Functional analysis of glycin rich- RNA binding protein, a suppressor of trehalose-6-phosphate mediating growth arrest in *Arabidopsis thaliana*

Mahnaz Aghdasi[1,2*], Henriette Schluepmann[2]

[1]Department of Biology, Faculty of Science, Golestan University, P.O. Box 155, Gorgan, I.R. Iran [2]Department of Molecular Plant Biology, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3584 CH, The Netherlands

Abstract
Metabolism of the alpha-1,1 glucose disaccharide, trehalose, is indispensable in plants. In the Murashige and Skoog (MS) medium, trehalose inhibits plant growth and allocation of carbon to roots. A suppressor of trehalose-6-phosphate (T6P) mediated growth arrest, *GR-RBP2,* is characterized in more detail. Phylogenetic analysis revealed that GR-RBP2 is a protein of likely prokaryotic origin. A knockout mutant of *GR-RBP2* was identified in the T-DNA insertion line SALK-059714, yet plants of this line were not altered with regard to growth on different carbon sources and on trehalose compared to WT. GUS expression analysis showed that *GR-RBP2* was detected in adult leaves, flowers and siliques. Expression was particularly high in root tips. *GR-RBP2* expression also is insensitive to 100 mM trehalose. TAP-tagged versions of this protein showed that GR-RBP2 is part of a protein complex in *planta*.
Keywords**:** *Arabidopsis*; GR-RBP2; Gus fusion; Tap-taged; Trehalose

INTRODUCTION

Trehalose is the alpha, alpha-1,1-linked glucose disaccharide, which is found in a wide variety of organisms (Elbein *et al*., 2003). Its metabolism has recently been recognized to play an important role in carbon signaling in plants (Paul *et al*., 2008; Rolland *et al*., 2006).

Correspondence to: **Mahnaz Aghdasi, Ph.D.**
Tel: +98 171 2327520*; Fax: +98 171 2322906*
E-mail: Aghdasi46@yahoo.com; M.aghdasi@gu.ac.ir

Genes coding for the components involving in the trehalose metabolism have been identified in Planta recently. Synthesis of trehalose in plants is typically via its phosphorylated intermediate, trehalose-6-phoaphate (T6P). Trehalose-6-phoaphate synthase (TPS) converts UDP-glucose and glucose-6-phosphate to T6P. Trehalose phosphate phosphatase (TPP) dephosphorylate T6P to trehalose. Trehalase cleaves trehalose to two glucose molecules. There are 11 TPS and 10 TPP homologues in *ArabidoApsis*. Whilst only one gene encodes trehalase (Vandesteene *et al*., 2010; Pramanic and Imai 2005; Leyman *et al*., 2001). Minor alterations of T6P steady states in plants, yield dramatic and pleiotropic phenotypic changes (Pramanic and Imai 2005; Pellny *et al*., 2004; Schluepmann *et al*., 2003). Additionally, deletion of the T6P synthase (TPS), *AtTPS1*, in *Arabidopsis* is lethal and can be overcome by complementation with active TPS enzyme (Schluepmann *et al*., 2003; Eastmond *et al*., 2002). Current evidences suggest an important regulatory role for T6P such as carbon utilization in *Arabidopsis* seedlings (Schluepmann *et al*., 2004). It is not understood however, how T6P controls carbon utilization and why changes in the steady state level of T6P yield such strong phenotypic changes.

Trehalose feeding to *Arabidopsis* seedlings inhibits root growth, emergence of priming leaves and allocation of carbon to the root and shoots by a negative feedback (Aghdasi and Schluepmann 2009; Ramon *et al*., 2007; Fritzius *et al*., 2001; Wingler *et al*., 2000). Increased trehalose and T6P also affect starch synthesis via the control of T6P over AGPase

redox activation (Kolbe *et al*., 2005). Trehalose feeding induces massive starch accumulation in sources tissues and inhibits starch accumulation in the columnella cells of root tips (Aghdasi and Schluepmann 2009; Ramon *et al*., 2007; Wingler *et al*., 2000).

To understand how trehalose-6-phosphate control over growth, mutants were identified that accumulate similar amounts of T6P as WT, but that grows on trehalose medium, unlike WT. The screening has been performed in the Leclere and Bartel mutant collection (Leclere and Bartel, 2001). Interestingly, two of the trehalose resistant (*trr*) mutants express glycin-rich RNA-binding protein2 (GR-RBP2). Results from expression analysis and re-transformation with the CaMV35S/cDNA constructs showed that over-expression of *GR-RBP2* suppress trehalose-*6*-phosphate mediated growth arrest (Aghdasi *et al*., 2007).

