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The small glycine-rich RNA binding protein AtGRP7 promotes floral transition in Arabidopsis thaliana

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Summary
The RNA binding protein AtGRP7 is part of a circadian slave oscillator in Arabidopsis thaliana that negatively autoregulates its own mRNA, and affects the levels of other transcripts. Here, we identify a novel role for AtGRP7 as a flowering-time gene. An atgrp7-1 T-DNA mutant flowers later than wild-type plants under both long and short days, and independent RNA interference lines with reduced levels of AtGRP7, and the closely related AtGRP8 protein, are also late flowering, particularly in short photoperiods. Consistent with the retention of a photoperiodic response, the transcript encoding the key photoperiodic regulator CONSTANS oscillates with a similar pattern in atgrp7-1 and wild-type plants. In both the RNAi lines and in the atgrp7-1 mutant transcript levels for the floral repressor FLC are elevated. Conversely, in transgenic plants ectopically overexpressing AtGRP7, the transition to flowering is accelerated mainly in short days, with a concomitant reduction in FLC abundance. The late-flowering phenotype of the RNAi lines is suppressed by introducing the flc-3 loss-of-function mutation, suggesting that AtGRP7 promotes floral transition, at least partly by downregulating FLC. Furthermore, vernalization overrides the late-flowering phenotype. Retention of both the photoperiodic response and vernalization response are features of autonomous pathway mutants, suggesting that AtGRP7 is a novel member of the autonomous pathway.

Keywords: Arabidopsis, flowering time, circadian clock, post-transcriptional regulation, autonomous pathway, RNA binding protein.

Introduction
The appropriate timing of the transition from vegetative to reproductive growth is controlled by a suite of signaling pathways responding to endogenous cues and tracking environmental signals, such as ambient temperature and light quality (Corbesier and Coupland, 2006; Kobayashi and Weigel, 2007; Putterill et al., 2004; Simpson and Dean, 2002). Arabidopsis thaliana flowers earlier in long photoperiods than in short photoperiods. This photoperiodic flower induction is mediated by the endogenous circadian clock. The zinc-finger protein CONSTANS (CO) plays a critical role in interpreting day length to initiate floral transition. CO mRNA oscillates with a circadian rhythm, and peaks at the end of the daily light phase in long days (LD), but after the light–dark transition in short days (SD) (Suarez-Lopez et al., 2001). In light, the CO protein is stabilized, and thus accumulates to a level sufficient to induce flowering (Valverde et al., 2004). CO, in turn, directly activates FLOWERING LOCUS T (FT) (Abe et al., 2005; An et al., 2004; Wigge et al., 2005). Movement of FT protein from phloem cells in the leaves to the apex induces flower formation (Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007).

FT and SUPPRESSOR OF CONSTANS (SOC1), encoding two of the earliest targets of the photoperiodic pathway (Borner et al., 2000; Samach et al., 2000), are also negatively regulated by the MADS-box protein FLOWERING LOCUS C (FLC), which is a key repressor of flowering (Lee et al., 2000; Michaels and Amasino, 1999; Michaels et al., 2005; Sheldon et al., 1999). The repressive effects of FLC can be overcome by prolonged cold treatment (vernalization) to ensure that flowering occurs when winter is over. During this process,
late-flowering phenotype, whereas transgenic plants ectopically overexpressing AtGRP7 flower early. Nevertheless, these plants with altered AtGRP7 levels retain a photocyclical response. The effect on flowering is in large parts mediated by FLC, and the late-flowering phenotype can be overcome by vernalization, implicating AtGRP7 in the autonomous pathway.

Results

An atgrp7 T-DNA insertion mutant is late flowering

Because post-transcriptional control emerges as an important mechanism in flowering time control, we asked whether AtGRP7 plays a role in floral transition. First, we investigated how the loss of AtGRP7 impacts on the flowering time in the atgrp7-1 mutant from the SALK collection with a T-DNA insertion in the 5′ region (Fu et al., 2007). Under SDs, atgrp7-1 plants formed about 61 leaves at the onset of bolting, compared with 54 leaves in wild-type (WT) plants, and under LDs, atgrp7-1 plants flowered with 15 leaves compared with WT flowering (13 leaves) (Table 1). Student’s t-tests revealed that this small increase in leaf number was significant. To monitor the floral transition at the molecular level, we investigated the floral meristem identity gene APETALA1 (AP1). Whereas in LD-grown WT plants AP1 transcript levels were increased by about sixfold from day 10 to day 21, the expression level rose more slowly in atgrp7-1 (Figure 1a). Thus, in plants that lack AtGRP7, the transition to flowering is weakly delayed with long photoperiods, and is more strongly delayed with short photoperiods.

