDB ID = 2M70, Z-score = 9.0, RMSD = 3.0 Å, 26% sequence identity),25 suggested to associate with the poly(A) tail of mRNA and, similarly to cytoplasmic PABP proteins, to regulate the translation of polyadenylated mRNAs and mRNA decay41,62 (Figure 5A,B). Sequence analysis also revealed that the amino acids within the putative nucleotide-binding site of HvGR-RBP1, NtGR-RBP1, and AtGRBP7 are identical, suggesting that all three orthologs may bind similar nucleic acid target sequences (Figure 6).

### In Vitro Assays Testing the Ability of HvGR-RBP1 to Bind to Various Nucleic Acid Sequences

**In Vitro Assays Testing the Ability of HvGR-RBP1 to Bind to Various Nucleic Acid Sequences.** In vitro gel electrophoretic mobility shift assays (EMSAs) were performed using single-stranded RNA (ssRNA), single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA) templates to assess the nucleic acid binding capabilities of HvGR-RBP1. Since AtGRP7 has been reported to bind to and regulate the splicing of its own pre-mRNA transcript, full-length genomic Hvgr-rbp1 was used to evaluate whether HvGR-RBP1 can bind to its own pre-mRNA transcript. Results of FL-HvGR-RBP1 binding to Hvgr-rbp1 ssRNA are shown in Figure 7A. In general, increasing concentrations of FL-HvGR-RBP1 (0–1 μM) protein shifted the electrophoretic mobility of the Hvgr-rbp1 ssRNA band. Addition of N-HvGR-RBP1 (which corresponds to the N-terminal RRM domain only of HvGR-RBP1) to the Hvgr-rbp1 ssRNA template resulted in no electrophoretic mobility shift of the ssRNA transcript (Figure 7B).

Similarly, truncated constructs of HvGR-RBP1 including 112-HvGR-RBP1 and 137-HvGR-RBP1 (the latter lacking only the 25 C-terminal residues of the full-length protein) also resulted in no band shift (Figure 7C). These data suggest that no interactions take place between these protein products and ssRNA under the conditions of these in vitro EMSA assays. The lack of ssRNA binding using either 112-HvGR-RBP1 or 137-HvGR-RBP1
Figure 6. Amino acid sequence alignment of HvGR-RBP1 with its most closely related plant homologues AtGRP7 and NtGR-RBP1. Conserved hydrophobic core residues are shaded in yellow. Fully conserved amino acid residues are highlighted by an asterisk. Residues that are very similar in chemical properties, but not identical, are denoted by a colon, and residues that are weakly similar are indicated by a dot. Absence of a symbol denotes that there is no common property between the amino acids. The Arg 49 residue (highlighted in blue) of AtGRP7 has been identified as being critical for the alternative mRNA splicing function of AtGRP7. A structurally equivalent residue (Arg 50) is found at that same location in the 3D structure of HvGR-RBP1 (highlighted in blue), and is part of the protein RNP1 consensus sequence. This suggests that R50 in HvGR-RBP1 may be important for this protein to function in alternative splicing.

HvGR-RBP1 protein constructs indicates that the complete C-terminal glycine-rich (GR) region of full-length HvGR-RBP1 is necessary to detect protein binding to ssRNA under the conditions of these in vitro nucleic acid binding experiments.

The different HvGR-RBP1 constructs were incubated with either dsDNA or ssDNA (not shown) coding for the full-length Hvg-rbp1 gene, no visible shift in gel mobility was observed (Figure 7D), suggesting that HvGR-RBP1 binds preferentially to ssRNA. The ability of N-HvGR-RBP1 and FL-HvGR-RBP1 to bind to an ssRNA affinity chromatography column composed of RNA isolated from barley plant leaf samples was also tested. As observed with EMSA experiments, we found that N-HvGR-RBP1 could not be retained on the ssRNA-bound affinity chromatography column, whereas FL-HvGR-RBP1 was retained on the column and could be eluted from the column using increasing ionic strength KCl salt solutions. The interaction of full-length HvGR-RBP1 with ssRNA, however, was determined to be nonspecific, as similar binding of full-length HvGR-RBP1 was observed with unrelated RNA transcribed from E. coli genomic DNA (data not shown).

