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*At*GRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in *Arabidopsis thaliana*

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ABSTRACT **The endogenous clock that drives circadian rhythms is thought to communicate temporal information within the cell via cycling downstream transcripts. A transcript encoding a glycine-rich RNA-binding protein, *Atgrp7*, in *Arabidopsis thaliana* undergoes circadian oscillations with peak levels in the evening. The *At*GRP7 protein also cycles with a time delay so that *Atgrp7* transcript levels decline when the *At*GRP7 protein accumulates to high levels. After *At*GRP7 protein concentration has fallen to trough levels, *Atgrp7* transcript starts to reaccumulate. Overexpression of *At*GRP7 in transgenic *Arabidopsis* plants severely depresses cycling of the endogenous *Atgrp7* transcript. These data establish both transcript and protein as components of a negative feedback circuit capable of generating a stable oscillation. *At*GRP7 overexpression also depresses the oscillation of the circadian-regulated transcript encoding the related RNA-binding protein *At*GRP8 but does not affect the oscillation of transcripts such as *cab* or catalase mRNAs. We propose that the *At*GRP7 autoregulatory loop represents a "slave" oscillator in *Arabidopsis* that receives temporal information from a central "master" oscillator, conserves the rhythmicity by negative feedback, and transduces it to the output pathway by regulating a subset of clock-controlled transcripts.**

An endogenous clock imposes rhythmicity on physiological processes in plants, animals, and some prokaryotes with an approximately 24-h period, reflecting the period of the Earth's rotation (1–4). During the last two decades, the analysis of mutants affected in circadian-regulated output has provided valuable insights into the central clock machinery. A concept has been developed where a "master" clock controls individual subordinated "slave" oscillators, each of which in turn controls overt rhythms (5).

Single genes regulating conidiation rhythms in *Neurospora crassa* and eclosion rhythms in *Drosophila melanogaster* have been isolated (6–9). Current understanding of the biochemical mechanism underlying circadian timekeeping is largely based on the characteristics of the *Neurospora* FREQUENCY (FRQ) and *Drosophila* PERIOD (PER) and TIMELESS (TIM) proteins (10–12). These clock proteins negatively regulate oscillation of their own transcripts, thereby forming an autoregulatory feedback circuit involving transcription of the clock gene, translation, posttranslational protein modification, and nuclear import (2, 9, 13, 14). The analysis of the *per* homologue in the giant silkmoth *Antheraea pernyi* recently demonstrated that this clock molecule can operate in a distinctly different way in the adult brain: PER oscillations are not the result of a transcriptional autoregulatory loop but rather generated by an endogenous *per* antisense RNA that oscillates antiphasic to *per* and might block *per* transcript function in a cyclic manner (15). It is generally accepted but not yet proven that the clock proteins transduce temporal information to generate the overt rhythms by causing downstream transcripts to cycle (3, 16).

In higher plants, a large number of transcripts undergo circadian oscillations (17–19). Because none of the corresponding gene products has been shown to be causally involved in the generation of rhythmicity, presumably these rhythms merely reflect outputs from the clock. So far only in *Arabidopsis thaliana*, clock-related mutants have been identified employing luciferase reporter gene activity under control of a circadian-regulated promoter as a screenable clock-output phenotype (20).

In contrast to this strategy of clock mutant analysis we have chosen a reverse-genetics approach to investigate the potential role of a circadian-regulated RNA-binding protein in the genesis of endogenous rhythmicity in *Arabidopsis thaliana*. This protein previously was identified in a screen for transcripts differentially expressed as a function of time of day (19, 21, 22).