The response to inductive LDs is determined by the phase of the key regulator CO (Suarez-Lopez et al., 2001). In atgrp7-1 plants, diurnal oscillations of the CO transcript were very similar to WT, with respect to phase and amplitude, both in LDs and SDs (Figure 1b,c), which is consistent with their retaining a photoperiodic response.

Because the lack of AtGRP7 in atgrp7-1 had a relatively mild effect on flowering time, we examined in detail the expression of AtGRP8, which shows 77% sequence identity to the amino acid level and oscillates more or less in phase with AtGRP7.

Notably, whereas the AtGRP7 transcript was undetectable, as expected (Fu et al., 2007), the AtGRP8 transcript level was elevated in atgrp7-1 plants compared with WT (Figure 1d). To be able to distinguish between the highly similar AtGRP7 and AtGRP8 proteins, anti peptide antibodies specifically

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Leaf number of short-day (SD) and long-day (LD) grown atgrp7-1 and wild-type (WT) plants ± SD at an inflorescence height of 0.5 cm: the total number of plants n and the P value determined by a Student’s t-test are indicated. A representative experiment of five independent replicates with n ≥ 30 each is shown.
directed against AtGRP7 or AtGRP8 were generated (see Experimental procedures). The specificity of the antibodies was tested using recombinant AtGRP7 and AtGRP8, respectively (Figure 1e). Weak cross-reactivity was only observed for the AtGRP7 antibody when as much as 2 μg of pure recombinant AtGRP8 protein was blotted. Whereas AtGRP7 protein was absent in atgrp7-1 plants, the AtGRP8 protein level was elevated compared with WT (Figures 1e and S1). This indicates that the release from negative regulation in the absence of AtGRP7 leads to higher AtGRP8 accumulation.

Thus, it remains possible that the elevated AtGRP8 levels may mask part of the effect of AtGRP7 on flowering time. To substantiate the floral-promotive function of AtGRP7 we decided to analyze flowering time in transgenic plants with reduced levels of AtGRP7 and AtGRP8 on the one hand, and with constitutively elevated AtGRP7 levels on the other hand.

Molecular analysis of AtGRP7 and AtGRP8 RNAi lines

To obtain transgenic Arabidopsis plants with reduced AtGRP7 or AtGRP8 expression, we performed RNA interference using the pKannibal system encoding a Cauliflower mosaic virus (CaMV) promoter-driven hairpin (hp) RNA, consisting of an inverted repeat of the gene fragment separated by the PdK intron (Wesley et al., 2001). Two types of constructs were designed: short (s) constructs comprising only the part encoding the N-terminal RNA recognition motif of AtGRP7 or AtGRP8, and long (l) constructs comprising additionally the part encoding the glycine-rich C terminus (Figure 2). The short construct directed against AtGRP7 could not be stably propagated in Agrobacterium tumefaciens, and thus was not used further (not shown).

Transformed plants were selected on phosphokinotrinic, and were checked for the presence of the transgene. Seedlings harboring the intact hp constructs were raised to maturity, and were then surveyed for endogenous RNA and protein levels in the next generation. To test whether the simultaneous presence of AtGRP7 and AtGRP8 and long (l) constructs comprising additionally the part encoding the glycine-rich C terminus (Figure 2). The short construct directed against AtGRP7 could not be stably propagated in Agrobacterium tumefaciens, and thus was not used further (not shown).

