Reciprocal regulation of glycine-rich RNA-binding proteins via an interlocked feedback loop coupling alternative splicing to nonsense-mediated decay in Arabidopsis

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ABSTRACT

The Arabidopsis RNA-binding protein AtGRP8 undergoes negative autoregulation at the post-transcriptional level. An elevated AtGRP8 protein level promotes the use of a cryptic 5’ splice site to generate an alternatively spliced transcript, as_AtGRP8, retaining the 5’ half of the intron with a premature termination codon. In mutants defective in nonsense-mediated decay (NMD) abundance of as_AtGRP8 but not its pre-mRNA is elevated, indicating that as_AtGRP8 is a direct NMD target, thus limiting the production of functional AtGRP8 protein. In addition to its own pre-mRNA, AtGRP8 negatively regulates the AtGRP7 transcript through promoting the formation of the equivalent alternatively spliced as_AtGRP7 transcript, leading to a decrease in AtGRP7 abundance. Recombinant AtGRP8 binds to its own and the AtGRP7 pre-mRNA, suggesting that this interaction is relevant for the splicing decision in vivo. AtGRP7 itself is part of a negative autoregulatory circuit that influences circadian oscillations of its own and the AtGRP8 transcript through alternative splicing linked to NMD. Thus, we identify an interlocked feedback loop through which two RNA-binding proteins autoregulate and reciprocally crossregulate by coupling unproductive splicing to NMD. A high degree of evolutionary sequence conservation of the introns retained in as_AtGRP8 or as_AtGRP7 points to an important function of these sequences.

INTRODUCTION

Post-transcriptional regulation has come into focus as an important mechanism to control gene expression in higher plants (1–4). It occurs at multiple levels including pre-mRNA maturation, mRNA transport, translation and breakdown. The major players are RNA-binding proteins that influence the fate of an mRNA molecule either directly by binding to defined RNA sequences and structural elements or indirectly through protein–protein interaction (5).

One important protein domain known to interact with RNA molecules is the RNA recognition motif (RRM) (6). It is composed of a four-stranded antiparallel β-sheet with two α-helices. The highly conserved octapeptide RNP1 and hexapeptide RNP2 sequence motifs are located in the β3 and β1 sheets and contain conserved aromatic residues making contacts to the RNA substrate. A systematic survey disclosed 196 RRM-containing proteins in the genome of Arabidopsis thaliana (5). Among those, the 16 kDa AtGRP8 (A. thaliana glycine-rich RNA-binding protein 8) protein combines a single N-terminal RRM with a C-terminal region enriched in glycine repeats with some interspersed serine, tyrosine and arginine residues (7–9). It is also known as GR-RBP8, GRP8 or CCR1 (cold and circadian regulated 1) (5,10,11). Both AtGRP8 and AtGRP7 encoding an orthologous RNA-binding protein that shares 77% sequence identity undergo circadian oscillations with a peak at the end of the daily light phase (9,11). Notably, AtGRP8 is subject to negative regulation by AtGRP7, as in transgenic plants ectopically overexpressing AtGRP7 under control of the CaMV (Cauliflower Mosaic Virus) 35S RNA promoter,

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\( \text{AtGRP8} \) oscillations are strongly depressed (9). This regulation occurs at the post-transcriptional level through reduction of constitutive splicing and stimulation of alternative splicing at a cryptic intronic 5′ splice site of the \( \text{AtGRP8} \) pre-mRNA, leading to an alternative splice variant (as \( \text{AtGRP8} \)) with a premature termination codon (PTC) in the retained part of the intron.

\( \text{AtGRP7} \) uses the same mechanism for negative autoregulation: rising protein levels promote alternative splicing by binding to its own pre-mRNA, and the alternatively spliced transcript (as \( \text{AtGRP7} \)) is degraded via a pathway involving the nonsense-mediated decay (NMD) components AtUPF1 (UP FRAMESHIFT PROTEIN 1) and AtUPF3 (12,13). Thus, \( \text{AtGRP7} \) is part of a negative feedback loop through which it influences its own oscillation at the post-transcriptional level. This feedback loop is thought to operate as a slave oscillator downstream of the circadian clock, transducing temporal information with a functional role. As the alternatively spliced \( \text{AtGRP7} \) and \( \text{AtGRP8} \) transcripts are retained in the unproductively spliced transcripts show an exon-like evolutionary preservation, pointing to a functional role. As the alternatively spliced \( \text{AtGRP8} \) and \( \text{AtGRP7} \) transcripts are bona fide NMD targets, it appears that the interlocked \( \text{AtGRP7}/\text{AtGRP8} \) feedback loops harness autogenous alternative splicing-activated decay via the NMD pathway to fine-tune the expression of their components.

