

# Isolation and Characterization of a Novel Drought Responsive Gene Encoding a Glycine-rich RNA-binding Protein in *Malus prunifolia* (Willd.) Borkh.

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**Abstract** Drought is one of the most severe environmental factors that impair plant growth and agricultural production. To investigate how *Malus prunifolia*, an excellent apple rootstock with strong drought tolerance, adapts to stress and to identify genes for improving this important trait, we constructed a suppression subtractive hybridization cDNA library from seedling leaves under water stress. The cDNA for a drought-inducible glycine-rich RNA-binding protein (GR-RBP) was isolated, and the gene was characterized for its role in the response of *Malus* seedlings to drought stress. cDNA clone *MpGR-RBP1* has 781 nucleotides, with an open reading frame of 516 nucleotides. The deduced 171 amino acids contain an amino-terminal RNA recognition motif and a carboxyl-terminal glycine-rich domain, with structural similarity to a class of stress-induced GR-RBP proteins found in other plants. Phylogenetic analysis confirmed that the *MpGR-RBP1* protein belongs to the plant GR-RBP family, members of which play important roles in posttranscriptional regulation of gene expression under various stress conditions. The expression profile of *MpGR-RBP1* transcripts was detected by quantitative real-time polymerase chain reaction (PCR) and semiquantitative reverse transcriptase PCR. *MpGR-RBP1* was expressed in

both roots and leaves, with expression being higher in the latter. This is the first report of this class of protein in *Malus* plants, and the putative role of *MpGR-RBP1* is discussed.

**Keywords** *Malus prunifolia* · Drought stress · Glycine-rich RNA-binding protein · Gene expression · Sequence characterization

## Abbreviations

GR-RBP	Glycine-rich RNA-binding protein
GRP	Glycine-rich protein
SSH	Suppression subtractive hybridization
RT-PCR	Reverse transcriptase polymerase chain reaction
ORF	Open reading frame
UTR	Untranslated region

## Introduction

Plant glycine-rich RNA-binding proteins (GR-RBPs) (Burd and Dreyfuss 1994; Fu et al. 2007) are characterized by at least one RNA recognition motif (RRM) present at the N-terminus (also known as an RNA-binding domain or ribonucleoprotein [RNP] domain) and a glycine-rich domain at the C-terminal (Bandziulis et al. 1989; Albà and Pagès 1998). Similarly, a group of structural proteins in the cell walls, i.e., glycine-rich proteins (GRPs), contain glycine-rich domains (Sachetto-Martins et al. 2000). Whereas GRPs are well-characterized as cell-wall proteins, GR-RBPs are involved in posttranscriptional RNA processing in the cytoplasm as well as in the nucleus (Ziemiencowicz et al. 2003; Fusaro et al. 2007). To distinguish between these two groups, Lorković and Barta (2002) have proposed that RRM-

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containing GRPs be renamed as GR-RBPs. An RRM has two short, highly conserved sequences—an octamer designated RNP1 and a hexamer designated RNP2—plus several other, mostly hydrophobic, conserved amino acids interspersed throughout the motif (Query et al. 1989). A different type of RNA-binding motif in the N-terminus of GR-RBPs is the cold shock domain (CSD), where only the RNP1 sequence is conserved (Sachetto-Martins et al. 2000). The CSD is proposed to be an ancient molecule that was present before the origin of single-cell life and is the most evolutionary conserved nucleic acid-binding domain within prokaryotes and eukaryotes (Nakaminami et al. 2006). Nevertheless, the function of GR-RBPs in eukaryotes, including plants, remains largely unknown (Kim et al. 2007a; Lorković 2009).

GR-RBPs have now been isolated from cyanobacteria and the cells of plants and mammals (Shinozuka et al. 2006). Some cDNAs that encode plant GR-RBPs have been identified from both angiosperms and gymnosperms and are highly conserved (Stephen et al. 2003). Bocca et al. (2005) have utilized ForEST database and the phylogenetic tree method to cluster plant GR-RBPs into groups based upon their combination of structural domains. Those in group 1 have an RRM conserved motif at the N-terminal end, followed by a glycine-rich region with GYG G repeats (Sachetto-Martins et al. 2000). Group 2 members, also known as RZ proteins, contain an RRM at the N-terminus and a glycine-rich region interspersed with a (CCHC)-type zinc finger at the C-terminus. This group appears to be plant-specific (Lorković and Barta 2002; Kim et al. 2007c). In group 3, GR-RBPs are organized with a CSD at the N-terminus plus one to seven CCHC zinc fingers in their glycine-rich region, which is also termed the cold shock proteins. Finally, group 4 presents two copies of the RRM motif followed by a C-terminal glycine-rich region. It is therefore interesting to determine whether these different structural features have a functional importance in plants. Although some GR-RBPs have been shown to play roles in posttranscriptional regulation of gene expression in *Arabidopsis* plants under cold, high-salinity, or dehydration stress, other functions must still be verified (Kim et al. 2005; Kim and Kang 2006; Kim et al. 2007a; 2008; Kim et al. 2007b).