Proteome analysis revealed that GR-RBP2 is localized into the mitochondria of *Arabidopsis* (Kruft *et al*., 2001). The GR-RBP2 protein is only 153 amino acids long. GR-RBP2 has a mitochondrial targeting sequence (amino acid residues 1-33). Amino acid residues 34-113 contain the RNA recognition motif (RRM) domain, with the consensus sequences ribonucleoprotein (RNP)-1 and RNP-2 RNA binding domains (Nomata *et al*., 2004). Residues 122-153 contain the glycin-rich domain thought to be involved in protein-protein interactions (Lorkovic and Barta, 2002). The typical RRM consists of four anti-parallel β-strands and two α-helices arranged in a β-β, α-β fold with side chains that stack with RNA bases. RRM proteins are essential for regulation of post-transcriptional processes. The *Arabidopsis* genome encodes 200 RRM-containing proteins, among which eight proteins are classified into GR-RBP1-8 (Lorkovic, 2009; Lorkovic and Barta, 2002). Expression of several of this GR-RBP is induced by various stresses. Meanwhile it has been shown that GR-RBP3 has non-specific RNA and DNA binding ability (Carpenter *et al*., 1994; Fang *et al*., 1991; Condit and Meagher, 1987).

By using a reverse genetic approach, we attempted to identify the processes that are controlled by T6P in plants. Phylogenetic analyses were carried out to identify GRRBP2 homologues in *Arabidopsis*. In an attempt to reveal GR-RBP2 protein function, identified T-DNA insertion lines were characterized. Transcriptional fusion constructs were made to visualize expression pattern of GR-RBP2. Tagged versions of GR-RBP2 were expressed to identify potential protein complexes with these proteins.

MATERIALS AND METHODS

Phylogenetic analyses: Protein sequences of GR-RBP2 were obtained from the TAIR database (WWW.Arabidopsis.org). The BLAST searches were performed, using the TAIR and NCBI BLAST programs at http:// *Arabidopsis*.org/ and http://ncbi.gov/, respectively. The multiple sequence alignment and the respective phylogenic tree were performed at http://www.ebi.ac.uk/clustalw/, using the ClustalW method, scoring matrix Blosum, gap penalty of 10.

Plant materials: Seeds obtained from *Arabidopsis* transformed plants were sterilized by the gas-sterilization method (Salinas-Jose and Sanchez-Serrato, 2006). *Arabidopsis* seedlings were grown in agarose solidified (0.8%) half strength Murashige and Skoog (Murashige and Skoog, 1962) medium supplemented with antibiotics as required for selection, 100 mg/l gentamycin, 100 mg/l spectinomycin, 12.5 µg/l hygromycin and 50 mg/l kanamycin. The plants were grown under long-day conditions at 22ºC in soil.

Vector construction and plant transformation: To detect the expression pattern of *GR-RBP2*, fragments containing 2 kb promoters and the 5′ UTRs of this gene was fused to the GUS sequence in vector pMDC162 (Curtis and Grossniklaus, 2003). The genomic sequences were PCR amplified from Col-0 using a two step procedure. In step one, amplification of template DNA was done by using template specific primers (Forward: 5′-AAAAAGCAGGCTTCGGTCTACTTACCTCCAGAT-3′ and Reverse: 5′-AGAAAGCTGGGTATCTACAAAGTTCCAAAAACAC-3′). Amplification conditions were performed as initial DNA denaturation at 95ºC for 2 min followed by 10 cycles of 15 second denaturation at 94ºC, 30 second annealing at 56ºC and 2 minutes of extension at 72ºC with a final extension time at 72ºC for 10 minutes. Then 10 µl of the first PCR product was used in the second PCR step, as a template. In this step, attB1 and attB2 adapter primers (Forward: 5′-GGGGACAAGTTTGTACAAAAAAGCAC-3′ and Reverse: 5′- AGGGACCACTTTGTACAAGAAAGCTGGGT-3′) were used. The PCR amplification is carried out in two phases. The initial denaturating was done at 95ºC for 2 min, followed by 5 cycles of 94ºC for 15 s, annealing at 45ºC for 30 second and extension at 72ºC for 2 min. The second amplification was done at 94ºC for 15 second, annealing at 55ºC for 30 second and extension at 72ºC for 2 min. and a final extension at

72ºC for 10 min. PCR product was separated on agarose gel and the specific band was cut and cleaned using a DNA purification kit (Amersham, Biosciences, England).

PCR amplified fragments were cloned into pDONR201 by using the GateWay^R cloning system and the sequence was confirmed by DNA sequencing. Transferring fragments from pDONR201 to pMDC162 (*GUS* reporter gene) vector was performed using the recombination reaction as described by the manufacturer (Invitrogen, Carlsbad, CA). The construct was transformed into WT Col-0 plants by the floral dip method (Clough and Bent 1998) using *Agrobacterium tumefaciens* pGV2260. Transformed seedlings were selected on ½ MS supplemented with 12.5 µg/l hygromycin. The transformed seedlings were further grown on soil in long day conditions (16 h light, 8 h dark) at 22ºC to obtain seeds.

Translational fusions with the Tandem Affinity Purification (TAPa)-tag: To generate C-terminal fusions with the TAPa-tag, the cDNA's encoding the full-length versions of GR-RBP2 were amplified by PCR and cloned into pDONR201 (Rubio *et al*., 2005). The transfer of genes from pDONR201 to TAPa vector was performed using the recombination reaction as described by the manufacturer (GateWay, Invitrogen, Carlsbad, USA). Constructs were transferred to wild-type plants (Col-0) by the floral dip method (Clough and Bent 1998). Transgenic seedlings were selected on solidified ½ MS medium supplemented with 100 mg/l gentamycin. The selected lines were grown on soil to obtain seed.