**MATERIALS AND METHODS**

**AtGRP8 overexpression in transgenic plants**

The protein-coding region of \( \text{AtGRP8} \) was amplified by PCR from the cDNA with the upstream primer 5′ GGCCATGGCTGAAAGTGAGT 3′ and the downstream primer 5′ CCGGATCCCTTACCAGCCGCCAC CAC 3′ covering the translation start and stop (bold) and comprising engineered NcoI and BamHI sites (underlined), respectively. The PCR product was inserted between the CaMV 35S RNA promoter with the duplicated enhancer fused to the Tobacco Mosaic Virus omega translational enhancer and the CaMV polyadenylation signal (9). To express \( \text{AtGRP8-RQ} \), the Arg47-Gln mutation was introduced by PCR-mutagenesis (see below), sequenced and reinserted into the original plasmid by BglII/SacI digestion. The entire cassettes were inserted into the binary vector pHPT1 that was obtained by replacing the promoter-less β-glucuronidase gene of pGPTV-HPT by the pUC19 polylinker (16). Arabidopsis \( t_{h} \)aliana L. Columbia plants were transformed by vacuum infiltration (17).

**Plant growth**

Seeds were germinated on one-half strength MS plates (18) containing 0.5% sucrose and the appropriate antibiotic and grown in 16 h light/8 h dark cycles at a constant temperature of 20°C. After 2 weeks, resistant plants were transferred to one-half strength MS plates without antibiotics.

**Recombinant GST-\( \text{AtGRP8} \) and GST-\( \text{AtGRP8-RQ} \)**

Recombinant GST-\( \text{AtGRP8} \) protein was constructed by inserting the \( \text{AtGRP8} \) coding region into NotI-EcoRI-cut pGEX-6P1 vector (GE Healthcare, Freiburg, Germany).

To generate the mutant variant GST-\( \text{AtGRP8-RQ} \), Arg47 in GST-\( \text{AtGRP8} \) was exchanged for glutamine by PCR with Phusion Polymerase (Finnzymes, Espoo, Finland) using the overlapping primers \( \text{RQ}_{\text{for}} \) CGA GAGTGGAAAGATCCCAAGGT-TCCGATTCTGCA and \( \text{RQ}_{\text{rev}} \) TGACGAATCCCGATCTTGGATCCTT CCACTTTCG. Silent mutations were introduced into neighbouring amino acids creating a diagnostic SfiI site (underlined). The mutation was verified by sequencing. The recombinant proteins were expressed in E. coli BL21 DE3. Affinity purification by chromatography on Glutathione Sepharose (GE Healthcare) and concentration of the eluate by centrifugation through Centricon® 30 filter devices (Millipore, Billerica, MA, USA) were done as described (12,13).

**RNA-binding assay**

Synthetic oligoribonucleotides (ORN) were purchased from Biomers (Ulm, Germany). RNA bandshifts with recombinant \( \text{AtGRP8} \) and the ORNs labelled at the 5′ end with \( \gamma-[32\text{P}] \) ATP were performed as previously described (8,19). Semiquantitative RT–PCR on retrotranscribed total RNA was done as described (15). Primers are listed in Table S2.

**Immunoblot analysis**

Protein extraction from Arabidopsis plants and incubation of protein gel blots with antipeptide antibodies raised against \( \text{AtGRP8} \) and \( \text{AtGRP7} \), followed by chemiluminescence detection were done as described (12).