Lastly, to establish whether the C-terminal GR domain of HvGR-RBP1 is sufficient to bind to ssRNA in vitro, a fusion protein comprising thioredoxin and the GR domain of HvGR-RBP1 was prepared and tested for its ability to bind to an unrelated ssRNA construct derived from a mRNA sequence isolated from barley plant leaf samples. The fusion protein comprising thioredoxin and the GR domain of HvGR-RBP1 resulted in an electrophoretic mobility change in the 2D1H−15N HSQC spectrum (Figure 7E), suggesting that the glycine-rich (GR) domain of HvGR-RBP1 is sufficient to modulate nonspecific ssRNA binding.

NMR Analysis of N-HvGR-RBP1 Binding to ssDNA. Although the N-terminal RRM domain of HvGR-RBP1 did not bind ssRNA in the in vitro EMSA assays conducted, these results are insufficient to unambiguously establish whether the RRM domain of HvGR-RBP1 is incapable of interacting with ssRNA. Lack of RNA binding would be surprising, as other isolated RRM from GR-BP proteins have been shown to bind, albeit very weakly, to single-stranded RNA targets.

Evidence from the literature would thus suggest that the ssRNA binding affinity of N-HvGR-RBP1 (containing only the RRM domain of HvGR-RBP1) may be too weak to be observable using the described in vitro EMSA experiments. Recently, a 6-nt DNA sequence (5′-TTCTGG-3′) has been used as a proxy for a 6nt ssRNA sequence identified as a putative nucleic acid binding site for NtGR-RBP1 based on sequence similarity with nucleotide sequences present within the 3′-UTR region of Atgrp7 and Ntgr-rbp1 pre-mRNA. Analysis of the genomic sequence of Hvg-rbp1 reveals the presence of two homologous sequences, 5′-TTCTGA-3′ and 5′-TTCTGT-3′, one located in the 3′-UTR region of Hvg-rbp1 pre-mRNA, and the second located upstream near the 5′-end of the intron. The locations of these sequences in the Hvg-rbp1 gene are analogous to Atgrp7 nucleotide binding sites identified in Atgrp7 pre-mRNA (Figure 8).

These observations led us to investigate whether or not the RRM domain of HvGR-RBP1 can bind to 6-nucleotide ssDNA sequences corresponding to segments of the 3′-UTR and intronic regions of Hvg-rbp1 pre-mRNA. Subsets of 2D 1H−15N correlation HSQC spectra resulting from these titration experiments are shown in Figure 9. In the absence of the nucleotide ssDNA ligand, the 2D 1H−15N HSQC spectrum of 15N-labeled N-HvGR-RBP1 is well-resolved and similar to the one reported in ref 26 and in Figure 2C of the present manuscript. Progressive titration of unlabeled 5′-TTCTGA-3′ up to a 1:1.5 molar ratio of 15N-labeled N-HvGR-RBP1 to ssDNA elicited small but noticeable spectral changes in the 2D 1H−15N HSQC spectrum of N-HvGR-RBP1 (Figure 9A). Most notable were changes in amide 1H chemical shifts of residues F12, W18, N19, T20, F52, and Q86, residues primarily within or adjacent to RNP1 and RNP2 consensus RNA-binding sequence sites. These residues correspond to the RNA-binding site of the RRM motif of
HvGR-RBP1, which includes the RNA-binding consensus sequences RNP1 and RNP2 (Figures 4 and 9C–D).

Similar $^1$H/$^{15}$N chemical shift changes were observed in the 2D $^1$H−$^{15}$N HSQC spectrum of $^{15}$N-labeled N-HvGR-RBP1 upon titration of the 6-nt fragment 5′-TTCTGT-3′ whose sequence is part of the 3′-UTR region of HvGR-RBP1’s pre-mRNA. Moreover, the magnitude of these chemical shift changes is similar to what is observed upon titration of the 6-nt fragment 5′-TTCTTGG-3′, which had been used in nucleic acid binding experiments of Negr-RBP1. These data thus indicate that differences within the last nucleotide position of these 6 nt ssDNA fragments contribute little if anything to the nucleotide binding specificity of the RRM domain.