Western blot analyses: Protein fractions separated by SDS-PAGE (12% polyacrylamide) were blotted to polyvinylidene membrane (Millipore, Bedford, USA) in running buffer (3g Tris, 14.2 g glycine, 200 ml EtOH, 0.8 g SDS per litter) for 1.5 hour at 100 V, on ice. The membranes were blocked in phosphate buffered saline (PBS) supplemented with 5% w/v Milk (PBSM), incubated overnight with a 3000 fold dilution of Anti-myc primary antibody from mouse (Santa Cruz, biotechnology, Santa Cruz, CA) in PBSM. Unbound antibody was washed 4 times with PBS supplemented with 0.1% v/v Tween 20 (Merck, Hohenbrunn, Germany). The blots were incubated 1.5 hours with a 3000 fold dilution of anti-mouse IgG secondary antibody conjugated to horseradish peroxidase in PBSM. Unbound antibody was washed 4 times in PBS with 0.1% v/v Tween 20, before a one minute reaction with enhanced chemi-luminescence reagent (Sigma, USA) at room temperature and exposure of the blots to X-ray film for 5 minutes (Kodak, Sigma-Aldrich, Germany).

Gel filtration on Sepharose S-300: Protein was extracted from frozen ground leaf material (0.5g) in 500 µl ice-cold buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% v/v glycerol, 0.1% w/v Nonidet P-40, 1 mM DTT, 1 mM PMSF, and 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The resulting crude extract was filtered through four layers of cheesecloth, and then centrifuged at 12,000 ×*g* (Eppendorf, UK) for 10 min at 4°C. The supernatant fraction (300 µg) was loaded onto a Superdex S-300 column (Amersham Biosciences, England) equilibrated in PBS and a flow rate of 0.1 ml/min. In each 1 ml, sixty fractions were collected. Proteins in each 3rd 1 ml fraction were concentrated using 10 ul Strataresin (Stratagene company,La Jolla, USA). Resin bound proteins (5 ul) was fractionated by SDS-PAGE using 12% w/v polyacrylamide gels, that were either stained with Coomassie blue (R250, Sigma) or blotted for detection of the TAP-tag as described in the previous section.

Tandem affinity purification: TAPa-tagged proteins were purified essentially as described by Rubio *et al*. (2005). For protein extraction, fifteen gram of fresh leaves was harvested, snap frozen ground in liquid nitrogen with mortar and pestle. Protein was extracted in buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 10% v/v glycerol, 0.1% w/v Nonidet P-40) supplemented with 1 mM PMSF, and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The crude extract was filtered through four layers of cheesecloth and centrifuged at 12,000 ×*g* for 10 min at 4°C. The protein concentration in the supernatant was determined by Bradford assay (Bradford, 1976). Extracts containing similar amounts of total protein were incubated with 500 µl IgG beads (Amersham Biosciences, UK) for 2 h at 4°C with gentle rotation. After centrifugation at 150 g for 3 min at 4°C, the IgG beads were recovered and washed three times with 10 ml of washing buffer (50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 10% v/v glycerol, 0.1% w/v Nonidet P-40) and once with 10 ml of cleavage buffer (50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 10% v/v glycerol, 0.1% w/v Nonidet P-40, 1 mM DTT). Elution from the IgG beads was performed by 3h incubation with 50 µl (100 units) of 3C protease

(Precission protease; Amersham Biosciences, UK) in 2 ml of cleavage buffer at 4°C with gentle rotation. Released protein was recovered in the supernatant after centrifugation at 150 g for 3 min at 4°C and, after addition of 1 ml cleavage buffer to the beads and re-centrifugation, in the second supernatant; the supernatants were pooled and stored at 4ºC. Pooled released protein was loaded onto 0.5 ml of Co-NTA resin (Qiagen, Valencia, CA, USA) packed into a 10-ml disposable polyprep chromatography column (Bio-Rad). To increase the likelyhood of binding, the flow-through was loaded again onto the Co-NTA column before washing with 5x 1 ml washing buffer (50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 10% v/v glyc-