**Secondary structure prediction**

Evolutionary conserved secondary structure elements were determined with the RNA-Decoder program (20,21). RNA-Decoder takes as input a fixed alignment
of evolutionarily related RNA sequences and an evolutionary tree relating them and predicts as output the conserved secondary structures supported by the evolutionary patterns detected in the input alignment. A set of nine pre-mRNA sequences comprising \textit{AtGRP8} orthologs with the same gene structure comprising a 5' UTR, two protein-coding exons of phase 0, a single intron and a 3' UTR (Table S1) were assembled. To generate the input alignment, we first compiled separate alignments for 5' UTRs, exon 1, intron, exon 2 and 3' UTRs using ClustalW (Version 1.83) (22), which we then combined manually into longer alignments. To maximize the alignment quality, the alignments of the pre-mRNA sequences of exons 1 and 2 were based on the ClustalW alignments of the corresponding encoded amino-acid sequences. As the resulting full-length pre-mRNA alignment (1407 nt) was too long to analyze in a single chunk, we generated an mRNA input alignment with UTRs (1055 nt) to investigate conserved secondary structures in or near the UTRs and a pre-mRNA input alignment without UTRs (1093 nt) to investigate conserved secondary structures in or near the intron.

\textbf{RESULTS AND DISCUSSION}

\textit{AtGRP8} negatively autoregulates its own pre-mRNA

To begin to investigate the molecular properties of the predicted RNA-binding protein \textit{AtGRP8}, we generated transgenic plants ectopically overexpressing the \textit{AtGRP8} coding region under control of the CaMV promoter with the duplicated enhancer (\textit{AtGRP8}-ox plants). Immunoblot analysis using a specific \textit{AtGRP8} antibody identified transgenic lines with strongly elevated \textit{AtGRP8} protein levels (Figure 1A). Compared to WT plants, the total \textit{AtGRP8} transcript level was strongly elevated in \textit{AtGRP8}-ox plants harvested at zt3 (zeitgeber time 3, that is 3 h after lights on), the circadian minimum of \textit{AtGRP8} oscillations, and zt11, the circadian maximum, due to the expression of the transgene (Figure 1C). The endogenous \textit{AtGRP8} transcript forms are selectively detected with a gene-specific probe derived from the 5' UTR that is not contained in the overexpression construct (Figure 1C). In WT plants, the endogenous \textit{AtGRP8} mRNA and a small amount of its pre-mRNA containing the 283-nt intron were present. In the \textit{AtGRP8}-ox plants, almost no mature endogenous \textit{AtGRP8} mRNA was detectable. Instead, an intermediate size transcript appears at a low level, corresponding to the alternatively spliced \textit{AtGRP8} transcript (as \textit{AtGRP8}) that is generated through the use of a cryptic 5' splice site within the intron. These data indicate that \textit{AtGRP8} exerts negative autoregulation on its own pre-mRNA.

In WT plants, as \textit{AtGRP8} is hardly detectable. Nevertheless, corresponding cDNAs have been isolated from cDNA libraries (DS, unpublished results). In response to the elevated \textit{AtGRP8} protein level, a shift to a cryptic 5' splice site occurs and as \textit{AtGRP8} accumulates to a low level at the expense of the mature mRNA in \textit{AtGRP8}-ox plants. Because the retained part of the intron contains in-frame stop codons, no full-length mRNA sequences comprising \textit{AtGRP7} would also influence its own circadian oscillations (12,13).

\textit{AGRP8} protein can be produced from the as \textit{AtGRP8} transcript. Thus, like \textit{AtGRP7}, \textit{AtGRP8} negatively autoregulates, and the molecular underpinnings of the \textit{AtGRP8} feedback loop are similar to the way \textit{AtGRP7} influences its own circadian oscillations (12,13).