EXPERIMENTAL PROCEDURES

RNA Isolation and RNA Gel Blot Hybridization. Isolation of total RNA, separation on 1.5% agarose-formaldehyde gels, and transfer to nylon membranes (GeneScreen, DuPont) were performed as described (21, 22). cDNA probes were radioactively labeled with the "Prime it II" kit (Stratagene). Gene- and strand-specific antisense probes that distinguish between the *Arabidopsis thaliana* genes *Atgrp7* (23), also designated *ccr2* (24), and *Atgrp8* (23), also designated ccr1 (24), both encoding glycine-rich RNA-binding proteins with 75% sequence identity (23, 24), were derived from the 5′ untranslated regions. They were obtained by replacing the nonamer primers in the labeling reaction by oligonucleotides covering the respective translation start sites. Hybridization was performed according to ref. 25. The Northern blots were quantitated using a PhosphorImager (Molecular Dynamics) and associated software. *Atgrp*7 signals were normalized to signals obtained by hybridization with a barley 26 S rDNA probe (26).

Protein Isolation and Immunoblots. Four-week-old plants were ground in liquid nitrogen. To minimize variation in the extraction, 100 μl of sample buffer (21, 22) was added per 10 mg of powder. Samples were boiled for 10 min and insoluble material was pelleted. The protein concentration of the su-

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Abbreviations: CaMV, cauliflower mosaic virus; zt, zeitgeber time; ct, circadian time; LD, light/dark cycles; LL, continuous light; *Sa*GRP, *Sinapis alba* glycine-rich protein; *At*GRP, *Arabidopsis thaliana* glycine-rich protein.

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pernatant was determined according to Esen (27). A test gel was stained with Coomassie blue to check for sample variation. Five micrograms of total protein was separated on 15% SDS polyacrylamide gels and electroblotted to Polyvinyliden difluoride membranes (Pierce). *At*GRP7 was assayed using an antiserum against bacterially expressed *Sa*GRP (21) at a 1:2,500 dilution. In some of the experiments, levels of plastid ATPase (α and β subunits) were assayed afterward using an antiserum raised against rye ATPase (28) at a 1:4,000 dilution, followed by chemiluminescence detection (POD chemiluminescence kit, Boehringer Mannheim). Following the immunodetection, the filters were stained with 0.1% amido black in 45% methanol/10% acetic acid and destained in 80% methanol/4% acetic acid to verify equal sample application. Scans of the immunoblot were evaluated using the NIH IMAGE 1.59 program. *At*GRP was normalized against loaded protein by densitometry of the amido black-stained filter.

Plasmid Construction and Transformation of *Arabidopsis thaliana*. The *Atgrp7* protein-coding region was amplified by PCR from the cDNA with primers 5′-GGCC**ATGG**CGTCCGGTGAT-3′ and 5′-GGGATCC**TTA**CCATCCTCCACC-3′ covering the translation start and stop (bold) and comprising engineered *Nco*I and *Bam*HI sites (underlined), respectively. The gel-purified 540-bp amplification product was inserted into the vector pRT104 (29) between the cauliflower mosaic virus (CaMV) 35S RNA promoter with the duplicated enhancer (30) fused to the tobacco mosaic virus omega element (31) and the CaMV polyadenylation signal. The entire expression cassette was transferred as a *Hin*dIII fragment to the binary vector pBin19 (32) and introduced into *Arabidopsis thaliana* strain C24 by *Agrobacterium*-mediated root-transformation (33). Calli resistant to 50 μg/ml kanamycin were regenerated, rooted, and allowed to set seeds.

For Northern blot kinetics, kanamycin-resistant F_2 seedlings were grown on ½ MS medium (34) in light/dark cycles, as indicated in the figure legends.

RESULTS

As a way to understand the molecular mechanisms underlying clock control of rhythmic endogenous processes, we previously isolated oscillating transcripts differentially expressed as a function of time of day in the long-day plant *Sinapis alba* (white mustard) by subtractive hybridization (21, 22). One of these transcripts, which reaches its maximal concentration in the evening, codes for a glycine-rich protein (*Sa*GRP) with an N-terminal RNA recognition motif. Based on the homology to RNA-binding proteins such as nucleolin (35) and the splicing factor hnRNPA1 (36), and its ability to interact with RNA (unpublished observation; ref. 24) as well as its localization within the nucleus (21), it seems reasonable to assume a regulatory role for this plant protein.