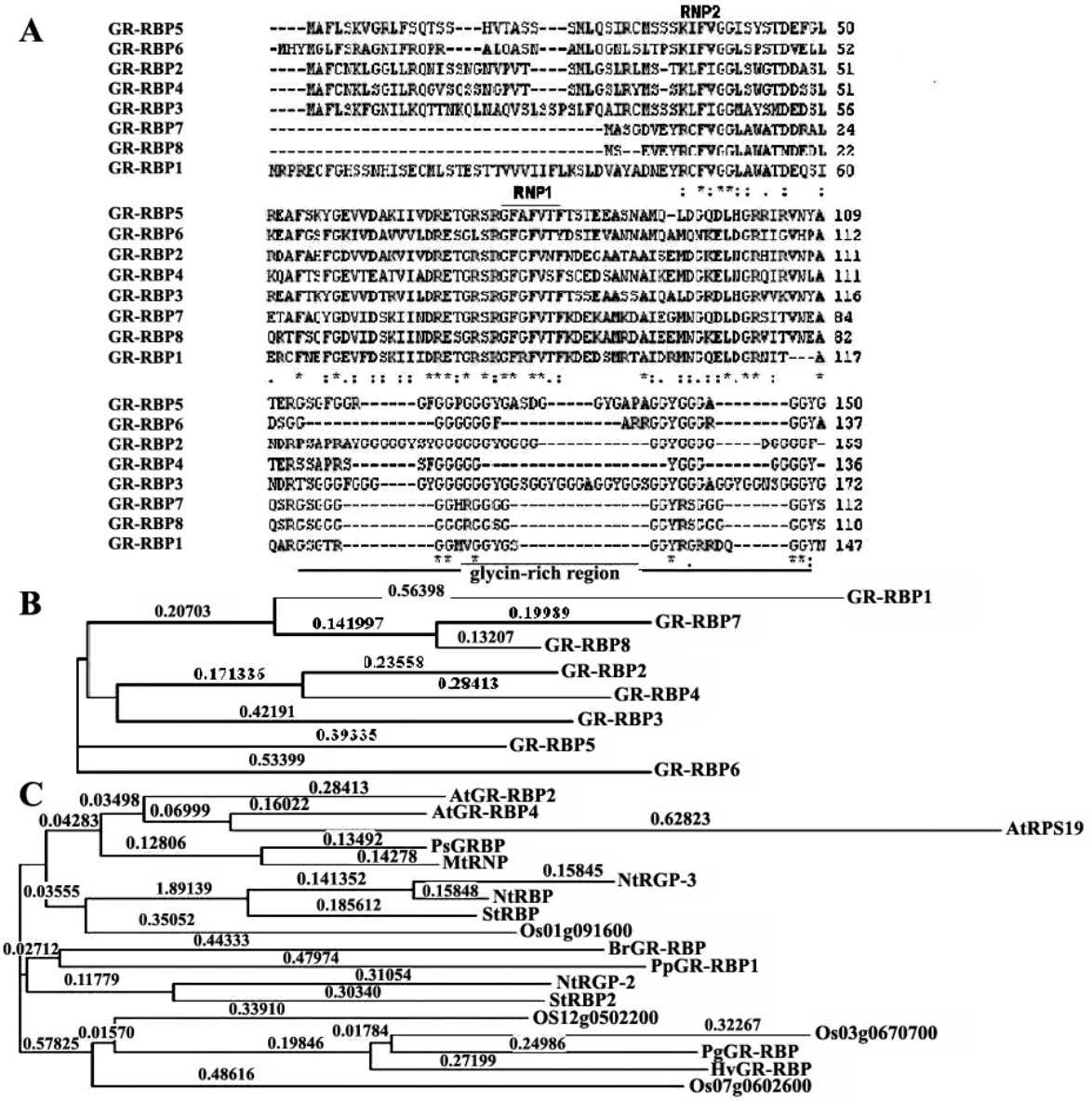

Figure 1. *Arabidopsis* GR-RBPs paralogs and ortologs. A: Aligned amino acid residues of GR-RBP protein in *Arabidopsis*. The lines indicate conserved motifs in the RRM (RNP1, RNP2) and the glycin-rich domain. B: Phylogram derived from alignement of the eight Arabidopsis GR-RBP2. The method used is the NJ (Neighbour Joining) method of Saitou and Nei, 1987. C: Phylogram derived from GR-RBP2's closest homologues in NCBI. AtGR-RBP2 and AtGR-RBP4, *Arabidopsis thaliana* GR-RBP protein 2 and 4; AtRPS19, *Arabidopsis thaliana* Ribosomal protein S19; PsGR-RBP, *Pisum sativum* GR-RBP protein; MtRNP, *Medicago truncatula* RNA binding protein; StRBP, *Solanum tubersum* RNA binding protein; BrGR-RBP, *Brassica rapa* GR-RBP protein; Os01g0916660, Os07g0602600 and Os12g0502200, proteins from loci in *Oriza sativa;* PpGR-RBP, *Physcomitrella patens* GR-RBP protein; PgGR-RBP, *Picea glauca* GR-RBP protein; HvGR-RBP, *Hordeum vulgare* GR-RBP.

erol, 0.1% w/v Nonidet P-40). Elution from the Co-NTA was performed using 5x 0.5 ml of imidazole containing buffer (50 mM Tris-HCl buffer pH 7.5, 150 mM NaCl, 10% v/v glycerol, 0.1% w/v Nonidet P-40, and 0.3 M imidazole). Protein in the fractions was bound to 10 ul strataresin (Stratagene company,La Jolla, USA), and separated by SDS-PAGE for subsequent staining with Coomassie Blue and for blotting and detection of the TAP-tagged proteins.