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AtGRP7 and AtGRP8 transcript levels were monitored in LD-grown plants harvested at zt12 (zeitgeber time 12, 12 h after lights on), with gene- and strand-specific probes derived from their 5′-untranslated regions (UTRs) that do not cross-hybridize with the transgenic mRNA (Figures 3
AtGRP7 transcript was almost undetectable (Figure 3c), and the AtGRP7 transcript was reduced (Figure 3a). In all AtGRP7i lines, the AtGRP7 protein level was strongly reduced or almost undetectable compared with WT and Cambia lines (Figure 3e), and in the AtGRP8 lines, the AtGRP8 protein was almost undetectable (Figures 3g and S1). Again, in the AtGRP7i lines, the AtGRP8 protein was only weakly affected, or remained at WT levels, whereas in the AtGRP8 lines the AtGRP7 protein level was clearly lower than in WT or Cambia lines (Figure 3g). Taken together, these data suggest that the AtGRP7i or AtGRP8i constructs are effective against endogenous AtGRP7 or AtGRP8, respectively, but also show some cross-regulation. The fact that in the AtGRP8i plants the AtGRP7 protein is also strongly affected, whereas in the AtGRP7i plants the AtGRP8 protein remains more or less at WT levels may indicate that the AtGRP8i constructs are more effective against AtGRP7 than vice versa. Alternatively, the effect of the AtGRP7i construct on AtGRP8 may be partially masked by enhanced AtGRP8 accumulation as a consequence of AtGRP7 down-regulation. In the crosses containing both hp constructs simultaneously, a further reduction of the AtGRP7 and AtGRP8 levels was only observed in line AtGRP7i×8i-21, compared with the parental lines (Figure 3e,g).

AtGRP7i and AtGRP8i plants are late flowering

Lines of all three genotypes AtGRP7i, AtGRP8i and AtGRP8-s, and of the respective crosses, developed more leaves before flowering than WT or Cambia plants in SDs (Figure 4a). Also, in LDs, the RNAi lines formed between one and three leaves more than the control plants (Figure 4b). One-way ANOVA followed by a post-hoc Dunnet test showed that these differences are statistically significant (Table S2). Taken together, in the RNAi lines the delay in the transition to flowering is more pronounced than in the atgrp7-1 mutant. Again, the late-flowering phenotype is more pronounced in LDs than in SDs.

Vernalization response of the RNAi and atgrp7-1 plants

The retention of a photoperiodic response is a feature of mutants in the autonomous pathway that flower late in LDs, like mutants in the photoperiodic pathway, but flower even later in SDs, i.e. irrespective of the photoperiod. Additionally, their late-flowering pheno-type can be corrected by vernalization. To determine whether the RNAi lines and atgrp7-1 also show this feature, their response to extended periods of cold in the early seedling stage was assessed (see Experimental procedures). Again, non-vernalized RNAi lines and atgrp7-1 flowered with more leaves than WT or C plants (Figure 5 and Table S3). After vernalization, RNAi lines flowered with a similar leaf number as the WT plants. atgrp7-1 mutants flowered even at a slightly reduced leaf
Values represent the means at bolting in non-vernalized plants (white bar) and vernalized plants (black bar). Values represent the means ± SD.

(a) Wild type (WT), Cambia-1 (C-1), AtGRP7i-I48 and AtGRP7i-I68, AtGRP8i-I71, AtGRP7i-x8i-21 and AtGRP7i-x8i-50 (left), and WT, C-5, AtGRP7i-I1 and AtGRP7i-I54, AtGRP8i-I14, AtGRP8i-s7, AtGRP7i-x8i-58 and AtGRP7i-x8i-109 (right), respectively, were grown in SDs.

(b) WT, C-5, AtGRP7i-I1, AtGRP8i-I64 and AtGRP7i-I68, AtGRP8i-I14 and AtGRP8i-s7 (left) and WT, C-5, AtGRP7i-x8i-21 and AtGRP7i-x8i-50 (right), respectively, were grown in LDs.

The number of rosette leaves (LN) produced at bolting is given as means ± SD. An ANOVA followed by a Dunnet test was performed to show statistical significance (see Table S2).

Figure 4. Flowering time of AtGRP7i and AtGRP8i lines grown in short-day (SD) and long-day (LD) conditions.