\textit{AtGRP8} promotes alternative splicing of the \textit{AtGRP7} pre-mRNA

To test whether \textit{AtGRP8} would also influence \textit{AtGRP7} transcript abundance, the \textit{AtGRP7} transcript level was
Table 1. Sequences of oligoribonucleotides used as RNA-binding substrates

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-UTR_WT</td>
<td>GUUUUGUUAAUAUAAUAAUUAUUAAAUUUUGUGU</td>
</tr>
<tr>
<td>8-UTR_G4mut</td>
<td>GUUUUGUUAAUAUAAUAAUAAUUAUUAAAUUGUGU</td>
</tr>
<tr>
<td>8-UTR_938</td>
<td>GUUUUGUUAAUAUAAUAAUAAUUAUUAAAUUGUGU</td>
</tr>
<tr>
<td>8-intron_WT</td>
<td>CUUCCAGAUGUUGUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU</td>
</tr>
<tr>
<td>8-intron_G4mut</td>
<td>CUUCCAGAUGUUGUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU</td>
</tr>
<tr>
<td>7-UTR_WT</td>
<td>AUUUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU</td>
</tr>
<tr>
<td>7-UTR_G4mut</td>
<td>AUUUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU</td>
</tr>
<tr>
<td>7-intron_WT</td>
<td>GUGCUAGUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU</td>
</tr>
<tr>
<td>7-intron_G4mut</td>
<td>GUGCUAGUUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU</td>
</tr>
</tbody>
</table>

Sequences of the AtGRP8 3’ UTR (8-UTR_WT and 8-UTR_938) and intron (8-intron_WT) ORN and the corresponding mutated 8-UTR_G4mut and 8-intron_G4mut with six G residues exchanged for A or C (in bold), respectively, as well as sequence of AtGRP7 3’ UTR (7-UTR_WT) and intron (7-intron_WT) ORN and the corresponding mutated 7-UTR_G4mut and 7-intron_G4mut with four or six G residues exchanged for A (in bold), respectively, are shown.

compared in WT and AtGRP8-ox plants (Figure 1C). In WT plants, the mature AtGRP7 mRNA and a small amount of the pre-mRNA were detected at the circadian maximum. When AtGRP8 was expressed at high levels, a low amount of the as_AtGRP8 transcript appeared at the expense of the mature mRNA. Accordingly, AtGRP7 protein was barely detectable in the AtGRP8-ox plants (Figure 1B).

AtGRP8 RNA and protein steady-state abundance previously have been shown to be under negative control by AtGRP7 (12,13). Consistent with this, AtGRP8 RNA and protein levels are elevated in the atgrp7-1 T-DNA insertion mutant due to relief of repression (15). Our data now show that not only does AtGRP7 influence AtGRP8 abundance, but also AtGRP8 in turn regulates AtGRP7. Similarly, another Arabidopsis splicing regulator, the Ser/Arg-rich (SR) protein atRSZ33 crossregulates alternative splicing of a conserved long intron in the atRSp31 gene, equivalent to the effect on its own pre-mRNA (23,24). The alternative atRSp31 form accumulates to high levels and thus is stable in contrast to as_AtGRP8.

Recombinant AtGRP8 binds to its own pre-mRNA

As AtGRP7 autoregulation involves AtGRP7 binding to its own 3’ UTR and intron, it is conceivable that AtGRP8 might similarly interact with its own pre-mRNA. Therefore, we performed RNA-bandshift assays to test the AtGRP7-binding sites determined previously (12) for interaction with recombinant AtGRP8 (Table 1). For the intronic binding site 8-intron_WT GST-AtGRP8 formed a retarded complex which was competed more efficiently by the ORN 8-intron_WT than by the mutated counterpart 8-intron_G4mut with exchanges of two G and two U residues (Figure 2A). For the 3’ UTR, a retarded complex was observed for 8-UTR_WT that was more efficiently outcompeted by unlabeled ORN 8-UTR_WT than by the mutated counterpart 8-UTR_G4mut with mutation of six G residues (Figure 2B). Sequence alignment of the AtGRP7-binding sites upon the AtGRP8 intron and 3’ UTR had uncovered a second motif with homology to the AtGRP7-binding site within the AtGRP8 3’ UTR (data not shown). However, determination of the Kd values revealed that AtGRP8 has a 10-fold lower affinity for the corresponding ORN 8-UTR_938 (Table 1) compared to 8-UTR_WT (Figure 2C). Furthermore, the complex was outcompeted by an excess of unlabeled 8-UTR_938 to the same degree as by a negative control ORN 7-UTR_G4mut (Table 1) (Figure 2D). Based on the low affinity and lack of specificity, 8-UTR_938 was excluded as binding site. Taken together, our data show that AtGRP8 binds to its own pre-mRNA and recognizes sequences in the second half of the intron and the 3’ UTR that also comprise targets for AtGRP7.