GUS activity detection: Seedlings and different parts of transgenic GUS plants were incubated in a solution containing 1 mM X-Gluc buffer, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.016 mM Triton X-100, 10 mM EDTA, and 100 mM of NaH_2PO_4 and Na_2HPO_4 pH 7.0 at 37°C for 10 h. To visualize the blue precipitate, chlorophyll was removed in 70% v/v ethanol after staining.

Isolation and characterization of homozygous T-DNA insertion lines: Seeds were obtained from the Nottingham *Arabidopsis* Stock Centre (Nottingham, UK). Homozygous *gr-rbp2* mutant plants were isolated from seeds of segregating T3 lines of the T-DNA insertion lines SALK-059714, SALK-13850 (*GR-RBP2*). The insertion in the gene was identified using T-DNA left border primer LBa (5′-TGGTTCACG-TAGTGGGCCATCG-3′) and specific primers for SALK-059714 (Forward: 5′-TTAGAATGCACC-TAATGGGG-3′ and Reverse: 5′-CTACTTGTTTTG-GATCAGCCC-3′), SALK-13850 (Forward: 5′-TTA-GAATGCACCTAATGGGGG-3′ and Reverse: 5′-CCCATCTCGTTGAATTGATG-3′).

RESULTS

Phylogenetic analyses of GR-RBP2: To determine the phylogenetic relationship, *GR-RBP2* homologous sequences were retrieved from NCBI and TAIR. Cluster analysis revealed that the protein is identical to the cDNA product GRP2. It was named earlier in the "plant gene register" section of Plant Physiology by Macknight (1997). Here to avoid confusion we use GR-RBP2 (At4g13850), as named by Lordovic and bartel (2002). Alignmemt of the eight GR-RBP paralogs in *Arabidopsis* reveals that the GR-RBPs are conserved in their RRM domains and more divergent in the glycine- rich domain (Fig. 1A). GR-RBP2, GR-RBP3, GR-RBP4, GR-RBP5 and GR-RBP6 have mitochondrial targeting sequences. The closest homologue to GR-RBP2 is GR-RBP4 (Fig. 1B). Alignment of GR-RBP2 with proteins from all organisms revealed that the protein is conserved in both monocotyledonous and dicotyledonous plants. Homologues to GR-RBP2 can be found in other eukaryotes and bacteria (Fig. 1C). Ribosomal protein RPS19 (RPS19) from *Arabidopsis* is close homologue to GR-RBP2. Homology is high in the single RRM sequence but S19 lacks the low complexity Glycine rich region.

Isolation and characterization of knockout mutants of GR-RBP2: Initially, T-DNA insertion lines of GR-RBP2 were considered for functional analysis. Homozygous T-DNA mutant lines were isolated from segregating T3 plants. The knockout phenotype in plants from SALK-059714 is caused by T-DNA insertion in the coding region of *GR-RBP2* (Fig. 2A). Transcript analysis of homozygous lines showed absence of expression of *GR-RBP2* in mutants (Fig. 2B). When wild type and knock out lines are growing on 100 mM trehalose, there was not any difference between them (Fig. 2C). Response of these seedlings

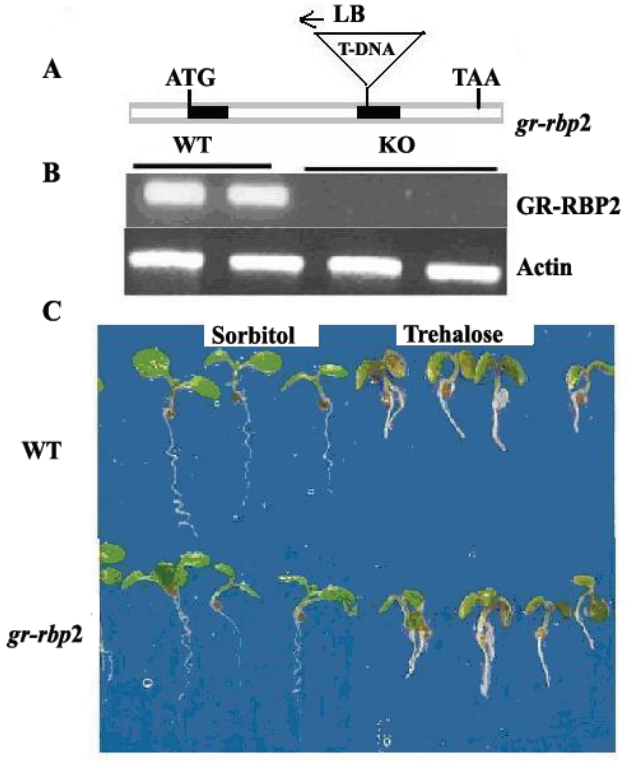

Figure 2. Knock out mutants of *GR-RBP2*. A: Schematic representation of T-DNA insertion in *gr-rbp2* mutant. B: Expression of GR-RBP2 in knockout lines of *gr-rbp2* (Salk-059714). "WT" wild type seedlings, "mutant" plant lines homozygous for insertions in *GR-RBP2*. *AtACTIN2* expression levels are shown as control. C: Seedlings from WT and lines with GR-RBP2-knockouts (*gr-rbp2*) after 14 d on 100 mM of either trehalose or sorbitol under long day conditions at 22°C.

to fructose, glucose or sucrose was similar to WT (data not shown). Taken together, *GR-RBP2* mutants were identified but failed to reveal phenotypic of GR-RBP2 with respect to trehalose sensitivity or response to other. Functional redundancy with other members of the multi-gene families of *GR-RBP2* may be one reason for the absence of altered phenotypes.