Plant GR-RBPs are induced by various stresses, especially low temperatures. Since the first gene encoding GR-RBP was identified in immature maize embryos (Gómez et al. 1988), other such cDNAs have been isolated from monocots, dicots, and gymnosperms, including *WhGRP-1* in wheat (Guiltinan and Niu 1996), *ngRBP* in tobacco (Naqvi et al. 1998), *SCRGP-1* in wild potato (Baudo et al. 1999), *PgRNP* in white spruce (Richard et al. 1999), *sbGR-RNP* in broomcorn (Aneeta et al. 2002), *LpGRP1* in perennial ryegrass (Shinozuka et al. 2006), and *NtGRP1*

in tobacco (Lee et al. 2009). All of the proteins encoded by these genes share similar structural features, including an RRM at the N-terminal half and glycine-rich domains at the C-terminal half. Their expression has been linked to both developmental (Bandziulis et al. 1989; Staiger et al. 2003) and physical influences, including cold, wounding, ABA, jasmonic acid, high salinity, water stress, viral infection, and pathogens (Sachetto-Martins et al. 2000). Although their occurrence has been reported in several species, only a few plant GR-RBPs have been studied for their functions (Lorković and Barta 2002; Fu et al. 2007). Some may act as key posttranscriptional regulators through the trafficking, stabilization, or processing of specific mRNAs in response to pathogens (Fu et al. 2007). In addition, several GR-RBPs circadian oscillations of transcript levels have been demonstrated to be regulated by the circadian clock. Notably, the clock-regulated *AtGRP7* (*AtGR-RBP7*) influences the amplitude of its transcript oscillation by negative feedback at the posttranscriptional level. This autoregulation relies on *AtGRP7* binding to its own pre-mRNA and cause alternative splicing (Staiger et al. 2003; Schöning et al. 2007; 2008). Several *Arabidopsis* GR-RBPs also serve as RNA chaperones for cold adaptation (Kim and Kang 2006; Kim et al. 2007a; b). *Arabidopsis GR-RBP7* is strongly expressed in the guard cells and regulates the opening and closing of stomata in plants under dehydration or salt stress (JS Kim et al. 2008). However, the mechanisms by which GR-RBPs contribute at the molecular level are still largely unknown (Lorković 2009).

Drought is one of the most serious problems for sustainable agriculture worldwide. Woody plants utilize mechanisms for resistance and adaptation to a limited water supply. Drought tolerance is a complex trait that is influenced by the coordinated expression of a network of genes. It is affected by numerous anatomical, physiological, biochemical, developmental, and environmental factors (Ramanjulu and Bartels 2002; Zhu 2002). For promoting agricultural and environmental sustainability, it is important to breed or genetically engineer crops with improved stress tolerance. To understand the molecular mechanism for such tolerance in fruit crops, we focused on *Malus prunifolia* (Willd.) Borkh., a wild species that is naturalized to the desert mountain regions of northwest China. This rootstock is important for the propagation of apples in China and displays strong disease resistance and stress tolerance. In this study, we described the isolation and identification of a new full-length cDNA, *MpGR-RBP1* (*M. prunifolia* glycine-rich RNA-binding protein1), using in silico cloning and reverse transcriptase polymerase chain reaction (RT-PCR). To get an insight into its behavior and regulation by drought stress, we have characterized the expression profile of mRNA from these apple leaves and roots during drought treatment.

## Materials and Methods

### Plant Materials and Drought Treatment

Two-year-old seedlings of *M. prunifolia* were grown in pots in a greenhouse at the College of Horticulture, Northwest A&F University, Yangling (34°20'N, 108°24'E), Shaanxi Province, China. Drought treatments were previously described by Ma et al. (2008). Field experiments were conducted between 20 March and 10 August 2009. After 3 months of development under well-watered conditions, control seedlings continued to receive this standard care while others were progressively stressed by withholding normal irrigation. Leaves and roots from both control and drought-stressed plants were sampled at day 0, 2, 4, 6, 8, 10, and 12 of treatment. All samples were rapidly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Total RNA Extraction and mRNA Isolation

Total RNA was extracted according to the method described by Chang et al. (1993). Poly(A)<sup>+</sup> RNA was purified with a poly(A)<sup>+</sup> Tract<sup>®</sup> mRNA Isolation Systems III kit (Promega, USA) according to the manufacturer's instructions. Three independent biological replications were performed for each experiment.

### Suppression Subtractive Hybridization cDNA Library Construction and EST Analysis

Total RNA was extracted from *Malus* leaves after 6, 8, and 10 days of drought treatment for suppression subtractive hybridization (SSH) and at all sampling time points for gene expression analysis. Total extracted RNA from stressed seedlings was mixed equally as the tester, whereas total RNA from control plants, collected at each corresponding time point, was used as the driver. Our SSH library was constructed according to the method of Diatchenko et al. (1996), using a PCR-select<sup>™</sup> cDNA Subtraction kit (Clontech, USA) and 2  $\mu\text{g}$  of Poly(A)<sup>+</sup> mRNA isolated from leaf tissues. Briefly, the tester and driver cDNAs were digested with *Rsa*I, a restriction enzyme that yields blunt-ended cDNA fragments. The tester cDNA pool was then divided into two portions, each of which was ligated with a different cDNA adapter sequence. Two sequential hybridizations were performed. First, an excess of driver was added to each tester subpool, and the samples were heat-denatured and allowed to anneal to each other. This resulted in the generation of several different hybrid sequences of cDNA. Second, the two tester subpools were mixed together in the presence of excess driver, but without denaturing, to form new hybrids. The ends of the differentially expressed cDNA sequences were

then filled in by DNA polymerase, and two rounds of PCR were performed to enrich these cDNA clones. During PCR, the nondifferentially regulated hybrid clones were either not amplified or did not exhibit exponential amplification. Thus, only differentially regulated genes were enriched by this process. After two rounds of subtractive hybridization and two rounds of suppressive PCR, the products were ligated into the pMD19-T cloning vector (TaKaRa, China), transformed into DH5 $\alpha$  cells (Tiangen, China), and plated onto Luria–Bertani agar plates containing ampicillin ( $100 \mu\text{g mL}^{-1}$ ). Based on their blue/white coloring, recombinant colonies were then selected from those plates. To identify which *M. prunifolia* genes are differentially expressed under drought treatment, all positive clones obtained from the subtraction libraries were subjected to sequencing, clustering, BLAST alignment, functional annotation, and classification into different categories (unpublished data).