To assess the secondary structure of these putative binding sites in the context of the entire pre-mRNA, AtGRP8 was searched for secondary structure elements conserved in plant orthologs with RNA-Decoder (20,21). Among the numerous existing secondary structure prediction programs, RNA-Decoder is unique in that it explicitly takes the known protein-coding regions of an input alignment into account. This feature is important when searching partly protein-coding sequences such as pre-mRNAs for conserved secondary structure elements, as the evolutionary pattern due to amino-acid conservation needs to be carefully distinguished from the evolutionary pattern due to secondary structure conservation (20,21).

In order to detect conserved secondary structures in the AtGRP8 pre-mRNA, we assembled a set of nine pre-mRNA sequences comprising AtGRP8 as well as eight AtGRP8 orthologs from the Brassicaceae mustard and oilseed rape, the other dicotyledoneous plants tobacco and Pelargonium, and the monocotyledoneous plants rice and maize (Table S1). As input tree to RNA-Decoder, we used the tree predicted by ClustalW (22) for the nine encoded protein sequences. The predicted secondary structures for the AtGRP8 and, for comparison, the AtGRP7 pre-mRNAs are shown in Figure S1. Interestingly, the predicted AtGRP8-binding sequences map to conserved regions, pointing to an important role of the secondary structure for the function of these motifs. 8-intron_WT and 8-UTR_WT are predicted to be single-stranded. In contrast, 8-UTR_938 is predicted to be partially double-stranded and thus may be less accessible to interacting proteins, in line with the very low binding affinity (Figure 2C).

Recombinant AtGRP8 binds to the AtGRP7 pre-mRNA

The data shown in Figure 1 indicate that AtGRP8 negatively influences AtGRP7 pre-mRNA splicing in the same way as AtGRP7 autoregulates. As the shift to the alternative splice form relies on AtGRP7 binding to its own pre-mRNA, we tested whether recombinant AtGRP8 would bind in vitro to the AtGRP7 target sites within the AtGRP7 pre-mRNA (Table 1). Complex formation was observed with 7-UTR_WT that was completely lost upon addition of 250 pmol of unlabeled 7-UTR_WT, but much less reduced with 500 pmol of the mutated 7-UTR_G4mut (Figure 3A). Also for the ORN spanning the AtGRP7-binding site within the second half of the
intron a strong interaction with GST-AtGRP8 was found which was abolished by 250 pmol of unlabeled 7-intron_WT but not affected by 500 pmol of 7-intron_G6mut (Figure 3B). This shows that AtGRP8 also binds to the 7-intron intron and 3' UTR with a certain specificity. Thus, AtGRP7 and AtGRP8 may recognize overlapping or identical motifs within both pre-mRNAs. To compare the binding affinities, $K_d$ values were determined for all interactions (Table 2). For the four binding sites, the $K_d$ values were in the same order of magnitude as those previously determined for GST-AtGRP7 protein (12). Therefore, presently we cannot infer which of the possible interactions between AtGRP7 or AtGRP8 and the respective target sites may prevail in vivo.

Mutation of AtGRP8 RNP1 Arg47 impairs RNA-binding activity in vitro

Binding of AtGRP8 to its own and the AtGRP7 pre-mRNA in vitro suggests that this interaction may initiate alternative splicing and down-regulation of endogenous AtGRP7 and AtGRP8 in vivo. To investigate whether this in vitro binding activity correlated with in vivo function, a mutation was introduced into the RRM of recombinant GST-AtGRP8. The conserved arginine (R) 47 predicted to lie at the beginning of the β3 strand that is part of the RNA-binding platform was exchanged for glutamine (Q) (25–27). Indeed, we have shown that recombinant GST-AtGRP7-RQ with an analogous mutation of R49 has a 6-fold lower affinity for its target sites than WT GST-AtGRP7 protein while folding of the protein is not affected (12).