GR-RBP2 is expressed in all developmental stages: To examine the organ specific expression of *GR-RBP2*, plant lines were transformed that express a transcriptional fusion of GUS using a 2 kb promoter and 5′ UTR of *GR-RBP2*. As a control, empty vector of pMDC162 was transformed in WT plants. Strong GUS expression specific to the *GR-RBP2* promoter was detected in the root tips of *Arabidopsis* seedlings (Fig. 3A). Gus activity was observed in adult leaves (Fig. 3B), flowers (Fig. 3C) and siliques (Fig. 3D), Whilst Plants expressing the empty control plasmid did not stain (Fig. 3E, F). The expression pattern of the GUS-fusions was affected by glucose, sucrose or trehalose treatment. These results indicated that the *GR-RBP2* promoter is not responsive to these sugars (data not shown).

Taken together, *GR-RBP2* appeared to be expressed in plants and at all developmental stage; its highest expression was in root tips. GR-RBP2 expression seems insensitive to trehalose or metabolisable sugars.

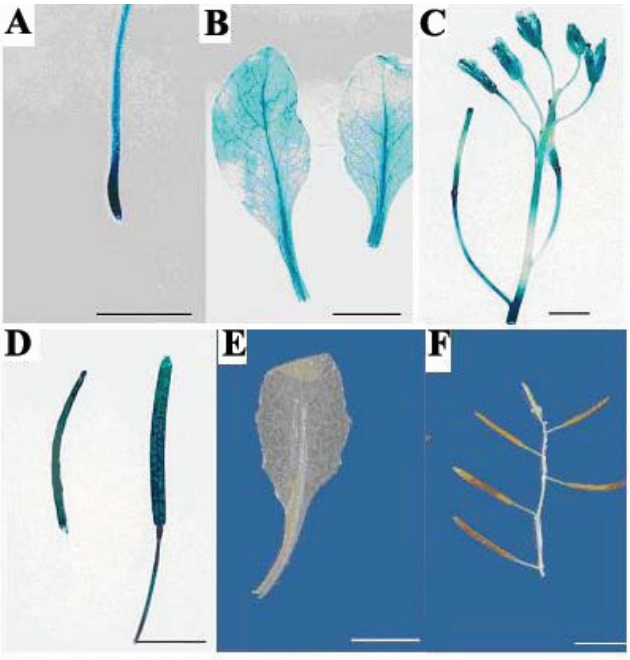

Figure 3. *GR-RBP2* gene expression and protein-localization. Typical GUS staining in plants containing the translational fusion in A: root tip, B: leaves, C: inflorescence, D: siliques, E and F: in a leaf and siliques of plants transformed with pMDC160 empty vector control. The typical staining pattern shown was found in all three independent transgenic lines tested from the nine lines raised. (Bar = 0.5 cm).

GR-RBP2 is likely found in a protein complex in planta: To identify complexes containing GR-RBP2 and eventually the factors that interact with GR-RBP2, plants were transformed that express a tagged version of GR-RBP2. GR-RBP2 was fused to the Tandem Affinity Tag (TAPa) at the C-terminal end (GR-RBP2: TAP); the TAP tag has protein A, myc- and his- epitopes (Rubio *et al*., 2005). To be able to test if the fusion protein is functional, the CaMV35S promoter was chosen to drive the expression of the tagged protein: over-expression of a functional protein leads to trehalose resistance. As control plants were transformed with empty vector. In addition, control plant lines were transformed with protein N-terminally tagged and empty plasmid. Plant lines were screened for expression of tagged GR-RBP2 by western blotting using anti-myc antibody. This antibody typically detected several protein bands in plants expressing *GR-RBP2:TAP*. But the N-terminally tagged protein or plants with empty vector control did not express any protein (data not shown). The highest molecular weight band corresponds to a protein of 43 kD, which is the expected size for the *GR-RBP2*:*TAP* fusion before its targeting into the mitochondrion.