As a first step toward the analysis of the RNA-binding protein in transgenic *Arabidopsis thaliana* plants, we have isolated the *Arabidopsis* counterpart of *Sagrp1*, which corresponds to both *Atgrp7* (23) and *ccr2* (24). As in *Sinapis alba*, an additional derivative of this cDNA was isolated. It contains a 300-bp insertion within the RNA recognition motif and corresponds to the unspliced 1-kb pre-mRNA (21).

Northern blot analysis using a gene-specific probe confirmed that the *Atgrp7* transcript, like *Sagrp1*, undergoes oscillations in young plants grown in light/dark cycles, with the highest levels occurring 8 to 12 h after onset of illumination and persisting thereafter in constant light (Fig. 1*A*) (21, 24). Peak quantities of *Atgrp7* transcript are about 40 times the amount at the trough (Fig. 1*D*). Immunoblot analysis demonstrated that the *At*GRP7 steady state concentration also oscillates (Fig. 1*B*). Most notably, *At*GRP7 protein peaks are delayed relative to the *Atgrp7* transcript peaks by about 4 h. When the *At*GRP7 protein has accumulated to high levels, the *Atgrp7* transcript level declines and does not rise again until the *At*GRP7 protein has reached its trough (Fig. 1*D*). This delayed oscillation of the RNA-binding protein relative to its transcript could reflect a translational control or could be the result of an autoregulatory feedback loop in which an elevated level of the protein negatively affects the accumulation of its own transcript. In the latter case, constitutive overexpression of this protein in transgenic plants should eliminate the detectable oscillation of its endogenous transcript.

To obtain high, constitutive *At*GRP7 expression, the coding sequence under control of the CaMV promoter with the duplicated enhancer (30) was introduced into *Arabidopsis thaliana*. *Atgrp7* steady-state transcript concentrations were assayed in F_2 seedlings grown in light/dark cycles (Fig. 2*A*). Three lines (RS13, RS15, and RS55) were identified that show equally high *Atgrp7* transcript levels in the morning (zt0; zt, zeitgeber time) as well as in the evening (zt12), in contrast to the wild-type plants. Immunoblot analysis confirmed a high *At*GRP7 protein level at the minimum (zt4) as well as at the