To assess the specificity of these single-stranded 6-nucleotide binding interactions, we also investigated the capability of N-HvGR-RBP1 to bind to a randomized 6-nt fragment 5′-CTGTGTG-3′, which retains the CTG binding element identified in the Negr-RBP1 studies, but is altered with respect to the nature of the flanking nucleotides. Titration of this ssDNA fragment into an $^{15}$N-labeled N-HvGR-RBP1 protein sample resulted in the observation of smaller $^1$H/$^{15}$N chemical shift changes in resulting 2D $^1$H$−^{15}$N HSQC spectra of $^{15}$N-labeled N-HvGR-RBP1 in complex with this ssDNA (Figure 9B). The equilibrium dissociation constant ($K_d$) for binding of this randomized 5′-CTGTGTG-3′ ssDNA to N-HvGR-RBP1 was determined to be $\sim$550 μM, much higher than the $K_d$ values ranging from ~4 to 43 μM for the other ssDNA ligands (see Supporting Information Table S1). These chemical shift perturbation experiments thus indicated that the nucleotides adjacent to the CTG binding element are important modulators of the interactions of N-HvGR-RBP1 with single-stranded 6-nucleotide ssDNA targets. Overall, these results demonstrate that the N-terminal RRM domain of HvGR-RBP1 can interact weakly with ssDNA fragments. The high nucleotide sequence identity and location of these nucleotide fragments within the 3′-UTR or intronic region of the pre-mRNA of Hvgr-rbp1 suggest that HvGR-RBP1 may regulate self-splicing of its own mRNA transcript, similar to what has been observed for Arabidopsis thaliana AtGRP7,53,64.

**Figure 8.** (A) Schematic representation of the genomic DNA sequence of Hvgr-rbp1. 5′- and 3′-untranslated regions (5′-UTR and 3′-UTR) are indicated by gray rectangles, exons 1 and 2 by white-shaded rectangles, and the intron by a solid black rectangle, with nucleotide positions indicated above. Locations of the CTG binding elements are indicated with solid arrows. (B) Partial DNA nucleotide sequences corresponding to the intron and 3′-UTR region of the Hvgr-rbp1 gene are shown to indicate the potential CTG binding elements (bold and underlined) that may be recognized and bind specifically to HvGR-RBP1 when transcribed to ssRNA.
environmental stresses. In particular, AtGRP7 has been shown to function as an RNA chaperone during the cold adaption process of *Arabidopsis thaliana* plants. To explore the effect of cold stress on *Hvgr-rbp1* gene expression, transcript data retrieved from the BarleyBase/PLEXdb database (experiment BB81) in which barley plants were subjected to a 4 °C chilling treatment were compared to transcript levels before treatment. It can be seen that *Hvgr-rbp1* transcript levels increased following the chilling treatment (Supporting Information Figure S3) indicating this gene is induced by cold stress.

**Influence of HvGR-RBP1 on *E. coli* Growth Following Cold Shock.** To examine whether *Hvgr-rbp1* functions as an RNA chaperone in response to cold stress, similar to what has been demonstrated for the glycine-rich RNA-binding protein aTRZ-1α of *Arabidopsis thaliana*, we investigated the growth of *E. coli* cells that were heterologously expressing FL-*HvGr-RBP1*, FL-*HvGr-RBP1(F54A)*, IsdB<sup>N1</sup> (an unrelated control protein), or truncated constructs containing either the N-terminal RRM domain (*N-HvGr-RBP1*) or the C-terminal glycine rich region of *HvGr-RBP1* (*HvGR*) following cold shock stress. *E. coli* cells were grown to mid log phase, induced for protein expression using IPTG, cold shocked by letting the cells grow at 10 °C for 24 h, and then returned to normal growth at 37 °C.