Influence of AtGRP7 on FLC

The autonomous pathway acts in parallel with vernalization to constitutively repress FLC (Michaels and Amasino, 2001). Because vernalization bypasses the requirement of AtGRP7 in the RNAi lines and the atgrp7-1 mutant, we investigated whether the floral promotive effect of AtGRP7 was mediated by FLC.

To this end, we first determined FLC levels by real-time PCR (Figure 6a). In the RNAi lines and in atgrp7-1, FLC was elevated by more than fivefold relative to WT or C plants. Vernalization strongly reduced the FLC levels in WT. Also, in the RNAi lines and atgrp7-1, FLC levels were reduced to a basal level similar to WT and C plants (Figure S2). Concomitantly, the floral integrator SOC1, which is negatively controlled by FLC, was upregulated upon vernalization both in WT and the RNAi lines (not shown). Thus, plants with reduced AtGRP7 levels or complete loss of AtGRP7 remain fully responsive to vernalization, indicating that FLC down-regulation in response to cold treatment does not require AtGRP7.

If the late flowering effect seen in plants with reduced AtGRP7 levels entirely resulted from a lack of FLC repression, it should be suppressed in a genetic background lacking FLC. Therefore, we crossed the late-flowering RNAi lines AtGRP7i-x8i-21 and AtGRP7i-x8i-50 with flc-3 mutants lacking active FLC because of a 107-bp deletion around the ATG (Michaels and Amasino, 1999). In the F2 generation we identified lines homozygous for both the RNAi construct and the flc-3 allele, by monitoring phosphinotricin resistance and PCR genotyping. Flowering time was investigated in the F2 generation. Again, AtGRP7i-x8i-21 and AtGRP7i-x8i-50 flowered later than WT and C plants both in SDs and LDs (Figure 6b,c and Table S4). This late-flowering phenotype was eliminated in AtGRP7i-x8i flc-3 plants, which flowered with a leaf number comparable with flc-3. As previously noted, flc-3 flowered with fewer leaves than WT in SDs (Michaels and Amasino, 2001). These data indicate that the lack of AtGRP7 does not manifest itself in the absence of FLC.

Constitutive overexpression of AtGRP7 promotes flowering

Because of the partial off-target effect of the AtGRP7i and AtGRP8i hp constructs on the AtGRP8 and AtGRP7 abundance, respectively, and the relief of AtGRP8 repression in the atgrp7-1 T-DNA line, the floral promotive effect could only be assigned tentatively to AtGRP7. Therefore, an effect of ectopic AtGRP7 overexpression on flowering time was investigated in a complementary approach. The AtGRP7-ox lines D and G express highly elevated levels of AtGRP7 protein, resulting in strongly reduced AtGRP8 protein levels (Figure 7a). In SDs, these plants formed about 10 leaves less than WT at bolting (Figure 7b). This clearly shows that AtGRP7 by itself is able to promote flowering. In the At-
GRP7-ox lines, FLC was downregulated to 10–20% of the level in WT (Figure 7c). After vernalization, AtGRP7-ox and WT plants formed about the same number of leaves at bolting (Table S5). In LDs, the AtGRP7-ox lines flowered only slightly earlier than WT plants (Table S5), indicating that the promotive effect of AtGRP7 has a stronger impact under non-inductive photoperiods.

To address the question of whether AtGRP7 may act via other autonomous pathway components, we analyzed selected transcripts encoding proteins associated with RNA metabolism, and their respective alternative splice
forms (http://www.plantgdb.org/ASIP/) in the AtGRP7 gain-of-function and loss-of-function plants. For FPA encoding a protein with three RRMs (Schomburg et al., 2001), a transcript retaining the 129-nt (nucleotide) intron 4 was detected, in addition to the spliced mRNA (Figure S3a). The ratio between the longer and shorter variants, and their levels, were not changed in the atgrp7-1 T-DNA line or representative RNAi lines compared with WT or the C line. Also, constitutive overexpression of AtGRP7 did not affect intron 4 retention. Furthermore, retention of a 99-nt intron located 40-nt downstream of the stop codon in the FPA 3’-UTR was found for all genotypes (Figure S3b).