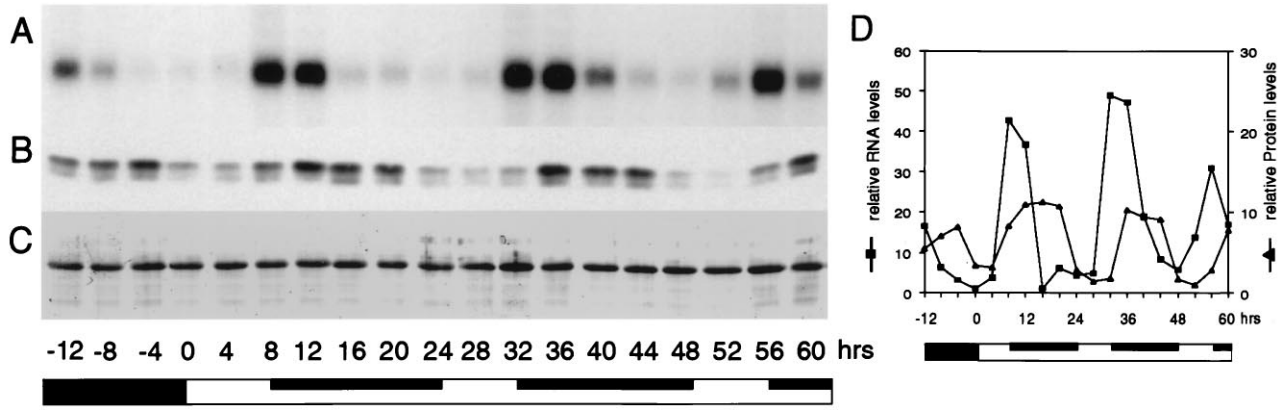

FIG. 1. *Atgrp7* mRNA and protein cycling in *Arabidopsis thaliana*: Delay of the protein peak relative to the transcript peak. Plants were first entrained to light/dark cycles (LD 8:16), harvested at 4-h intervals and subsequently left in LL for the indicated number of hours after "lights on" on the final day in LD. (*A*) RNA gel blot with 10 μg of total RNA. *Atgrp7* transcript was detected with a gene-specific probe derived from the 5′ untranslated region. (*B*) Protein gel blot of the same plants. Immunodetection of *At*GRP (*B*) and amido black staining of the filter (*C*). (*D*) Relative *Atgrp7* transcript and *At*GRP protein levels. *Atgrp7* RNA levels were normalized to controls obtained with rDNA hybridization. Densitometric evaluation of the stained filter in *C* was used to normalize protein levels in *B*. Values are expressed relative to the minimal level defined as 1. The solid and open bars represent dark and light periods, respectively. The inserted dark bars indicate subjective night. hrs, hours before and after onset of illumination on the last day in LD.

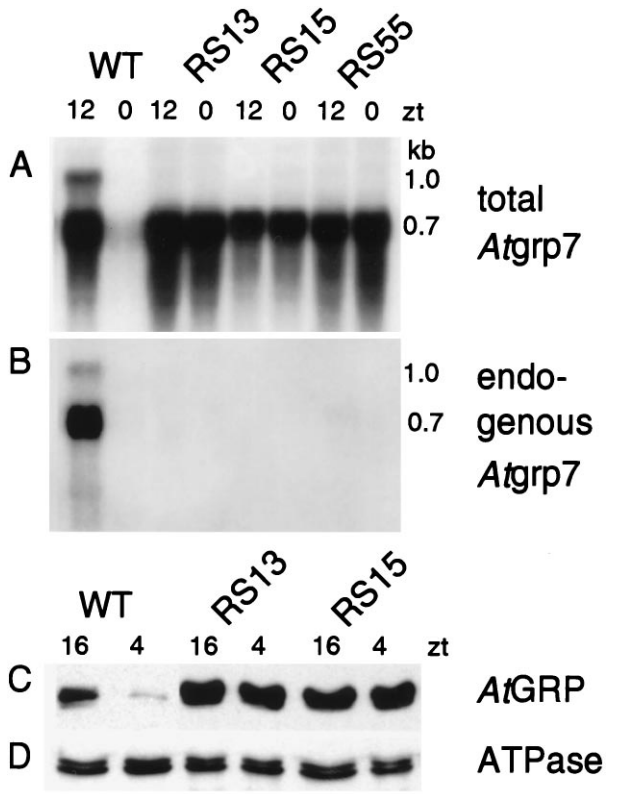

FIG. 2. *At*GRP7 overexpression suppresses the endogenous *Atgrp7* transcript. RNA was isolated from three independent transgenic lines and wild-type controls grown in light/dark cycles at zt0 and zt12. The Northern blot with 20 μg of RNA was hybridized with the *Atgrp7* cDNA to measure total *Atgrp7* transcript levels (*A*). Note that due to the higher loading the 1-kb pre-mRNA is clearly detectable in the wild-type plants in contrast to Fig. 1*A*. After stripping of the membrane, expression of the endogenous *Atgrp7* transcript was monitored by hybridization with a gene-specific probe derived from the 5′ untranslated region not contained within the transgene (*B*). (*C* and *D*) Immunoblot analysis of wild-type plants and the transgenic lines RS13 and RS15, harvested at the time of the protein trough (zt4) and the protein peak (zt16) with antibodies against *Sa*GRP (*C*) and the α and β subunits of ATPase (*D*).

maximum (zt16) due to transgene expression in contrast to the wild-type plants (shown for lines RS13 and RS15 in Fig. 2*C*).