Figure 10 displays the observed growth patterns and demonstrates that *E. coli* cells transfected with the pET46-FL-*HvGr-RBP1* vector grow more rapidly than cells harboring a control pET46-IsdB<sup>N1</sup> vector, following return to normal growth at 37 °C. Similar results are observed for *E. coli* cells harboring the pET46-N-*HvGr-RBP1* vector, which leads to increased expression of the protein N-terminal RRM domain. In contrast, *E. coli* cells containing the pET32-C-*HvGr-RBP1* vector encoding only the C-terminal glycine-rich region of *HvGr-RBP1* (*HvGR*) following cold shock stress grew at a slower rate, similar to *E. coli* cells harboring the control vector pET46-IsdB<sup>N1</sup>.

Interestingly, cells transformed with the pET-FL-*HvGr-RBP1* (F54A), in which Phe 54 is replaced by Ala in the full-length protein and corresponding to a mutation within the consensus RNPI RNA-binding site of...
RNA binding, with amino acid side chains of residues within 3 and 5 and span the results suggest that interaction to other potential post-transcriptional regulatory functions RRM-containing glycine-rich RNA-binding proteins, in addition, a function that has been associated with other plant activity, while the C-terminal glycine-rich domain of HvGR-RBP1 (a full-length HvGR-RBP1 variant with a Phe to Ala amino acid substitution at residue position 54, which is located within the RNP1 consensus sequence of the RRM domain of HvGR-RBP1), and IsdBNI (a control protein expressed in the same pET46 plasmid as the ones encoding the different HvGR-RBP1 protein constructs), following cold shock growth conditions (10°C for 24 h) followed by a return to normal growth conditions at 37°C.

**Figure 10.** Growth curves of *E. coli* cells expressing FL-HvGR-RBP1 (i.e., full-length protein), N-HvGR-RBP1 (N-terminal RRM domain), C-HvGR-RBP1 (C-terminal glycine-rich domain), FL-HvGR-RBP1-F54A (a full-length HvGR-RBP1 variant with a Phe to Ala amino acid substitution at residue position 54, which is located within the RNP1 consensus sequence of the RRM domain of HvGR-RBP1), and IsdBNI (a control protein expressed in the same pET46 plasmid as the ones encoding the different HvGR-RBP1 protein constructs), following cold shock growth conditions (10°C for 24 h) followed by a return to normal growth conditions at 37°C.

RRMs lose their ability to grow well following cold shock treatment (Figure 10).

Together, these results demonstrate that the N-terminal RRM domain of HvGR-RBP1 is necessary for enhanced *E. coli* growth following cold shock treatment, while the C-terminal glycine-rich domain of HvGR-RBP1 does not appear to play a significant role in this cold adaptation function. Furthermore, and analogous to findings about AtGRP7 and other *Arabidopsis thaliana* GRPs, these results suggest that HvGR-RBP1 may possess RNA chaperone activity, a function that has been associated with other plant RRM-containing glycine-rich RNA-binding proteins, in addition to other potential post-transcriptional regulatory functions such as alternative splicing, which has been established in its closest *Arabidopsis* ortholog AtGRP7.

**DISCUSSION**

In this manuscript, we report the detailed structural characterization and nucleic acid binding properties of the barley HvGR-RBP1 protein whose transcript has been identified as being >40-fold up-regulated in early senescing “10_11” barley plants. We have shown that the N-terminal domain of HvGR-RBP1 adopts a β1-α1-β2-β3-α2-β4 structural topology which is characteristic of canonical RNA Recognition Motifs (RRMs). The structural fold of N-HvGR-RBP1 is further augmented by the presence of two β-hairpin structures that form within loops 3 and 5 and span the β2-β3 and α2-β4 structural elements of the protein, respectively. While the two hairpins are not typically present in the loop regions of other RRMs, a single β-hairpin bridging α2 and β4 is often observed and is involved in RNA binding, with amino acid side chains of residues within the hairpin participating in H-bonding to bases of nucleic acid targets.