### cDNA Synthesis and In Silico Cloning of *MpGR-RBP1*

A 371-bp cDNA fragment of SSH clone D1403, showing high similarity to *GR-RBP*, was used as a query probe to search the EST database in GenBank. Homologous EST clones were retrieved and assembled. To verify this assembly, we used forward primer Mpf (5'-GTCCCATTTGTCAGGGT-TACTG-3') and reverse primer Mpr (5'-ATCT-CAAAAGTCCCAAACCACCTAA-3') to amplify the open reading frame (ORF) of the full-length cDNA. Complementary DNA was synthesized with 2  $\mu\text{g}$  of total RNA, using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega) and the random hexamer primer. PCR was performed with an initial denaturation step at  $95^{\circ}\text{C}$  for 10 min; followed by 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing at  $60^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 90 s; then a final extension at  $72^{\circ}\text{C}$  for 10 min. Products were gel-purified and cloned into the pMD19-T cloning vector. Afterward, both strands of the DNA fragments from positive clones were sequenced with an ABI 3730 sequencer.

### Analyses of Sequences and Phylogenetics

DNA sequence similarities were analyzed using the programs provide by NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast/>). The protein theoretical molecular weight and isoelectric point were predicted using compute pI/Mw (<http://au.expasy.org/tools/>). A hydropathic index was calculated according to the Kyte–Doolittle scale (<http://www.expasy.org/tools/protscale.html>). The signal peptide of the putative protein was forecasted using SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>), and prediction of protein localization sites was analyzed using PSORT (<http://psort.ims.u-tokyo.ac.jp/form.html>). The protein conserved

domain was predicted using SMART ([http://smart.embl-heidelberg.de/smart/change\\_mode.pl](http://smart.embl-heidelberg.de/smart/change_mode.pl)) network services. The protein structure homology modeling prediction and analysis were performed using 3D-JIGSAW (<http://bmm.cancerresearchuk.org/3djigsaw/>) and SWISS-MODEL (<http://swissmodel.expasy.org/>) (Arnold et al. 2006). The deduced amino acid sequence was aligned and the phylogenetic tree was constructed by the ClustalW method using MEGA4.0 software (<http://www.megasoftware.net/>).

#### Quantitative Real-Time PCR

To remove any contaminating genomic DNA prior to cDNA synthesis, we treated the RNA with RNase-free DNase I (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was quantified on a NanoDrop™ 1000 spectrophotometer before and after this DNase I treatment, and its quality and integrity were checked by electrophoresis through agarose gels stained with ethidium bromide. For real-time RT-PCR, first-strand cDNA was synthesized with 2 µg of total RNA in a volume of 20 µL, using a SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa) plus random hexamers and oligo(dT) primers. After reverse transcription, the reaction product was diluted tenfold with sterile water. Real-time PCR was performed on an iQ5.0 instrument (Bio-Rad, USA) using SYBR Green qPCR kits (TaKaRa) according to the manufacturer's instructions. Primer sequences for *MpGR-RBP1* and reference genes were as follows: *MpGR-RBP1*, forward primer 5'-CGTCG TGAGGGTGGCTATG-3' and reverse primer 5'-AAAGT CCCAAACCACCTAACAC-3' (119-bp product); apple *EF-1α*, forward 5'-ATTCAAGTATGCCTGGGTGC-3' and reverse 5'-CAGTCAGCCTGTGATGTTCC-3' (174-bp product); and apple *actin*, forward 5'-CCAAAGGCT AATCGGGAGAA-3' and reverse 5'-ACCACTGGCGTA GAGGAAAG-3' (105-bp product). Real-time PCR reactions were done in 20-µL volumes containing a 10 µM concentration of each primer, 40 ng of cDNA, and 10 µL of SYBR Premix Ex Taq™ II. Thermal cycling conditions included an initial heat-denaturing step at 95°C for 3 min, and then 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s. Fluorescence was measured at the end of each cycle. A melting curve analysis was performed by heating the PCR product from 58 to 95°C. Expression data for *MpGR-RBP1* were presented as relative units after normalization to the *EF-1α* control, using the  $2^{-\Delta\Delta CT}$  method. Values for mean expression and SD were calculated from the results of three independent experiments.

#### Semiquantitative RT-PCR

The relative amount of gene transcription by cloned cDNAs was quantified via RT-PCR. Primer sequences and first-

strand cDNA were the same as those used for quantitative real-time PCR. A PCR Master Mix Kit (TaKaRa) was used with 2 µL (10-dilution) of first-strand cDNA as template plus 0.2 µmol L<sup>-1</sup> of each primer (final concentration) in a total volume of 25 µL. The linear range of detection for each transcript was monitored after 35 cycles for *MpGR-RBP1* in the leaves or roots, or 30 cycles each for *EF-1α* or *actin*. Except for cycle numbers, all other reaction conditions were identical. A 15-µL sample of each PCR product was detected on a 1.2% (w/v) agarose gel, then visualized and photographed under UV light after staining with ethidium bromide. Three repetitions were compared for each sample.

## Results

#### Isolation of Full-Length *M. prunifolia* GR-RBP cDNA

Screening of our SSH cDNA library for drought-stressed seedling leaves from *M. prunifolia* revealed a 371-bp fragment containing a short polyA-tail, D1403, which shared high sequence identity with genes that encode GR-RBPs. Based on this finding, we used in silico cloning and RT-PCR to isolate a novel full-length cDNA sequence (781 bp) with a complete ORF (Fig. 1).

The gene has been submitted to the GenBank database with accession number HM042682. The sequence surrounding the first ATG codon at nucleotide position 32 was in the context of GCCATGG, thus agreeing with that of the Kozak (1987) consensus initiator A/GXXATGG. The cDNA contained a 31-bp 5'-untranslated region (UTR), a 516-bp ORF, and a 234-bp 3'-UTR. The ORF of the *MpGR-RBP1* gene encodes 171 amino acids with an estimated molecular mass of 16.69 kDa and an isoelectric point of 6.36. The deduced protein, MpGR-RBP1, has 38.01% glycine (G), which is due to the glycine-rich region. Transmembrane and signal peptide prediction show that MpGR-RBP1 might belong to nonsecretory protein.