In the wild-type plants, an additional 1.0-kb *Atgrp7* transcript species is visible (Fig. 2*A*). It corresponds to the unspliced pre-mRNA which, due to inefficient removal of the intron, accumulates to levels that can be detected on blots with high amounts of RNA (21, 24). In the *Arabidopsis* lines overexpressing *At*GRP7, this 1-kb pre-mRNA is absent. The high *At*GRP7 level from the CaMV-*At*GRP7 construct might thus have either selectively decreased the level of the unspliced pre-mRNA or repressed the level of all of the endogenous *Atgrp7* transcript species. To discriminate between these two alternatives, the blot was reprobed with the *Atgrp7* 5′ untranslated region that distinguishes between the transcripts of the endogenous gene and the transgene (Fig. 2*B*). Almost no endogenous *Atgrp7* transcript could be detected in the transgenic lines at zt12, whereas both the fully spliced *Atgrp7* transcript and the pre-mRNA were detectable in the wild type. Thus, overexpression of *At*GRP7 greatly suppresses the abundance of all of the endogenous *Atgrp7* transcripts.

To examine this molecular phenotype in greater detail, a time course of *Atgrp7* expression in transgenic and control plants was made over the entire circadian cycle (Fig. 3). The *Atgrp7* transcript showed a high, relatively uniform level in light/dark cycles (LD) as well as in continuous light (LL) in the

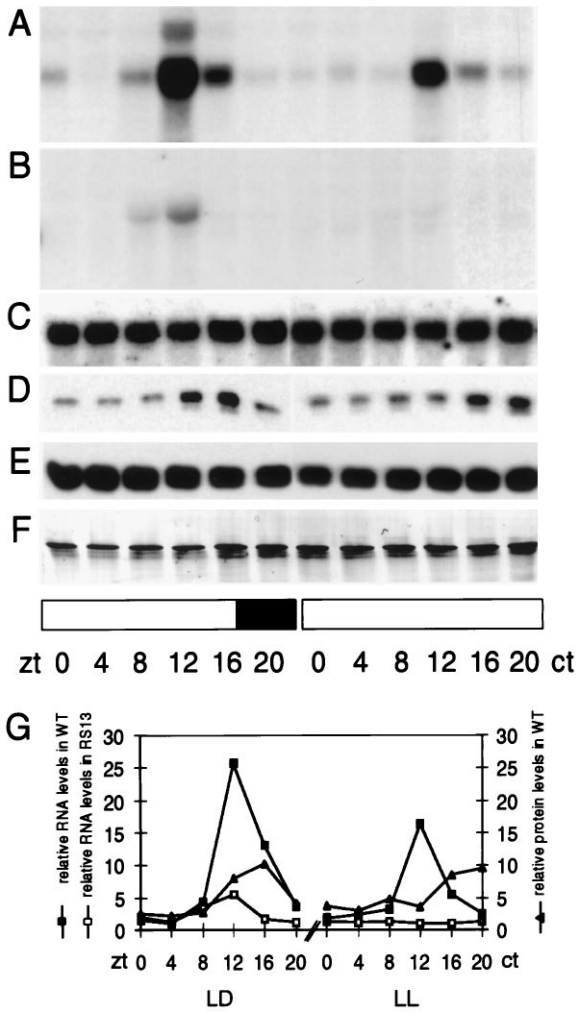

FIG. 3. *Atgrp7* transcript and *At*GRP protein time course in wild-type plants and the transgenic line RS13. Plants were harvested at 4-h intervals during one light/dark cycle and on the second day after transfer to LL. Expression of the endogenous *Atgrp7* transcript was monitored with a gene-specific probe derived from the 5′ untranslated region, which also included part of the promoter to increase specific activity of the hybridization probe, in wild-type plants (*A*) and in the representative line RS13 (*B*). (*C*) Total *Atgrp7* transcript level in the transgenic line RS13 measured by hybridization with the *Atgrp7* cDNA. (*D*) Immunoblot analysis of wild-type plants with the antibody against *Sa*GRP. (*E*) Immunoblot analysis of the transgenic line RS13 with the antibody against *Sa*GRP and amido black staining of the filter (*F*). (*G*) Quantitation of the endogenous *Atgrp7* transcript profile in wild-type plants, shown in *A*, and the transgenic line RS13, shown in *B*, and *At*GRP protein oscillations in wild-type plants, shown in *D*. *Atgrp7* RNA levels were normalized to controls obtained with rDNA hybridization. Protein levels were normalized to the densitometric evaluation of the amido black-stained blot. Identical results were obtained with the transgenic line RS15. The solid and open bars represent dark and light periods, respectively.