The RRM motif is found ubiquitously in all kingdoms of life and is one of the most common protein folds found in eukaryotes. Functionally, RRM-containing proteins participate in all post-transcriptional aspects of gene expression, e.g., RNA splicing, transcript stability and degradation, RNA transport, capping, translation, termination, and RNA chaperone activity. Further, RNA-binding proteins with one or more RRMs can be adapted to incorporate a variety of auxiliary motifs such as Arg/Asp-repeats, Ser/Arg-repeats, Arg-rich motifs, zinc finger motifs, and Gly-Tyr-Arg rich motifs as observed in HvGR-RBP1.

Interestingly, RNA-binding proteins comprising an N-terminal RRM domain and a C-terminal glycine-rich region are found ubiquitously but preferentially in plant species, which possibly highlights a specific functional role of the glycine-rich C-terminal domain, separate from the RRM domain function. Analysis of the RRM fold of HvGR-RBP1 shows that it possesses a high degree of structural similarity to the recently solved RRM domain of the *Nicotiana tabacum* NtGR-RBP1 protein, which, combined with the high sequence identity of HvGR-RBP1 to *Arabidopsis thaliana* AtGRP7, suggests that these proteins share similar functional properties.

NMR investigations of the glycine rich region of HvGR-RBP1 indicate that it is unstructured. A recent study of *Nicotiana tabacum* NtGR-RBP1 showed that despite the disorder, the glycine-rich region of NtGR-RBP1 can interact in trans with the RRM domain of another NtGR-RBP1, indicating the potential for self-association. A similar effect is observed with barley HvGR-RBP1. Full-length HvGR-RBP1 was found to be prone to aggregation and/or intermolecular association at high concentrations resulting in broadened NMR signals and line-shapes. Moreover, we found that the 1H/13N amide resonances corresponding to the RRM domain of HvGR-RBP1 could be clearly distinguished from random coil signals corresponding to HvGR and that the former experience small but significant chemical shift changes in the presence of HvGR, suggesting that the two domains of the protein can interact. Interactions between HvGR and RRM could be, however, abrogated by varying the temperature or pH conditions of the samples. These results suggest that the interactions between HvGR and the RRM domain are weak and, most likely, very dynamic.

A truncated construct lacking the 25 most C-terminal residues of the full-length protein, 137-HvGR-RBP1, also eliminated these chemical shift perturbation effects, suggesting that the very C-terminal end of HvGR is required for interactions with the RRM domain of HvGR-RBP1. The fact that we observed chemical shift changes for resonances of the full-length protein could indicate that the full-length protein may adopt a more compact structure in the absence of binding to nucleic acid polymers.