For the 3’ end processing factor, FY, the use of an alternative acceptor site at intron 8 leads to the inclusion of four additional nt in intron 8, causing a frameshift and premature in-frame termination codon. Using flanking primers, a fragment of the same size as in WT and C lines was detected on a polyacrylamide gel for the RNAi lines, the atgrp7-1 T-DNA line and the AtGRP7-ox line (Figure S3d). Also for FCA, no obvious variations were detected for the FCA-γ mRNA, encoding active FCA protein, and the alternatively spliced, prematurely polyadenylated FCA-β form (not shown). The autonomous pathway component LUMINDEPENDENS (LD) is a homeodomain protein that may interact with DNA or RNA (Aukerman et al., 1999). The steady-state abundance of LD is not significantly different from WT levels in the RNAi lines, the atgrp7-1 mutant and the AtGRP7-ox lines (Figure S3e).

Discussion

The RNA-binding protein AtGRP7 promotes flowering

Here, we demonstrate the participation of a small glycine-rich RNA binding protein with a single RRM in the regulation of flowering time in Arabidopsis. The loss of function of AtGRP7 delays the transition to flowering, whereas the gain of function through ectopic overexpression of AtGRP7 promotes flowering.

The atgrp7-1 T-DNA line flowers later than WT plants. In LDs, the delay is small, yet statistically significant. These data are supported by the observation that the rise in AP1, indicative of floral induction, occurs later in development than in the WT. The retardation becomes more pronounced in SDs, with a reduced impact of the photoperiodic inductive signal.

The well-described prominent regulation of AtGRP7 by the circadian clock may have pointed to a role in the photoperiodic pathway. Photoperiodic mutants like co or gi, however, very strongly delay flowering in LDs, in contrast to atgrp7-1 (Koornneef et al., 1991). Thus, AtGRP7 is dispensable for measuring day length. Accordingly, diurnal oscillations of the key photoperiodic regulator CO, the presence of which during the light phase initiates floral transition, are similar to WT.

The subtle effect that the loss of AtGRP7 has on the transition to flowering presumably has precluded its identification as a floral promoter in conventional screens for flowering time mutants. Alternatively, one may envisage that AtGRP8, the closest homolog of AtGRP7 that shares much of its regulatory properties, may act redundantly. In fact, by taking advantage of specific antipeptide antibodies, we uncovered an elevated AtGRP8 protein level in the atgrp7-1 mutant compared with WT. Also, the AtGRP8 transcript level is elevated in the absence of AtGRP7 protein. This is consistent with our previous observation that AtGRP7 negatively regulates AtGRP8 oscillations (Schönig et al., 2007; Staiger et al., 2003b). Thus, it remains possible that the elevated AtGRP8 level in atgrp7-1 may partly obscure the loss of AtGRP7 as a result of overlapping functions. Therefore, we aimed to generate plants with reduced AtGRP7 and AtGRP8 levels through RNA interference, as well as plants ectopically overexpressing AtGRP7.

Using hp constructs targeted against the respective RRMs, or the entire coding region, we obtained a series of transgenic plants in which AtGRP7 and AtGRP8 are downregulated to a varying degree. These RNAi lines showed a more pronounced late-flowering phenotype in LDs, and particularly in SDs, compared with atgrp7-1. On the other hand, plants constitutively overexpressing AtGRP7 flower with 10 leaves less than WT plants in SDs. In LDs, the advance is only very small, but is nevertheless significant. These data clearly indicate that AtGRP7 by itself promotes flowering, as AtGRP8 is almost completely downregulated in AtGRP7-ox plants as a result of the generation of an unproductively spliced transcript with a premature termination codon, which rapidly decays via an UPF1- and UPF3-dependent pathway (Schönig et al., 2007).

Nevertheless, it remains possible that AtGRP8 also promotes floral transition to some degree. For example, the flowering of AtGRP7-148, which displays WT levels of AtGRP8 but almost no AtGRP7, is only weakly retarded, and AtGRP7/x8i-50, with a weak reduction of AtGRP7, but strong reduction of AtGRP8, flowers very late. So plants with selective reduction of AtGRP8 or strong ectopic overexpression of AtGRP8 will be needed to unequivocally resolve this issue.