overexpressing line RS13 (Fig. 3*C*). Similarly, the *At*GRP protein level did not show circadian oscillations in the transgenic line (Fig. 3*E*) in contrast to wild-type plants (Fig. 3*D*). Whereas normal *Atgrp7* cycling was observed both in LD and in LL in wild-type plants (Fig. 3*A*), oscillations of the endogenous *Atgrp7* transcript in transgenic plants were severely depressed in LD (Fig. 3 *B Left* and *G*) and no longer detectable in LL (Fig. 3 *B Right* and *G*).

The presence of an RNA recognition motif suggests that *At*GRP7 might interact with other transcripts and in this way may confer circadian rhythmicity on them. Therefore we compared the steady-state concentrations of selected oscillat-

ing transcripts, representing different circadian phases, in wild-type plants with those in the *At*GRP7 overexpressing lines. No significant differences between control plants and the transgenic line RS13 were observed for the *cab* (chlorophyll a/b binding protein) transcripts that peak around noon (Fig. 4*A*) (37), for a germin-like protein, *Atger3*, that peaks in the late evening (Fig. 4*B*) (38), and for catalase3 (39) (not shown) in light–dark cycles, as well as in constant light.

However, circadian oscillations of *Atgrp8*, a transcript encoding a related glycine-rich RNA-binding protein (23, 24) that cycles in phase with *Atgrp7* in the wild-type plants (Fig. 4*C Left*), were almost fully suppressed in the transgenic plants in LD and not detectable any more in LL (Fig. 4*C Right*).

DISCUSSION

In the present study, we identify the RNA-binding protein *At*GRP7 as the first component of a circadian-regulated feedback loop in higher plants. Our data indicate that *At*GRP7 serves a 2-fold role both as target and modulator of circadian regulation, because it also influences cycling of a heterologous transcript.

We show that the robust *Atgrp7* transcript oscillation leads to circadian cycling of the *At*GRP7 protein. Increasing *At*GRP7 protein concentration coincides with the decay of *Atgrp7* mRNA quantity, and, conversely, the *Atgrp7* mRNA level does not rise again before *At*GRP7 has reached its trough level. These findings are consistent with *Atgrp7* and *At*GRP7 being part of a negative autoregulatory circuit. Moreover, the observed phase difference of about 4 h might provide a delay to generate a stable oscillation. Without such a delay the oscillation in the loop would damp out rather quickly and come to equilibrium (4, 40). To explain the phase of *At*GRP7 protein expression, an as yet undetermined posttranscriptional mechanism in addition to mRNA cycling has to be assumed. Persistent 24-h oscillations of the *Drosophila* clock components PER and TIM, for example, are assumed to depend on delayed nuclear entry of the PER and TIM proteins. In this manner, *per* and *tim* expression could proceed for several hours until PER and TIM repress accumulation of their cognate transcripts within the nucleus (11, 14, 41, 42).