Despite the unstructured nature of the GR domain, nucleic acid binding to FL-HvGR-RBP1 shows that this region can interact with ssRNA in EMSA experiments. However, this ssRNA binding appears to be nonspecific. A hybrid fusion protein composed of HvGR and thioredoxin retained ssRNA-binding ability, while truncation of the 25 most C-terminal residues of the full-length protein is sufficient to eliminate this effect. Interestingly, we observed frequently during the course of our experiments that the glycine-rich region is quite susceptible to protease degradation, which eliminated the ability of resulting protein products to bind ssRNA in the EMSA experiments. This susceptibility for degradation could indicate a control level for HvGR-RBP1 whereby the activity of the protein is rapidly turned off by protease cleavage of C-terminal residues within the GR domain of the protein.
The apparent absence of ssRNA binding to the RRM domain of N-HvGR-RBP1 in the in vitro EMSA assays is attributed to the specificity requirements of the RRM domain for well-defined nucleotide sequences, combined with the inherently low affinity of isolated RRMs for ssRNA targets, as has been observed for most RRMs when isolated from the rest of their protein scaffolds. We did, however, find that the RRM domain of HvGR-RBP1 interacts with several nucleotide target sequences using 2D 1H/15N chemical shift experiments. Similarly to what was observed by Khan et al. in their studies of NtGR-RBP1, titration of ssDNA 6nt oligonucleotides 5′-TTCCTGA-3′ and 5′-TTCCTGT-3′, which are part of the 5′-intronic region and 3′-UTR of the Hvgr-rbp1 gene, caused several 1H/15N chemical shift changes of Nt-HvGR-RBP1 amide resonances. Residues affected by the binding of the 6-nucleotide ssDNAs to N-HvGR-RBP1 corresponded to amino acid residues positioned within the RNP2 and RNP1 consensus RNA-binding sites of the RRM motif. The two oligonucleotide sequences that are naturally present in the Hvgr-rbp1 pre-mRNA transcript contain the same CTG element that was identified by Khan et al. to be a key element within the 5′-TTCCTGG-3′ oligonucleotide sequence observed to bind to NtGR-RBP1. In our study, we investigated the binding of a third ssDNA oligonucleotide fragment (i.e., 5′-CTGTGG-3′) to N-HvGR-RBP1, in which the CTG element was retained but the other nucleotides scrambled. Such a “scrambled” 6-nt oligonucleotide ssDNA sequence resulted in significantly weaker binding to N-HvGR-RBP1 (~550 μM Kd compared to equilibrium dissociation constants of ~4–43 μM for other ssDNA ligands), indicating that the nucleotides adjacent to the CTG element are indeed important modulators of the RRM binding affinity for its ssDNA target. These data support that the nucleotide binding surface of the RRM domain of HvGR-RBP1 is at least ~6–8 nt long, consistent with what has been reported for NtGR-RBP1.

Overall, the binding of HvGR-RBP1 to these two specific 5′-TTCCTGA-3′ and 5′-TTCCTGT-3′ sequences strongly suggests that HvGR-RBP1 may also function as an alternative splicing regulator of its own pre-mRNA transcript, similar to what has been observed for the Arabidopsis AGRP7 protein. Based on these results, we propose a nucleotide-binding model for HvGR-RBP1 in which the synergistic interactions between the protein RRM and GR domains enhance the affinity of HvGR-RBP1 for its target single-stranded nucleic acid sequences.

To date, the expression profiles of several GR-RBPs have been studied under different environmental stress conditions, whereby the data clearly indicate that these proteins are involved in plant responses to various environmental factors. In our studies, we observed that Hvgr-rbp1 transcript expression is up-regulated following cold stress treatment (Supporting Information Figure S3). Our cold stress experiments suggest that HvGR-RBP1 harbors RNA folding chaperone activity, similarly to what has been observed for AGRP7, although the glycine-rich domain of HvGR-RBP1 does not appear essential for this function. Our results are consistent with previous studies which have shown that only the RRM domain of GR-RBPs is necessary for RNA chaperone activity in bacteria. Similarly, DNA melting experiments, used as a proxy for demonstrating RNA chaperone function, have shown that the single RRM domain of NtGR-RBP1 is sufficient to induce DNA melting of CTG-containing fragments. Recent studies using photoelectric transfer (PET) fluorescence spectroscopy to investigate the interaction of AGRP7 with its ssRNA target have suggested that RRM domain binding to an extended form of its ssRNA target prevents formation of RNA hairpin structures. Analogies to the mode of function of AGRP7 and NtGR-RBP1 thus suggest that the mechanism of RNA chaperone function of HvGR-RBP1 resides in its ability to prevent the formation of undesirable RNA secondary structure. Interestingly, it has been noted that plant GR-RBPs involved in cold adaptation share sequence homology to cold-induced cyanobacteria proteins, which are composed of one or more N-terminal RRM motifs and a C-terminal glycine-rich domain.