#### High Conservation of *MpGR-RBP1*

We compared the predicted amino acid sequence of *MpGR-RBP1* with known GenBank sequences through BLASTP and PSI-BLAST searches. These revealed that our overall amino acid sequence was very similar to stress-responsive plant GR-RBPs such as Pa-RRM-GRP1 from sweet cherry (Stephen et al. 2003). This apple protein sequence showed high identity to sweet cherry (93.0%, AAL13082), soybean (86.0%, AAD48471), geranium (84.9%, AAB63581), tobacco (84.3%, ACD03270), potato (82.9%, ABB87126), citrus (82.6%, BAA92156), *Arabidopsis* (80.2%, AAM62447), and alfalfa (80.7%, AAF06329), while it



**Fig. 1** Nucleotide sequence of *MpGR-RBP1* cDNA and deduced amino acid sequence. Predicted protein sequence is shown beneath nucleotide sequence. RRM at N-terminal is shaded, and glycine-rich domain within C-terminal is underlined. RNP1 and RNP2 motifs are underlined and marked below sequence. Repeats of Tyr–(Gly)<sub>n</sub>–Arg–Arg–Glu–(Gly)<sub>2</sub> and Tyr–Ser–Arg–(Gly)<sub>4</sub> motif are underlined with arrows. Numbers at left indicate positions in recovered nucleotide sequence, those at right show amino-acid positioning from N-terminus. Initiation codon (ATG) and stop signals (TAG) are underlined in bold. Stop codons are represented by asterisk

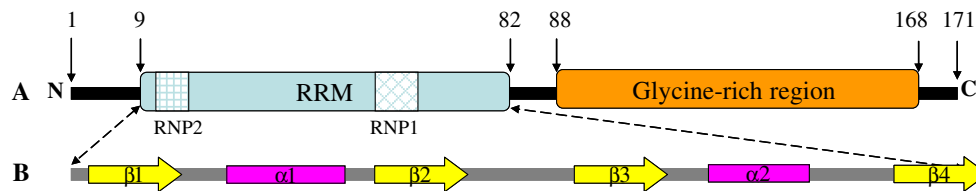
1	GTCCCATTGTCTACTAGGGTTACTGGTAGCC	<b>ATG</b>	GCC	TCT	GCC	GAG	ATC	GAG	TTC											
			M	A	S	A	E	I	E	F										
56	CGT	TGC	TTC	GTC	GGA	GGG	CTC	GCA	TGG	GCC	ACC	GAC	AAT	GAG	GCG	CTG	GAG	AGG	GCC	TTT
	R	C	F	V	G	G	L	A	W	A	T	D	N	E	A	L	E	R	A	F
	RNP2																			
116	TCC	CAG	TAT	GGT	GAG	ATC	ATC	GAA	TCG	AAG	ATC	ATC	AAC	GAC	CGT	GAG	ACT	GGG	AGG	TCC
	S	Q	Y	G	E	I	I	E	S	K	I	I	N	D	R	E	T	G	R	S
176	AGG	GGC	TTC	GGC	TTC	GTC	ACC	TTC	GGC	AGC	GAG	CAG	GCG	ATG	AGG	GAC	GCG	ATC	GAA	GGC
	R	G	F	G	F	V	T	F	G	S	E	Q	A	M	R	D	A	I	E	G
	RNP1																			
236	ATG	AAC	GGC	CAG	AAC	CTC	GAC	GGC	CGC	AAC	ATC	ACC	GTC	AAC	GAG	GCT	CAG	TCT	CGC	GGA
	M	N	G	Q	N	L	D	G	R	N	I	T	V	N	E	A	Q	S	R	G
296	TCC	GGT	GGT	GGT	GGT	GGC	GGA	GGT	GGC	TAC	AGC	CGC	GGA	GGC	GGC	GGT	GGC	GGC	TAC	GGA
	S	G	G	G	G	G	G	G	G	Y	S	R	G	G	G	G	G	G	Y	G
356	GGT	GGC	GGC	GGT	GGA	TAC	GGA	GGT	GGC	CGT	CGC	GGA	GGC	GGT	GGT	GGT	GGG	TAC	AGC	
	G	G	G	G	Y	G	G	G	G	R	R	E	G	G	G	G	G	Y	S	
416	CGT	GGA	GGT	GGT	GGC	GGT	GGA	TAC	GGA	GGT	GGT	GGT	GGT	CGT	CGT	GAG	GGT	GGC	TAT	GGA
	R	G	G	G	G	G	Y	G	G	G	G	G	G	R	R	E	G	G	Y	G
476	GGC	GGT	GAA	GGT	GGC	GGT	GCT	CGC	TAC	TCC	AGA	GGA	GGT	GGT	GGC	GCT	GAA	GGC	GGC	AGC
	G	G	E	G	G	A	R	Y	S	R	G	G	G	G	A	E	G	G	S	
536	TGG	AGG	AGT	<b>TAG</b>	ATTAGT	GTTAGG	TGGT	TTGGG	ACTTTT	GAGAT	GTTAGT	GTTGCC	GTTT	CTCG	TGGT	TGTT	TTA			
	W	R	S	*																
611	GTGTCATGGTCTGTTCTGGTGTGGT	TTCCGGT	AGTTCGAT	GGTATTGGT	GTGTGTGTT	TTGTCTCGATA	ATTTTGAT													
690	CGATGATCCTATCTTTACTTTTCCAT	GTATTTTGGT	TTCCAAAGA	CAAAAAAT	TGGTGT	TTGAGAAAA	AGTTGTT	CGTT												
769	CGCATAAAAAAAA																			

exhibited lower identities to maize (74.4%, AAM16020), broomcorn (73.3%, AAG23220), and wheat (72%, BAF30986). In addition, the putative MpGR-RBP1 protein was almost the same size as GR-RBP protein from broomcorn (AAG23220) and barley (CAA88558) (Table 1). Because of these apparent structural characteristics, we designated the gene as *MpGR-RBP1* (*M. prunifolia* glycine-rich RNA-binding protein1).