Transgenic lines expressing *GR-RBP2*:*TAP* were also tested for their ability to grow on 100 mM trehalose. Seedling roots of the transgenic lines were not significantly longer than WT. Extract of plants expressing *GR-RBP2*:*TAP* was separated by gel filtration and the fractions analyzed by western blotting using anti-myc antibody (Fig. 4). Absorption of proteins after separation on the column at 280 nm revealed that the major peak of Rubisco eluted at the size expected from calibration of the system with protein standards (Fig. 4A). Dominant 43 kD precursor protein in crude extracts was detected by Anti-myc antibodies (Fig. 4B, line T). Anti-myc further detected two major processed or degraded forms of GR-RBP2: TAP in fractions spanning E6 to E9 that correspond to complexes of approximately 95-100 kD (Fig. 4B, lines E6-E9). A very faint signal was observed in fraction F3 that corresponds to proteins of approximately 45 kD, close to the monomeric size of tagged GR-RBP2 (Fig. 4C, line F3). Gel-filtration experiments with extracts from control plants did not reveal any components cross-reacting with anti-myc antibody (data not

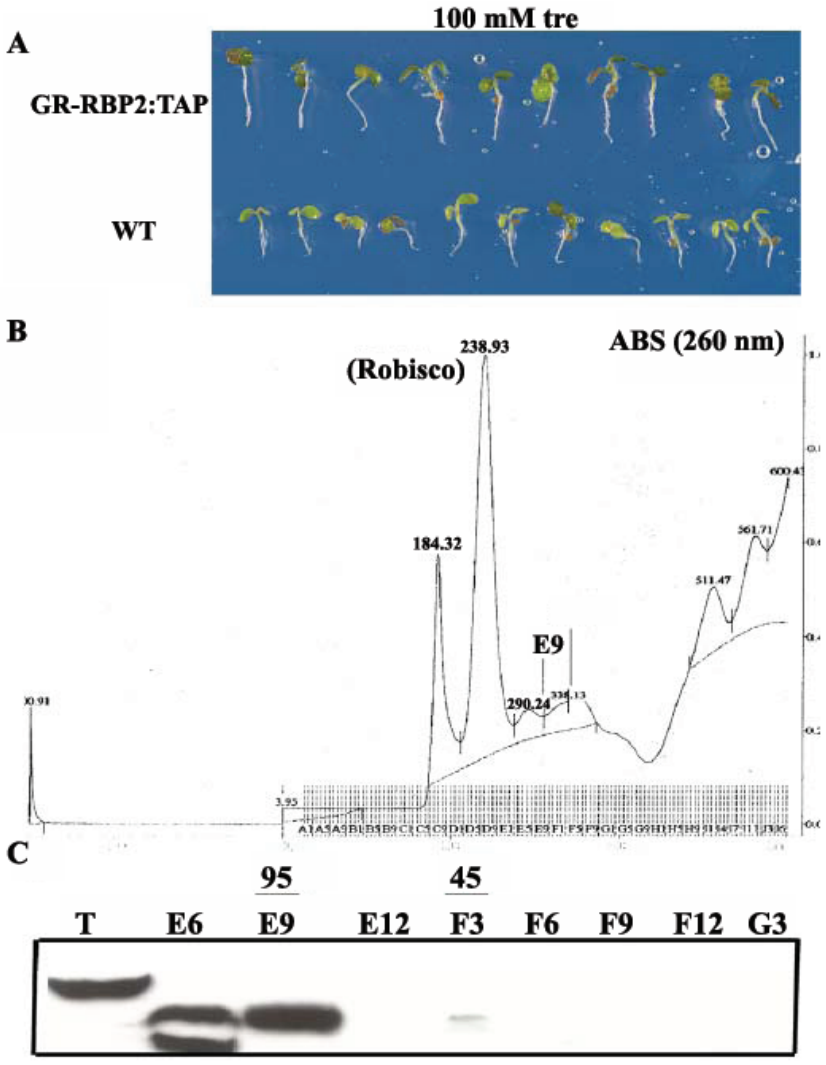

Figure 4. GR-RBP2 complex *in planta.* A: Overexpressors of GR-RBP2:TAP on 100 mM trehalose. Seedlings from a line over-expressing *GR-RBP2:TAP* and WT were grown on half strength MS medium with 100 mM trehalose for 2 weeks in long day conditions. B: Sepharose S-300 gel filtration of an extract of plants expressing *GR-RBP2:TAP.* Absorption at 280 nm of proteins after separation on the column: Rubisco, absorbance peak corresponding to rubisco protein; E9, F3 fractions that contain the GR-RBP2 fusion as detected in C. C: Detection of GR-RBP2:TAP by western blotting using anti-myc antibody in fractions E6 to G3 obtained during gel filtration. T, total crude extract; 95 and 45, fractions corresponding to molecular weight 95 and 45 kD, respectively.

shown). Therefore, GR-RBP2: TAP appears to occur in a complex of at least twice its molecular weight (95-100 KD). Tandem affinity purification of the complex was then performed according to Rubio *et al*. (2005). Preliminary results showed that the procedure allowed enriching a specific set of proteins including protein corresponding to processed GR-RBP2: TAP (data not shown).

DISCUSSION

In *Arabidopsis* seedlings, exogenously supplied trehalose inhibits root growth and emergence of leaves (Wingler *et al*., 2000). This growth inhibition is due to T6P accumulation (Schluepmann *et al*., 2004). Evidence is accumulating that suggests an important regulatory role for T6P. It was shown that T6P determines carbon utilization in *Arabidopsis* seedlings. However it is not understood how T6P controls carbon utilization and why changes in the steady state level of T6P yields such strong phenotypic changes. In current report we characterized a suppressor of T6P mediating growth arrest using a genetic approach.