Lastly, it remains to be established whether HvGR-RBP1 possesses regulatory activity influencing or controlling multiple cellular functions, similar to what has been observed for Arabidopsis GRPs. For example, Arabidopsis thaliana glycinine-rich RNA-binding proteins 7 and 8 (AtGRP7 and AtGRP8 which share 65% and 63% sequence identity with HvGR-RBP1) have been implicated in numerous biological processes including but not limited to circadian clock-controlled mechanisms, plant pathogen defense, and plant flowering control, in addition to a possible role in senescence. Furthermore, Streitner et al. demonstrated that Arabidopsis thaliana lines without AtGRP7 functionality flower somewhat later under long days, and considerably later under short days when compared to corresponding wild-type germplasm. In contrast, Arabidopsis plants overexpressing AtGRP7 flower earlier. These results parallel observations with early senescing barley line “10_11” that expresses high levels of Hvgr-rbp1 transcripts, suggesting that HvGR-RBP1 and AtGRP7 share at least some regulatory functions in plant development. Studies aimed at identifying additional regulatory functions for HvGR-RBP1 in barley plants are currently ongoing.

In summary, we have solved the 3D solution NMR structure of barley HvGR-RBP1, which has been identified as a potential regulator of barley leaf and whole-plant senescence. We have shown that FL-HvGR-RBP1 is a two-domain protein, composed of a well-folded N-terminal RRM motif and an unstructured C-terminal GR domain. We have shown that the RRM domain of HvGR-RBP1 binds nucleotide fragments containing the consensus nucleotide sequence 5′-TTCCTG-3′ with low micromolar affinity in vitro. We have also demonstrated that the C-terminal GR domain of HvGR-RBP1 can interact with ssRNA in vitro in a nonspecific manner. Based on protein expression analysis and E. coli growth studies following cold shock treatment, we conclude that HvGR-RBP1 can function in a manner similar to that of cold-shock proteins and may harbor RNA chaperone activity that confers cold-stress tolerance to barley plants.

**ASSOCIATED CONTENT**

*Supporting Information*

Supplementary Figures S1–S5 and Supplementary Table S1: Figure S1 depicts a multiple sequence alignment of HvGR-RBP1 with plant, protozoan, microbial, and eukaryotic RRM-containing proteins. Figure S2 represents a structural overlay of HvGR-RBP1 with mammalian RRM domains. Figure S3 is a graphical representation of the effect of cold stress in barley on Hvgr-rbp1 transcript expression. Figure S4 represents an overlay of the 2D 1H–15N HSQC spectra of FL-HvGR-RBP1 (in black) and N-HvGR-RBP1 (in red) recorded at pH 7.2 and temperature of 303 K. Figure S5 presents the 2D 1H–15N HSQC spectra of the truncated constructs 137-HvGR-RBP1 (Figure SSA) and 112-HvGR-RBP1 (Figure SSB) recorded at
pH 6.8 and temperature of 298 K. Table S1 reports equilibrium dissociation constants ($K_d$) obtained from titration experiments whereby varying concentrations of unlabeled 6-nucleotide ssDNA ligands were added to $^{15}$N-labeled N-HvGR-RBP1 NMR samples followed by the recording of series of 2D $^1$H−$^{15}$N HSQC NMR spectra, and the analysis of $^1$H/$^{15}$N chemical shift changes upon ligand binding. These data support the notion that the RRM domain of HvGR-RBP1 exhibits micromolar binding affinity for 6nt ssDNA targets in these in vitro experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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**ABBREVIATIONS**
AtGRP7, *Arabidopsis thaliana* glycine-rich RNA-binding protein 7; AtGRP8, *Arabidopsis thaliana* glycine-rich RNA-binding protein 8; BMRB, BioMagResBank database; CSD, cold shock domain; dpa, days past anthesis; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; EDTA, ethylene-diaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; GPC, grain protein content; GRP, glycine-rich proteins; HSQC, heteronuclear single quantum coherence spectroscopy; HvGR-RBP1, *Hordeum vulgare* L. Glycine-Rich RNA-Binding Protein 1; IPTG, isopropyl β-thiogalactoside; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser effect; PDB, Protein Data Bank; RNA, robust multiarray average; RSMDS, root-mean-squared deviation; RRM, RNA recognition motif; ss/ds RNA/DNA, single-stranded/double-stranded RNA/DNA; SSM, secondary structure matching; TBE buffer, Tris/Borate/EDTA buffer

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