Constitutive *At*GRP7 overexpression in transgenic *Arabidopsis* leads to a dramatic depression of *Atgrp7* transcript oscillations, proving that *At*GRP7 indeed exerts a negative feedback onto its own transcript. In LD-grown plants, the residual low-level *Atgrp7* oscillation in LD suggests that there is input from an unknown external factor. The level of *At*GRP7 repressor activity obtained in the transgenic plants is not sufficient to completely stop the oscillation of *Atgrp7* and can still be overridden by this positively acting factor. In LL, *Atgrp7* expression can no longer be detected, very likely reflecting the damping effect of these light conditions on *Atgrp7* oscillations that is also evident in wild-type plants (Fig. 3*A*). Recently, in the short-period *toc1* (timing of *cab* expression) mutant, the *ccr2* transcript corresponding to *Atgrp7* has been demonstrated to oscillate with a period that is significantly shorter than in wild-type plants (43). Therefore, the toc1 gene product could be one candidate for such an external factor that affects the *At*GRP7 feedback loop.

Atgrp7 mRNA cycling is mainly generated at the transcriptional level, as the *Atgrp7* promoter confers circadian rhythmicity. In transgenic *Arabidopsis* plants carrying the β-glucuronidase gene under control of a 1.5-kb fragment upstream of the transcription start site, the β-glucuronidase mRNA peaks in the subjective evening, whereas it is barely detectable in the subjective morning (D.S. and M.N., unpublished observation). Therefore, in wild-type plants *Atgrp7* transcript levels seem to be elevated through rhythmic transcriptional activation by the clock in the subjective evening. After a lag phase, *Atgrp7* mRNA accumulation seems to be repressed by the *At*GRP7 protein, resulting in a stable high-amplitude oscillation. The molecular basis of the *At*GRP7 autoregulatory circuit remains to be determined. On the one hand, *At*GRP7 could interfere with transcriptional activation of its own gene by a central oscillator. This may occur indirectly via association of *At*GRP7 with a transcription factor. Such a mechanism has been proposed to account for feedback inhibition of *per* transcription by the *Drosophila* clock protein PER (2, 3). Direct action of *At*GRP7 as a transcriptional repressor is also conceivable, because a potential interaction of an RNA-binding protein with DNA is not without precedent: hnRNPK has been shown to interact with a polypyrimidine tract in the c-*myc* promoter and to act as a transcription factor (44). Also, the RNA recognition motif-containing protein mRNP4/FRG Y2 from Xenopus oocytes stimulates transcription from specific promoters (45). Alternatively, *At*GRP7 might limit transcript accumulation by influencing transcript stability. In this case, the oscillatory feedback loop cannot solely be