The predicted amino acid sequence had two main features, an amino-terminal RRM and a glycine-rich carboxy-terminal domain. The RRM was characterized by 77 amino-acid residues, with a hydrophobic segment of six residues (RNP2) and an octapeptide motif of eight residues (RNP1) (Fig. 2a) (Stephen et al. 2003). The glycine-rich domain ranged from 88 to 168 amino-acid residues interspersed with two short, highly conserved sequences—

**Table 1** Comparison of the deduced amino acid sequences from *MpGR-RBP1* with known related protein sequences

Species	Accession no.	Identity (%)	Length (amino acid)
<i>M. prunifolia</i> (apple)	HM042682	100.0	171
<i>P. avium</i> (sweet cherry)	AAL13082	93.0	178
<i>Rumex obtusifolius</i> (bitter dock)	CAD29693	86.7	168
<i>Glycine max</i> (soybean)	AAD48471	86.0	160
<i>Gossypium hirsutum</i> (cotton)	ACJ11730	86.0	168
<i>Pelargonium hortorum</i> (geranium)	AAB63581	84.9	166
<i>Nicotiana tabacum</i> (tobacco)	ACD03270	84.3	157
<i>Ricinus communis</i> (castor bean)	CAC80549	83.7	165
<i>Solanum tuberosum</i> (potato)	ABB87126	82.9	178
<i>Citrus unshiu</i> (citrus)	BAA92156	82.6	167
<i>Medicago sativa</i> (alfalfa)	AAF06329	80.7	105
<i>Arabidopsis thaliana</i>	AAM62447	80.2	175
<i>Hordeum vulgare</i> (barley)	CAA88558	78.3	173
<i>Oryza sativa</i> (rice)	CAA05728	77.6	160
<i>Picea glauca</i> (white spruce)	AAD28176	75.6	155
<i>Zea mays</i> (maize)	AAM16020	74.4	155
<i>Sorghum bicolor</i> (broomcorn)	AAG23220	73.3	170
<i>Triticum aestivum</i> (wheat)	BAF30986	72.0	163
<i>Cryptomeria japonica</i> (Japanese cedar)	BAE92287	70.9	181



**Fig. 2** Putative conserved domain structure and schematic model for predicted amino acid sequence. **a** Model shows amino-terminal RRM and carboxyl-terminal glycine-rich domain. Within RRM are two conserved regions, hydrophobic six-residue RNP2 motif, and eight-residue RNP1. Numbers at top refer to amino-acid positioning from

N-terminal methionine. **b** Putative conserved domain structure of RRM. Secondary-structure prediction indicates that RRM structure comprises four antiparallel  $\beta$ -strands and 2  $\alpha$ -helices arranged in  $\beta$ - $\alpha$ - $\beta$ - $\beta$ - $\alpha$ - $\beta$  fold with side chains

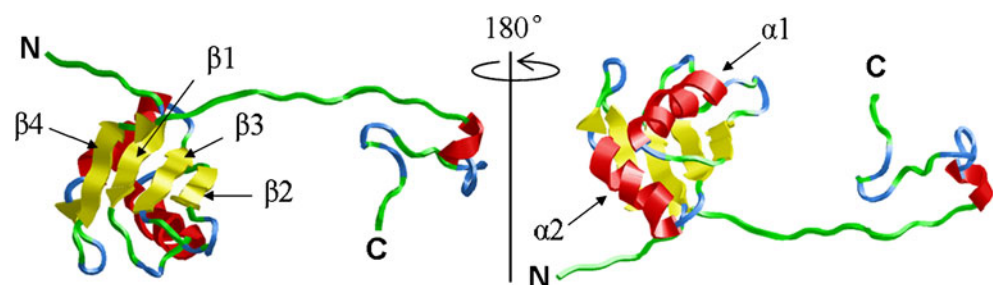
Gly/Gly/Tyr/Gly/Gly(GGYGG) and Arg/Arg/Glu(RRE)—both of which are also abundant in an ABA-inducible GR-RBP from maize embryos (Gómez et al. 1988). Furthermore, the C-terminal glycine-rich domain was mainly interposed with arginine and tyrosine (the Arg/Gly/Tyr(RGY)-rich domain) (Kumaki et al. 2004). The classic RGY-rich domain contained two similar peptides (YGGGGRREGG and YGGGGRREGG) and three consensus repeats (YSRGGGG) (Fig. 1). The predicted secondary structure provided long stretches of extended conformation. Such a structural pattern for this RRM (Fig. 2b) fit well with a model of four antiparallel  $\beta$ -strands and two  $\alpha$ -helices arranged in a  $\beta_1$ - $\alpha_1$ - $\beta_2$ - $\beta_3$ - $\alpha_2$ - $\beta_4$  fold with side chains (Birney et al. 1993). The three-dimensional (3-D) model of the protein was built using 3D-JIGSAW program (Fig. 3), and the protein structure homology modeling alignment and analysis were performed using SWISS-MODEL (Arnold et al. 2006). Depending on the SWISS-MODEL Web server, we built and evaluated the MpGR-RBP1 protein homology model based on homologous template: 3bs9B.pdb. Accordingly, the 3-D model of MpGR-RBP1 showed that the N-terminal RRM folds ( $\beta\alpha\beta\beta\alpha\beta$  topology) of MpGR-RBP1 structure most closely matched the RRM2 of the RNA binding protein TIA-1 (T-cell-restricted intracellular antigen-1) (Kumar et al. 2008; PDB-code=3bs9B; RMSD=1.95 Å and 38.5% sequence identity for residues 7–84). Therefore, these results suggested that MpGR-RBP1 is a member of the GR-RBPs family and is structurally conserved.