The AtGRP8-RQ mutant protein was expressed as GST-fusion and tested for binding to the AtGRP7 and AtGRP8 target sites. Only weak interaction was observed with $K_d$ values about one order of magnitude higher for GST-AtGRP8-RQ compared to GST-AtGRP8 (Table 2). Competition assays revealed that 8-UTR_WT competed much better for binding of GST-AtGRP8-RQ than 8-UTR_G6mut and 7-UTR_WT competed much better for binding of GST-AtGRP8-RQ than 7-UTR_G4mut (data not shown). Thus, mutation of the conserved R47 reduces the binding affinity rather than the specificity of the interaction.

The AtGRP8 R47Q mutation impairs but does not abolish promotion of alternative AtGRP8 and AtGRP7 splicing

To test the impact of the RQ mutation on AtGRP8 activity in vivo, plants constitutively overexpressing the mutated AtGRP8-RQ protein were generated (AtGRP8-RQ-ox) and compared to plants overexpressing WT AtGRP8 protein (AtGRP8-ox). Transgenic plants were
compared to WT plants, consistent with the idea that the residual binding activity of AtGRP8-RQ still causes some production of as AtGRP7 at the expense of the AtGRP7 mRNA. However, the alternatively spliced transcript is hardly detectable due to its short half life (13). Accordingly, almost no AtGRP7 protein was detected in AtGRP8-ox plants, whereas a small amount of AtGRP7 protein was detectable in AtGRP8-RQ-ox lines (Figure 4D). Altogether, the interaction of AtGRP8 with the AtGRP7 and AtGRP8 pre-mRNAs in vivo is relevant for the negative autoregulation and crossregulation. Presumably, binding of AtGRP8 and AtGRP7 triggers additional factors that act in concert to regulate the choice of splice sites (12).

**Distinct cold response of AtGRP8 and AtGRP7**

The reciprocal regulation between AtGRP7 and AtGRP8 uncovered here may suggest that the proteins are able to fully substitute for each other. Therefore we compared their expression patterns. According to publically available microarray data, AtGRP7 and AtGRP8 are expressed at a similar level across plant tissues except for a lower AtGRP8 abundance in siliques and later stages of seed development (Figure S2) (28). Moreover, the circadian maximum of AtGRP8 transcript oscillation is only slightly advanced relative to the AtGRP7 peak (29). Both AtGRP7 and AtGRP8 transcripts have been described as being upregulated by cold (11,30). We investigated steady-state protein levels under these conditions using specific antibody antigens. Two-week-old Col plants were transferred to 4°C at light onset and harvested without cold treatment and after 1, 2, 4 and 7 days, respectively. AtGRP8 protein abundance rose marginally at best after transfer to 4°C, whereas AtGRP7 protein abundance steadily increased up to day 7 (Figure 5A, B). The AtGRP8 transcript level was only weakly increased at the circadian minimum (zt2) after 1 day of cold treatment but declined afterwards (Figure 5C). Also at the circadian maximum (zt10), the level in plants transferred to 4°C was not elevated beyond that of control plants. These data indicate that AtGRP7 and AtGRP8 differ in their response to cold

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**Table 2. K_d values for recombinant GST-AtGRP8-RQ and GST-AtGRP8**

<table>
<thead>
<tr>
<th>ORN</th>
<th>Protein</th>
<th>K_d [M]</th>
<th>K_d RQ/K_d WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-UTR_WT</td>
<td>GST-AtGRP8-WT</td>
<td>2.05 ± 0.03 × 10^-7</td>
<td></td>
</tr>
<tr>
<td>7-UTR_WT</td>
<td>GST-AtGRP8-RQ</td>
<td>1.31 ± 0.35 × 10^-6</td>
<td>6.4</td>
</tr>
<tr>
<td>8-intron_WT</td>
<td>GST-AtGRP8-WT</td>
<td>3.18 ± 2.27 × 10^-7</td>
<td></td>
</tr>
<tr>
<td>7-intron_WT</td>
<td>GST-AtGRP8-RQ</td>
<td>3.22 ± 0.97 × 10^-6</td>
<td>10.1</td>
</tr>
<tr>
<td>7-UTR_G4m</td>
<td>GST-AtGRP8-WT</td>
<td>1.64 ± 0.65 × 10^-6</td>
<td></td>
</tr>
<tr>
<td>7-UTR_G4m</td>
<td>GST-AtGRP8-RQ</td>
<td>2.29 ± 0.36 × 10^-5</td>
<td>14.0</td>
</tr>
<tr>
<td>7-intron_G4m</td>
<td>GST-AtGRP8-WT</td>
<td>4.26 ± 0.52 × 10^-7</td>
<td></td>
</tr>
<tr>
<td>7-intron_G4m</td>
<td>GST-AtGRP8-RQ</td>
<td>3.59 ± 2.02 × 10^-6</td>
<td>8.4</td>
</tr>
</tbody>
</table>