AtGRP7 is a novel autonomous pathway component

Because the lack of AtGRP7 does not affect the photoperiodic response, we investigated whether AtGRP7 may share other features with components of the autonomous pathway. Three lines of evidence indeed place AtGRP7 in the autonomous pathway.

(i) The late flowering of AtGRP7 loss-of-function plants in SDs correlates with an elevated FLC level, whereas the early
flowering of AtGRP7 gain-of-function plants correlates with a reduction in FLC.

(ii) Whereas FLC is also a target of the vernalization pathway, in AtGRP7 loss-of-function plants, prolonged cold treatment in the young seedling stage leads to a reduction of FLC, to a level similar to WT plants. Thus, vernalization downregulates FLC in the absence of AtGRP7, and, in fact, vernalization completely overrides the late-flowering phenotype of atgrp7-1 and the RNAi lines in SDs, as previously observed for mutants in the autonomous pathway (Koornneef et al., 1991; Michaels and Amasino, 2001; Sheldon et al., 2000).

(iii) Moreover, crosses between RNAi lines and flc-3 mutants lacking active FLC provide genetic evidence that AtGRP7 influences the floral transition through FLC, as the plants flower with a similar number of leaves as flc-3 mutants. However, AtGRP7 may also have FLC-independent effects, as observed for other flowering-time genes (Doyle et al., 2005). So far we have not detected consistent changes in the alternative splicing pattern and/or steady-state abundance of transcripts encoding other autonomous pathway proteins implicated in RNA processing. Consistent with this, transcript profiling of LD-grown AtGRP7-ox lines and WT plants using the ATH1 GeneChip did not reveal significant changes in FCA, FY or LD steady-state abundance, whereas the FLC levels were lowered in AtGRP7-ox plants (C. Streitner, F. Rudolf and D. Staiger, unpublished data). Thus, based on the present data, impaired expression of FCA, FY, FPA or LD does not seem to be the major cause for the influence of AtGRP7 on floral transition. To further define the position of AtGRP7 within the autonomous pathway, however, we must analyze whether a lack of AtGRP7, and/or AtGRP8, impacts on the late-flowering phenotype of the respective autonomous pathway components in double mutant combinations. For the other RNA binding proteins of the autonomous pathway, it had been suggested that they act in parallel, and ultimately control FLC levels independently (Quesada et al., 2005).

RNA binding proteins in the regulation of flowering time

Several autonomous pathway genes encode RNA binding proteins or RNA processing factors, comprising different types of RNA binding modules and associated domains implicated in protein–protein interaction (Quesada et al., 2005). Although these loci had been identified early on because of their late-flowering phenotype when mutated (Koornneef et al., 1991), it is now thought that they regulate additional processes in the plant. In a microarray analysis, several transcripts with abnormal expression pattern in fca mutants were identified in which expression was also changed in fy mutants (Marquardt et al., 2006). This points to a more general function of FCA and FY.

Other proteins involved in various aspects of RNA metabolism have also been associated recently with flowering. The ABA HYPERSENSITIVE 1 (abh1) mutant defective in the large subunit of the CAP-binding complex, CBP80, flowers early in SDs and LDs (Bezerra et al., 2004; Hugouvieux et al., 2001). The HUA2 protein involved in the processing of AGAMOUS intron 2 is required for the correct regulation of FLC and the related repressors FLM1/MAF1 or MAF2 (Cheng et al., 2003; Doyle et al., 2005). Mutants defective in the tetratricopeptide repeat protein AT PRP39-1, with similarity to a yeast pre-mRNA processing protein, are late flowering (Wang et al., 2007). Notably, all these RNA binding proteins, like the well-known autonomous pathway components and AtGRP7, affect flowering time in a large part by influencing FLC levels. Based on the domain structure of these proteins, it has been inferred that they impact on FLC levels by a post-transcriptional mechanism (Kuhn et al., 2007; Quesada et al., 2005). Recently, however, the downregulation of FLC abundance by FCA has been shown to be dependent on FLD (Liu et al., 2007). Nascent FLC transcripts accumulate to higher levels in fca and fld mutants, suggesting that FCA and FLD actually silence FLC expression at the transcriptional level through H3K4 demethylation.