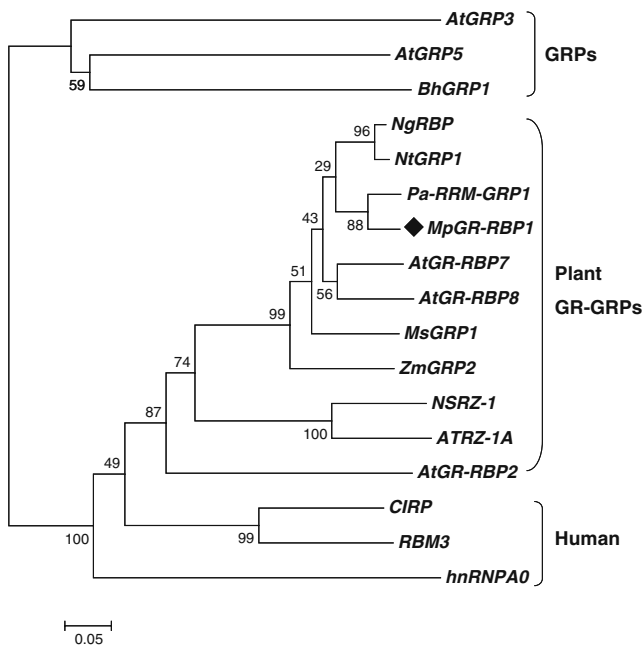
#### Phylogenetic Analysis of the Predicted MpGR-RBP1 Protein

In order to investigate the evolutionary relationships of MpGR-RBP1 protein among plant GR-RBPs, GRPs, and human hnRNPs, we constructed a phylogenetic tree (Fig. 4) that enabled us to group all of them into three distinct clusters. MpGR-RBP1 protein belonged to a cluster that does not include GRPs or human hnRNPs. MpGR-RBP1 was closely related to Pa-RRM-GRP1 with 80.6% nucleotide identity, which was consistent with our observations described above. Phylogenetic analysis demonstrated that plant GR-RBPs within the same species are more closely related to each other than to GR-RBPs from different species, e.g., NgRBP and NtGRP1 from tobacco or AtGR-GBP7 and AtGR-GBP8 from *Arabidopsis*, with Pa-RRM-GRP1 (*Prunus*) and MpGR-RBP1 (*Malus*) being subordinate to the Rosaceae family. ATRZ-1A is phylogenetic closeness to NSRZ-1 in that they have one zinc fingers in their glycine-rich domain, while the *Arabidopsis* cold induced proteins AtGR-GBP2 (namely AtGRP2) has two zinc fingers as described above. A recent research shows that AtGR-RBP2 is localized into mitochondria of *Arabidopsis* and may play a role in the multiple posttranscriptional mechanisms involved in mitochondrial gene expression (Vermel et al. 2002).

Another phylogenetic cluster contains CIRP (cold-inducible RNA-binding protein; A18 hnRNP), RBM3 (also known as *IS1*-RNPL or RNPL), and hnRNPA0 (also known

**Fig. 3** Structural mode of MpGR-RBP1, with RRM folds ( $\beta\alpha\beta\beta\alpha\beta$  topology).  $\alpha$ -Helices are colored red,  $\beta$ -sheet are colored yellow, and strands are colored blue–green, respectively. Three-dimensional representations are done with RasTop (<http://www.geneinfinity.org/rastop/>)





**Fig. 4** Phylogenetic tree of *MpGR-RBP1* with other plant GR-RBPs, GRPs, and human hnRNPs was reconstructed by the neighbor-joining method. The tree is based on an alignment corresponding to full-length amino acid sequences, using ClustalW and MEGA 4.0. Branches are labeled with gene names, and *MpGR-RBP1* is indicated by asterisk. The number shown at the branches denote the bootstrap majority consensus values of 1,000 replicates. The GenBank accession numbers for the sequence designations are as follows: *A. thaliana* (AtGRP3, S47409), *A. thaliana* (AtGRP5, S47414), *Boea hygrometrica* (BhGRP1, EU003996), *Nicotiana glutinosa* (ngRBP, AF005359), *N. tabacum* (NtGRP1, EU569289), *A. thaliana* (AtGR-RBP7, AAD23639), *M. sativa* (MsGRP1, AF191305), *P. avium* (Pa-RRM-GRP1, AY050483), *A. thaliana* (AtGR-RBP8, NP\_195637), *Z. mays* (ZmGRP2, NP\_00115156), *A. thaliana* (ATRZ-1A, NM\_113549), *Nicotiana sylvestris* (NSRZ-1, BAA06012), *A. thaliana* (AtGR-RBP2, CAB36849), *Homo sapiens* (CIRP, NP\_001271), *H. sapiens* (RBM3, NP\_006734), and *H. sapiens* (hnRNPA0, NP\_006796)