K_d values were determined as described in (12). The ratio between the K_d value for the mutated protein and the WT protein is indicated.
and thus do not act entirely redundant, as their expression pattern is not fully congruent.

To determine whether the exposure to low temperatures may influence the alternative splicing, the pattern of the alternative splice forms and mature mRNAs in 2-week-old plants exposed to cold for 1, 2 or 5 days was compared to that in untreated plants (Figure S3), as _AtGRP7_ accumulated in parallel with the RNA. While the _AtGRP8_ mRNA only weakly and transiently increased, a stronger upregulation was observed for _AtGRP7_. Thus, only for _AtGRP8_ a slight change in the ratio of the alternative splice forms is observed in the cold. Previous studies have demonstrated changes in the alternative splicing pattern of Arabidopsis SR genes upon cold treatment which may give rise to proteins with different domains and, consequently, changes in splicing of downstream targets (31,32).

**as** _AtGRP8_ and **as** _AtGRP7_ are direct targets of the NMD pathway

Degradation of **as** _AtGRP8_ and **as** _AtGRP7_ is dependent on AtUPF1 and AtUPF3, key components of the NMD pathway of mRNA surveillance that ensures clearance of PTC-containing mRNAs from the cellular transcriptome (12). Recently, NMD has emerged as a widespread regulatory mechanism of physiological gene expression (33–35). In the Arabidopsis _lba_ (low β-amylase) mutant which has a point mutation in the _UPF1_ gene a suite of transcripts show higher steady-state abundance, as expected for NMD substrates (36). But additionally several transcripts are down-regulated with a high proportion of sugar-inducible mRNAs, implicating AtUPF1 in sugar signalling (36).

By analogy, the elevated **as** _AtGRP8_ and **as** _AtGRP7_ levels in _upf1_ and _upf3_ mutants (12) may be an indirect consequence of the reduced AtUPF1 or AtUPF3 levels. To distinguish such an indirect effect of AtUPF1 and AtUPF3 on transcription from a direct effect on transcript abundance, we assayed the pre-mRNA levels by RT–PCR. _AtGRP8_ pre-mRNA steady-state abundance was not changed in the _upf1_ and _upf3_ mutants (Figure 6A) and also the _AtGRP7_ pre-mRNA level was indistinguishable from WT (Figure 6B) in contrast to the strongly elevated _as_ _AtGRP7_ and _as_ _AtGRP8_ levels found in _upf1_5, _upf3_1 and _upf3_2 (12). This indicates that the AtUPF1- and AtUPF3-dependent reduction of _as_ _AtGRP8_ and _as_ _AtGRP7_ is due to post-transcriptional destabilization rather than an indirect consequence of transcriptional inhibition. In higher plants, an increasing number of PTC-containing transcripts have been found to be stabilized when UPF1 and/or UPF3 functions are impaired (36–39). It has not been investigated whether some of them may be influenced either through an NMD-independent function of AtUPF1 and AtUPF3 or as a consequence of a cognate transcription factor undergoing NMD. In humans, in a survey of potential NMD targets more than 5% of the genes detected on Affymetrix GeneChips were affected by the presence or absence of UPF1 (40). For 15 out of 16 selected transcripts the pre-mRNA level was also changed, however, suggesting that the vast majority of those transcripts are affected indirectly through altered transcription rather than being bona fide NMD targets (40).