AtGRP7 was originally identified on the basis of its clock regulation and cold responsiveness (Carpenter et al., 1994; Heintzen et al., 1994). The AtGRP7 feedback loop is a molecular slave oscillator within the circadian system, regulating both rhythmic and non-rhythmic target transcripts (Heintzen et al., 1997; Schöning et al., 2007). Furthermore, AtGRP7 is involved in pathogen defense (Fu et al., 2007), and has been implicated in ABA and stress signaling, as an atgrp7 mutant accumulated higher levels of the ABA- and stress-inducible RD29A transcript (Cao et al., 2006).

Presumably AtGRP7 plays a more general role in pre-mRNA processing, and the flowering phenotype we identify here reflects the dependence of floral transition on fine-tuned FLC levels. The relatively mild phenotype, particularly in LDs, may have precluded the identification of AtGRP7 in screens for flowering-time genes so far.

It has long been suggested that the circadian clock is an integral part of the photoperiodic sensory device (Bünning, 1936). Experimental proof came from the identification of mutants in Arabidopsis with disturbances in both circadian and photoperiodic timekeeping (Fowler et al., 1999; Hicks et al., 1996; Park et al., 1999; Schaffer et al., 1998; Somers et al., 1998; Staiger et al., 2003a; Suarez-Lopez et al., 2001; Wang and Tobin, 1998).

Following the observation that FLC lengthens the period of the circadian clock at 27°C, the autonomous pathway mutants fca, ld and fve have been investigated for clock phenotypes, and have been found to moderately increase the period of leaf movement rhythms (Edwards et al., 2006; Salathia et al., 2006).
Our results now establish a link between the slave oscillator component AtGRP7 operating downstream of the circadian clock and floral transition, through the autonomous pathway. This points to extensive crosstalk between the circadian system and the floral-promoting network, beyond photoperiodic timekeeping.

Experimental procedures

Plant materials

The fcl-3 mutant was kindly provided by Dr. Amasino (Michaels and Amasino, 1999). The atgrp7-1 T-DNA mutant was kindly provided by Drs. Fu and Guo (Fu et al., 2007). Seeds were obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info).

Constructs for hairpin RNA-mediated silencing of AtGRP7 and AtGRP8

Fragments of AtGRP7 and AtGRP8 cDNAs were amplified using forward primers, while simultaneously introducing XhoI and EcoRI sites. The amplification products were inserted into pTOPOII (Invitrogen, http://www.invitrogen.com), verified by sequencing and moved to pKanIIa in the antisense orientation after XhoI-EcoRI restriction, and in the sense orientation after Clal-Xbal restriction (Wesley et al., 2001). The entire cassette was cut out using NotI, blunted by Klenow fill-in and subcloned into Smal-cut pCAMBIA3300.

The following primers were used: for construct AtGRP7-l, comprising part of the 5'-UTR, the RRM and the glycine-rich part (from position –22 to position –504, relative to the ATG start codon), p7i-F (5'-CTCGAGTCTAGATCCTTTTT-3') and p7i-S23R (5’-ATCGATGAATTCAGGCC-3’); for constructs AtGRP8-l, comprising the RRM (positions 13-231 relative to the ATG), p8i-F (5’-TTTCTAGACTCGAGTACCGG-3') and p8i-218R (5’-TTGAAATTCTCGATGACAGC-3'); for constructs AtGRP8-s, comprising the RRM and the glycine-rich part (positions 13-507 relative to the ATG) p8i-F and p8i-496R (5’-TTATCGAATTCCAGGCC-3').

Transgenic plants

The RNAi constructs and the Cambia vector were introduced into A. thaliana L. Columbia by Agrobacterium-mediated vacuum infiltration (Bechthold et al., 1993). Primary transformants were selected on plates with agar-solidified half-strength MS medium (Duchefa, http://www.duchefa.com) with 0.5% sucrose, adjusted to pH 5.7, and containing 25 mg l-1 phosphinothricin.

The genomic DNA of transformants was isolated according to the protocol of the Wisconsin KO facility (Sussman et al., 2000). The presence of the hp constructs was confirmed by PCR using primers for the OCS terminator (Table S6).