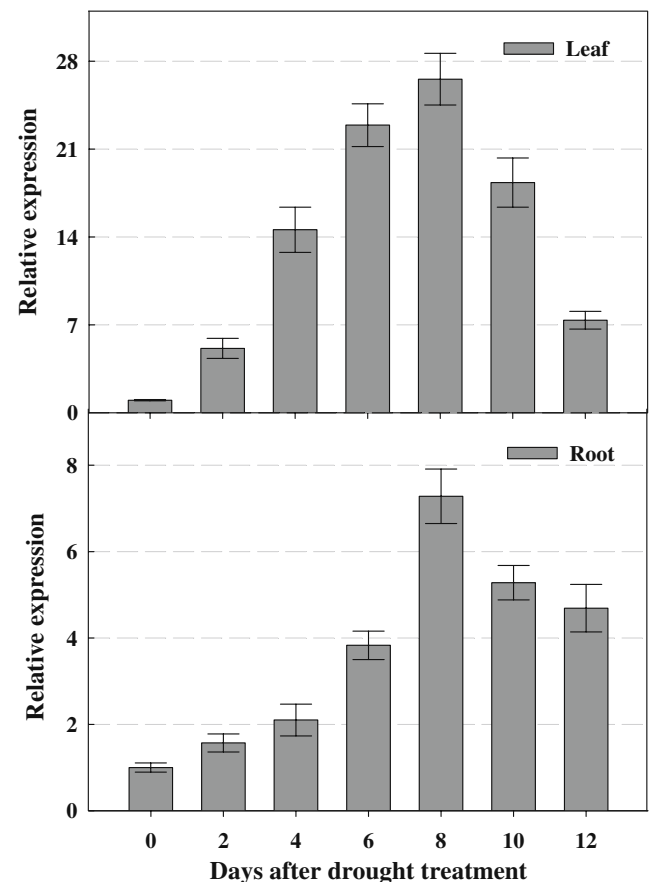
as hnRPA0). The first two hnRNPA0s have a single RRM domain. In contrast, the third one has two repeats of quasi-RRM domains that bind RNAs and a glycine-rich C-terminus (Rousseau et al. 2002). Independent clusters are used to distinguish among GRPs, BhGRP1, AtGRP5, and AtGRP3. Our results indicated that the current clustering of *MpGR-RBP1* is based on its complete primary sequences and species.

#### Stress-Responsive Expression Pattern of *MpGR-RBP1*

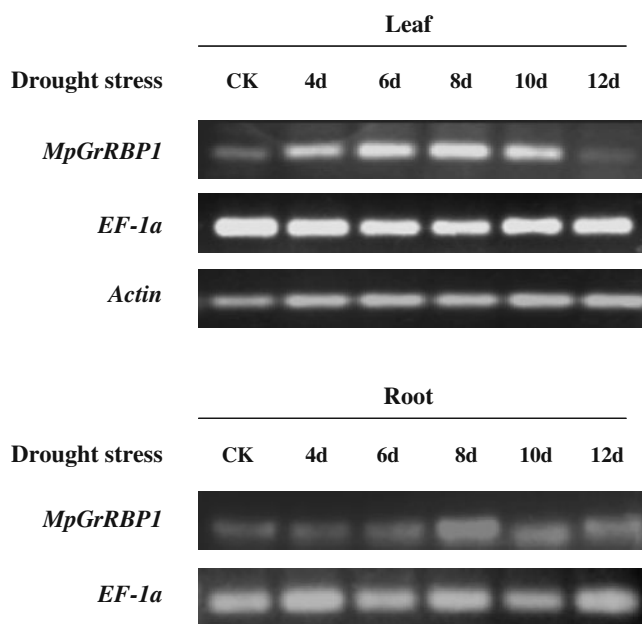
To investigate the effect of drought stress on *MpGR-RBP1* expression, we performed quantitative real-time RT-PCR. This gene was induced in both roots and leaves during treatment. Transcripts within the latter were initially increased up to 26-fold over that of the control (as

measured on day 0) in the first 8 days of drought stress before decreasing by approximately sevenfold at day 12. In contrast to this strong up-regulation in the leaves, expression in the roots increased by only about sevenfold during the first 8 days of treatment, followed by a slight decrease by day 10 (Fig. 5). Real-time PCR results showed that transcription levels were much higher in the leaves than in root tissues.

Expression was analyzed at the mRNA level at different time points during the stress period. Semiquantitative RT-PCR showed that transcripts of *MpGR-RBP1* were elevated in both leaves and roots between days 4 and 8 (Fig. 6). After peaking, this expression began to decline from day 10 to 12. By comparison, no significant changes were observed in the transcript levels of *EF-1 $\alpha$*  and *actin*. These RT-PCR results were in agreement with data from our quantitative real-time PCR analysis.



**Fig. 5** Quantitative real-time RT-PCR analysis of the *MpGR-RBP1* expression level in leaves and roots from *Malus prunifolia* under drought stress. Two-year-old potted seedlings were grown in 14-h light/10-h dark cycles during water treatment period. Leaves and roots of plants were harvested at midday, respectively. Transcript levels in seedlings stressed for 0, 2, 4, 6, 8, 10, or 12 days were plotted as expression (*n*-fold) relative to nonstressed control (day 0). Amount of transcript was normalized to apple *EF-1 $\alpha$*  expression level. Mean values and standard errors (bars) were obtained from three independent experiments per time point



**Fig. 6** Semiquantitative RT-PCR analysis of *MpGR-RBP1* expression under well-watered conditions (CK) and in response to drought (4, 6, 8, 10, or 12 days). Leaves and roots were harvested at indicated times after stress period. Accumulation of *MpGR-RBP1* was monitored by semiquantitative RT-PCR. *EF-1 $\alpha$*  or *actin* was used as constitutive controls

## Discussion

Plant GR-RBPs play an important role in normal developmental processes and also participate in adaptations to various environmental conditions. Although a few such GR-RBPs involved in abiotic stress responses have been identified in *Arabidopsis*, rice, and tobacco (Sahi et al. 2007; JS Kim et al. 2008; Lee et al. 2009), little information has been reported for GR-RBPs in *Malus* plants against water stress. Therefore, it is necessary to investigate this relationship because the development of drought-resistant rootstocks through the modification of stress-related genes can lead to enhanced fruit crop productivity.

Here, we reported the isolation and characterization of a novel GR-RBP gene, *MpGR-RBP1*. Like the orthologous genes in human and other plants, *MpGR-RBP1* protein displays considerable sequence conservation in the RRM motif and the glycine-rich region, suggesting conservation of functional properties. The RRM conserved motif at the N-terminal end contains two short sequences, RNP1 and RNP2. Evidence indicates that RRMs are present in a variety of RBPs and are also found in a few single-stranded DNA binding proteins (Birney et al. 1993; Lorković and Barta 2002). Proteins with RRM are involved in the posttranscriptional regulation of gene expression and pre-rRNA processing, as well as in RNA processing at several levels (Burd and Dreyfuss 1994; Guiltinan and Niu 1996).