If the unproductive _AtGRP8_ and _AtGRP7_ splicing elicited by elevated levels of _AtGRP7_ and _AtGRP8_ protein indeed is functionally relevant, this may be reflected at the level of sequence conservation. The gene structure of the small glycine-rich RNA-binding proteins with a single RRM is well conserved among different plant species, harboring a single intron of similar size between RNP2 and RNP1, and a predicted cryptic 5′ splice site (8,19).
Notably, the retained upstream part of the intron exhibits a higher degree of conservation than the downstream part spliced out in the as_@GRP7 and as_@GRP8 variants (Figure 7). A comparison of @GRP8 with @GRP7 and orthologs from mustard, oilseed rape, tobacco and Pelargonium shows that the degree of conservation reaches that of the surrounding exons (Figure 7, Table S1). The open reading frames contain an in frame termination codon within the first half of the intron and code for predicted 5 kDa polypeptides comprising only the RNP2 moiety of the RRM. As intact RNP1 is important for high-affinity RNA binding of @GRP2 moiety of the RRM. As intact RNP1 is important for high-affinity RNA binding of @GRP2 moiety of the RRM. As intact RNP1 is important for high-affinity RNA binding of @GRP2 moiety of the RRM. As intact RNP1 is important for high-affinity RNA binding of @GRP2 moiety of the RRM. As intact RNP1 is important for high-affinity RNA binding of @GRP2 moiety of the RRM. As intact @GRP1 and @GRP7 antibodies and an LHCP antibody (A and B, bottom) as loading control. (C) Semiquantitative RT-PCR of @GRP8. (D) Semiquantitative RT-PCR of as_@GRP7. (E) Semiquantitative RT-PCR of elf-4A as constitutive control. The exponential range was determined by comparing the signal with increasing number of cycles. The absence of genomic DNA was confirmed with nonretrotranscribed RNA.

Figure 5. Differential regulation by cold of @GRP8 and @GRP7. (A) Col plants grown for 2 weeks in 16 h light/8 h dark cycles at 20°C were transferred to 16 h light/8 h dark cycles at 4°C and harvested on day 0, 1, 2, 4 and 7 at zt2 and zt10, respectively. (B) Immunoblots with total protein extracts from the same plants were probed with the @GRP8 (A, top) and @GRP7 (B, top) antibodies and an LHCP antibody (A and B, bottom) as loading control. (C) Semiquantitative RT-PCR of @GRP8. (D) Semiquantitative RT-PCR of @GRP7. (E) Semiquantitative RT-PCR of elf-4A as constitutive control. The exponential range was determined by comparing the signal with increasing number of cycles. The absence of genomic DNA was confirmed with nonretrotranscribed RNA.

Figure 6. Effect of upf1 and upf3 mutations on steady-state abundance of the @GRP8 and @GRP7 pre-mRNAs. RNA from the upf1-5, upf3-1 and upf3-2 mutants and WT harvested at zt10 was reverse-transcribed. PCR amplification of the @GRP8 (A) and @GRP7 pre-mRNA (B) was performed using specific primers and 24 cycles. The gel with the PCR products was blotted and hybridized with the @GRP8 cDNA (A) or @GRP7 cDNA (B). Amplification with ACTIN primers served as control (C).
newly identified \textit{AtGRP8} feedback loop is interconnected with the \textit{AtGRP7} feedback loop previously identified as a circadian slave oscillator (9). The connection between the two regulatory circuits may serve to integrate input by diverse stimuli, and the crossregulation may fine tune and balance the expression of both proteins.

In the future it will be important to determine common target transcripts of \textit{AtGRP7} and \textit{AtGRP8} and to determine the relative influence both proteins have on such downstream transcripts.

**CONCLUSION**

We show here that the clock-controlled RNA-binding protein \textit{AtGRP8} forms an interlocked post-transcriptional negative feedback loop with the \textit{AtGRP7} autoregulatory circuit. Both proteins negatively autoregulate and reciprocally crossregulate by binding to their pre-mRNAs and promoting unproductive splicing coupled to degradation via the NMD pathway (Figure 8). Thus, we extend the examples of quantitative post-transcriptional control by
AS-NMD to autoregulatory loops in output pathways of the circadian clock.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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