Determination of flowering time

Plants were grown in a randomized fashion on soil in LDs (16-h light) or SDs (8-h light) at 20°C in Percival AR66-L3 incubators (CLF Laboratories, http://www.cff.de). The flowering time was determined by counting the rosette leaves once the bolt was 0.5-cm tall. Mean values ± SD were calculated.

For vernalization treatments, seeds were stratified at 4°C for 3 days in the dark. Germinated seeds were transferred to SDs at 20°C for 7 days, returned to 4°C in SDs for 46 days, and were subsequently transferred back to SDs at 20°C. Control plants were germinated at 4°C for 3 days and were immediately transferred to SDs at 20°C.

Statistical analysis of flowering time data

Statistical analysis was performed using STATISTICA 6.0 (http://www.statsoft.com). Mean values and standard deviation were calculated for each data set. P < 0.05 was considered significant. In experiments analyzing differences between two lines, a Student’s t-test was used when normal distribution and homogeneity of variances were proven by the Kolmogorov-Smirnov and the F-test, respectively. If normal distribution was not given, the Mann-Whitney U-test was used to analyze the significance of differences. If a normal distribution, but not the homogeneity of variances, was given, Welch’s test was used for analysis. When analysing means of more than two different samples, an ANOVA was performed. If significant differences were shown, the Dunnet test helped us to consider which samples were different from the control lines.

RNA analysis

Total RNA was isolated using the Trizol reagent. Hybridizations of RNA gel blots with 32P-labeled gene-specific antisense probes covering the 5'-UTR of AtGRP7 and AtGRP8, respectively, were performed as described by Heintzen et al. (1997).

For real-time PCR, duplicate samples were analyzed in an MJ research Opticon DNA Engine (http://www.bio-rad.com). Total RNA was treated with DNaseI and reverse-transcribed using Superscript II (Invitrogen). A 20-ng portion of retrotranscribed RNA was amplified with the Eppendorf Real MasterMix kit (Eppendorf, http://www.eppendorf.com) using an initial denaturation step of 2 min, followed by 45 cycles of 20 sec at 94°C, 30 sec at 60°C and 40 sec at 88°C. Threshold cycle (Ct) values were determined, and relative expression levels for the analyzed transcripts were calculated based on non-equal efficiencies for each primer pair (Czechowski et al., 2004; Pfaffl, 2001). Data were normalized to a transcript encoding 68S RNA gel blots against recombinant glutathione-S-transferase fusion proteins after the release of the AtGRP7 and AtGRP8 moiety by PreScission protease (GE Healthcare, http://www.gehealthcare.com) cleavage.

Generation of antibodies against AtGRP7 and AtGRP8, and protein analysis

Antibodies were raised against synthetic polypeptides spanning amino acids 22-31 of AtGRP7 and amino acids 20-29 of AtGRP8, respectively, which are divergent between the two proteins (Pineda Antibokper Service, http://pineda-abservice.com). The specificity of the antibodies for AtGRP7 and AtGRP8 was monitored by immunoblots against recombinant glutathione-S-transferase fusion proteins after the release of the AtGRP7 and AtGRP8 moiety by PreScission protease (GE Healthcare, http://www.gehealthcare.com) cleavage.
Protein extraction and immuno-blotting with chemiluminescence detection were performed as previously described (Heintzen et al., 1997).

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Supporting Information
Additional Supporting Information may be found in the online version of this article:
Figure S1. Molecular characterization of further AtGRP7i and AtGRP8i lines.
Figure S2. FLC levels in vernalized RNAi and atgrp7-1 lines.
Figure S3. Influence of altered AtGRP7i and AtGRP8i levels on autonomous pathway components.
Table S1. Crosses between AtGRP7i and AtGRP8i lines.
Table S2. Flowering time of AtGRP7i and AtGRP8i lines in short-day (SD) and long-day (LD) growth conditions.
Table S3. Effect of vernalization on the flowering time of the wild type (WT), Cambia, AtGRP7i and AtGRP8i lines and atgrp7-1.
Table S4. Flowering time in crosses between ftc-3 and AtGRP7i × AtGRP8i lines.
Table S5. Flowering time in AtGRP7-ox lines.
Table S6. Primers used for PCR.
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References
AtGRP7 and the floral transition in A. thaliana


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