GR-RBP2 is a small protein with binding sites for RNA, through its RRM domain, and for protein-pro-

tein interaction through its glycine-rich domain. GR-RBP2 is a protein of likely prokaryotic origin that is part of a protein complex. *GR-RBP2* expression is found throughout the plant and is not responsive to metabolisable sugars such as glucose, fructose or sucrose. *GR-RBP2* expression also does not respond to 100 mM trehalose. A T-DNA insertion mutant of *GR-RBP2* was identified in the T-DNA insertion line SALK-059714. These mutants did not show different phenotype from WT, when grown on different carbon sources. The data obtained did not reveal a function for GR-RBP2 with respect to growth in relation to the carbon metabolism.

Gus expression data revealed that *GR-RBP2* is highly expressed in young seedlings, flowers and root tips. High expression of *GR-RBP2* in young seedlings suggests that may have a capability to play functions in RNA metabolism in actively proliferating organs (Kwak *et al*., 2005). Transcript analysis data were in accordance with the data from gene expression databases such as Genevestigator (Zimmermann *et al*., 2004), suggesting that the 2kb promoter sequence contains the regulatory sequences driving *GR-RBP2* expression *in planta*.

TAPa-tagged fusion and gel-filtrration data showed that GR-RBP2 is likely in a complex of 95-100 kD in plants. A major lower molecular weight bands may correspond to the 40 kD processed protein that had been imported in the mitochondrion or to degraded products. Further work is however needed to analyze proteins co-purifying with GR-RBP2:TAP.

GR-RBPs are involved in abiotic stress response and their functions were recently indicated (Kim *et al*., 2008; Kim *et al*., 2007; Kwak *et al*., 2005). Previous studies showed that *GR-RBP* 1, 2, 3, 4, 7 and 8 expression are increased by cold stress. Meanwhile, *GR-RBP1* expression was strongly induced by dehydration and cold stress (kim *et al*., 2007; Kwak *et al*., 2005).

An improvement of cold tolerance was noted in over-expressing *GR-RBP2* plants (Kim *et al*., 2007; Vernel *et al*., 2002). It was also demonstrated that *Arabidopsis* GR-RBP2 is able to suppress the cold sensitivity of *E. coli* at low temperatures, which suggests that GR-RBP2 exhibits an RNA chaperone activity during cold adaptation. Plant over-expressing GR-RBP2 showed induced catalase and peroxidase but decreased superoxide dismutase activities. The closest homologue to GR-RBP2, GR-RBP4, is unable to complement *E. coli* BX04. Accordingly, the RNA chaperone activity of GR-RBP2 is specific (Kim *et al*., 2007). This specificity may arise from complex formation with other factors (Fig. 4). Further characterization of the proteins in the complex with GR-RBP2 will be necessary to understand GR-RBP2 function. Possibly, *GR-RPP2* over-expression leads to the growth on trehalose due to its stress protection function. T6P accumulation might generate metabolic stress that can be alleviated by GR-RBP2. In *GR-RBP2* over-expressors starch accumulation on trehalose is similar to WT. Therefore, GR-RBP2 is an indirect target of T6P. GR-RBP2 likely affects process downstream of T6P.

Kwak *et al*. (2005) suggested that GR-RBPs originate from cyanobacteria where they replace the function of cold-shock proteins as found in other bacteria such as in *E. coli*. GR-RBPs are also present in mammalians, e.g. the cold induced RNA binding proteins (Nishiyama *et al*., 1997). Cold induced RNA binding proteins (CIRP) of the mouse is involved in growth inhibition under reduced temperature (Nishiyama *et al*., 1997). Over-expression of CIRP leads to prolongation of the G1 phase of the cell cycle. Immuno-fluorescence studies located CIRP in the nucleus, unlike GR-RBP2 that is located in mitochondria (Fig. 3). Surprisingly the products of this gene are found in mitochondria whilst T6P biosynthesis is thought to occur in the cytosol (TAIR annotation of TPS genes, March, 2007). None of the proteins of T6P metabolism in *Arabidopsis* have a mitochondtial targeting sequence (Schluepmann *et al*., 2004; Leyman *et al*., 2001) and none have been found associated with mitochondrial proteome (Kruft *et al*., 2001). The link with reactions in mitochondria through GR-RBP2 is novel. The function of GR-RBP2 as an RNA chaperone (Kim *et al*., 2007) suggests that it may alter transcription of mitochondrial proteins that affect T6P targets in the cytosol or chloroplast.

In conclusion, the present work provides new information that increases our knowledge about the roles of GR-RBP2 in response of plants to trehalose. GR-RBP2 likely exerts its effect upon growth by changing mitochondrial metabolism. Taken together, GR-RBP2 links mitochondrial stress adaptation and T6P metabolism and this link may begin to explain how trehalose metabolism mediates stress protection in plants. This protein is likely modulating a component down stream of T6P.

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