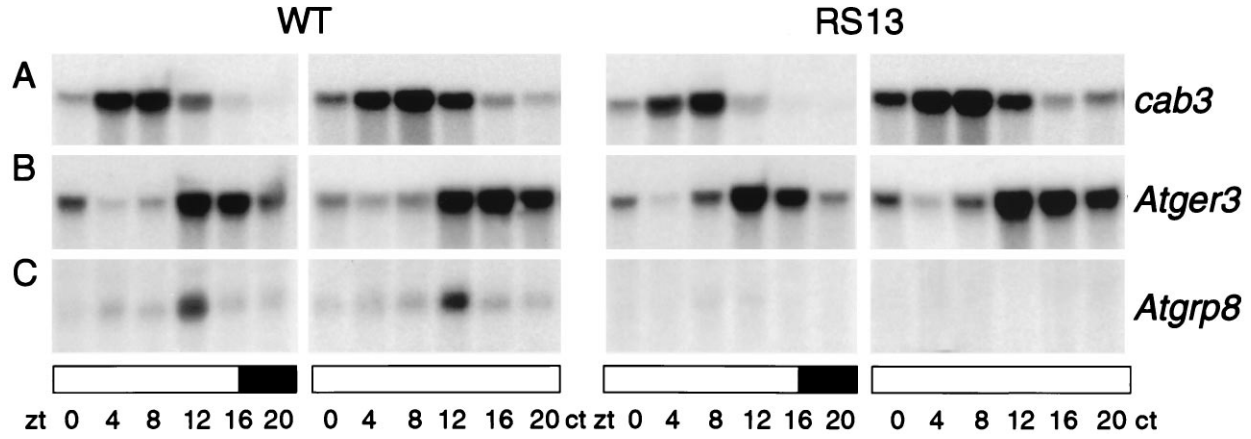

FIG. 4. Influence of *At*GRP7 overexpression on other circadian-regulated transcripts. RNA was isolated from wild-type plants and the representative transgenic line RS13, which were harvested at 4-h intervals in a light/dark cycle (LD 16:8) and on the second day after transfer to continuous illumination. The Northern blot with 10 μg of RNA was hybridized with a *cab3* probe (37), which recognizes all the *cab* transcripts (*A*), a probe for a germin-like protein, *Atger3*, (38) (*B*), and a gene-specific probe derived from the 5′ untranslated region of the *Atgrp8* gene (23) (*C*), respectively. Note that transcript peaks are delayed due to the LD 16:8 conditions used in this experiment compared with the phase of maximal transcript accumulation observed in LD 8:16 (cf. Fig. 1 and our unpublished observation). Identical results were obtained for the transgenic line RS15. The solid and open bars represent dark and light periods, respectively.

described by molecules involved in activation and repression of *Atgrp7* transcription. Measuring the effect of a high, constitutive *At*GRP7 level on reporter gene constructs with various cis-regulatory parts of the *At*GRP7 gene will allow us to discriminate between these different mechanisms.

Whereas direct proof for a function in the generation of circadian physiological processes will require the generation of *Atgrp7* mutants, its homology to splicing factors suggests that *At*GRP7 might transfer its rhythmic activity to other transcripts by means of RNA maturation processes. We demonstrate that *At*GRP7 negatively regulates another circadian-regulated transcript encoding the RNA-binding protein *At*GRP8 (23), although other investigated circadian-regulated transcripts, such as *cab*, a germin-like protein, or catalase mRNAs, are not affected by *At*GRP7 overexpression.

Because there is precedent for the existence of more than one master oscillator in plants (46), at present it is conceivable that *At*GRP7 might be part of one of such central oscillators that controls a limited set of rhythmic phenomena. Based on Pittendrigh's (5, 47) concept that the temporal organization within a cell is established by a central "master" pacemaker governing multiple "slave" oscillators, another interpretation arises: The circadian *At*GRP7 feedback loop could also represent one of several "slave" oscillators in *Arabidopsis* that acquires a circadian period by receiving impulses from the "master" clock. The suboscillator would conserve the rhythmicity by feedback regulation and would transduce it to the output pathway, thus controlling a subset of circadian-regulated processes.

Consistent with this view is the observation that in the overexpressing line, despite a high *At*GRP7 repressor concentration, there is external input into the feedback loop, allowing for a residual low-level oscillation in LD. Furthermore, the *Arabidopsis toc1* mutant shortens the period of cycling of the *cab2* promoter activity, leaf movement rhythms, as well as *Atgrp7* oscillations (20, 43), indicating that the *At*GRP7 autoregulatory circuit receives temporal information from the toc1 gene product. Assuming that toc1 is part of an oscillator, it may govern a possibly multiple-branched pathway, one regulating *cab* rhythms peaking at midday and another regulating *Atgrp7* rhythms peaking in the evening.

Although components of circadian output pathways that affect subsets of clock-controlled processes have been described in other organisms, there is no indication for their function as a slave oscillator, as either no rhythmicity or no feedback regulation have been demonstrated. For instance, the *Drosophila* lark factor, a putative RNA-binding protein, negatively regulates eclosion but does not affect adult locomoter activity rhythms. Its mRNA does not oscillate in abundance, and it is not known whether the encoded protein displays rhythmic activity (48). In the cyanobacterium *Synechococcus*, a mutation in a sigma^{70}-like transcription factor results in a low-amplitude rhythm phenotype. This factor seems to be part of a clock output pathway, because its loss affects the rhythmic transcription of a subset of clock-controlled genes (49). However, no negative autoregulation has been demonstrated yet for this gene.

The search for additional genes whose expression is affected by *At*GRP7 should allow us to define more precisely the physiological role of this oscillatory feedback loop within the circadian network in *Arabidopsis*.

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