The 3-D model of *MpGR-RBP1* shows significant homology with human RNA binding protein TIA-1. Human TIA-1 regulates alternative pre-mRNA splicing in the nucleus, and mRNA translation in the cytoplasm, by recognizing uridine-rich sequences of RNAs (Kumar et al. 2008). This suggests that *MpGR-RBP1* might have similar functions to the human protein.

Database searches of our sequence demonstrated that *MpGR-RBP1* has significant homology to GR-GRP protein family genes that are responsive to abiotic stress. Phylogenetic analysis further demonstrated that *MpGR-RBP1* can be highly homologous to some other plant GR-RBPs, in particular, the one encoded by *Pa-RRM-GRP1* (AY050483 from *Prunus avium*), rather than to *Arabidopsis* GR-RBP7 and GR-RBP8. These GR-RBP proteins are distinct from members of the group of GRPs, which lack a conventional RRM, such as a drought-induced cell-wall GRP protein AtGRP3 and a vacuole-located GRP AtGRP5 (Mangeon et al. 2009). The current data indicate that the *Arabidopsis* genome encodes eight GR-RBPs (AtGR-RBP1–AtGR-RBP8), all characterized by the presence of an N-terminal RRM domain and a C-terminal tail of varying lengths and all rich in glycine residues. Among them, AtGR-RBP7 and AtGR-RBP8 are homologous to each other (76.9% identity) and probably perform related functions (Fu et al. 2007; Lorković 2009). However, AtGR-GRP7 differs from AtGR-GRP2 and AtGR-GRP4 in that it has a much shorter N-terminal region and a much longer glycine-rich C-terminal region (JS Kim et al. 2008).

Plants produce a large number of kingdom-specific RNA-binding proteins. RBPs contain one or more RRMs at the N-terminus and a variety of auxiliary motifs at the C-terminus, such as glycine-rich, arginine-rich, SR-repeat, RD-repeat, and acidic domains (JS Kim et al. 2008; Lee et al. 2009). Plant GR-RBPs belong to the family of RNA-binding proteins that has in common a protein domain that is necessary and sufficient for RNA binding. Over time, only those proteins required for basic mechanisms in posttranscriptional regulation of gene expression have been preserved in all eukaryotic lineages (Lorković and Barta 2002; Fu et al. 2007). Our results further support that *MpGR-RBP1* protein is in that plant GR-GRP family. However, because GR-RBPs are encoded in the genomes of different species, certain variations do exist, and so researchers must still determine whether functioning of a particular protein is similar or equivalent to other plant GR-RBPs.

Abiotic stresses, such as drought, modulate expression at the posttranscriptional level as well as at the transcriptional level (Sunkar and Zhu 2004). Posttranscriptional mechanisms for the regulation of gene expression involve a control of pre-mRNA processing, transportation to cytoplasm, and mRNA stability. These regulatory mechanisms are mediated by specific interaction between RNA-binding



proteins and RNA molecules (Shinozuka et al. 2006). Plant GR-RBPs are involved in posttranscriptional RNA processing in response to various stress conditions. For example, transcripts of rice *Osg-rbp4* are constitutively expressed as well as regulated by high temperatures (Sahi et al. 2007). A recent report shows that *AtGR-GRP7* and *AtGR-GRP8* are rapidly up-regulated in response to oxidative stress (Schmidt et al. 2010). Here, we show that *MpGR-RBP1* gene expression is strongly up-regulated upon drought stress. This observation is consistent with the results shown by Lee et al. (2009), where tobacco *NtGRP1* is also up-regulated by drought, albeit to a much lower degree compared to flooding stress. On the contrary, the transcript level of *GR-RBP4* in *Arabidopsis* decreases slightly with dehydration stress (Kwak et al. 2005). Recent studies have shown that *Arabidopsis* GR-RBP2, -4, and -7, as well as atRZ-1a, are differentially involved in plant responses to abiotic stresses also, but may be either ABA independent (*AtGR-RBP2* and *AtGR-RBP7*) or dependent (*atRZ-1a*). Thus, both transcriptional reprogramming and posttranscriptional mechanisms are utilized for coping with stress (Lorković 2009).

Furthermore, to avoid bias while investigating *MpGR-RBP1* transcription, we selected two internal control genes, *actin* and *EF-1 $\alpha$* , both of which are consistently expressed in apple and grapevine during biotic or abiotic stress (Nicot et al. 2005; Paris et al. 2009). Our results show that *EF-1 $\alpha$*  gene used as a standard is more validated than *actin*, which is consistent with the results that *EF* gene proved to be the most stable among all the housekeeping genes tested (Nicot et al. 2005). Expression of *MpGR-RBP1* was increased in the roots and even more so in the leaves. Similarly, in *Arabidopsis*, real-time RT-PCR and GUS expression analyses have shown that *AtGR-RBP4* is abundantly expressed in young plants, root tips, and flowers, but only weakly in mature leaves and stems (Kwak et al. 2005). Overexpression of *AtGR-GRP2* and *AtGR-GRP7* enhances cold and freezing tolerances, and *AtGR-RBPs* exert RNA chaperone activity during the process of cold adaptation (Kim et al. 2007a). Therefore, we can speculate that the elevated expression of *MpGR-RBP1* also plays a critical role for the drought adaptation of *Malus* plant.

In conclusion, the results of the current study provide clear evidence that *MpGR-RBP1* is structurally conserved and may be involved in posttranscriptional RNA processing in response to water stress. Although we have not yet fully described its functioning, our results suggest that this protein is active in the adaptation process because its mRNA level increases following exposure to drought treatment. Therefore, it will be important to further evaluate the regulatory function of *MpGR-RBP1* in *Malus* plants and the molecular mechanisms that control its expression under different environmental conditions.

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