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The small glycine-rich RNA binding protein *At*GRP7 promotes floral transition in *Arabidopsis thaliana*

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Summary

The RNA binding protein *At*GRP7 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 *At*GRP7, and the closely related *At*GRP8 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 *At*GRP7, 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 *At*GRP7 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 *At*GRP7 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, *FLC* is silenced through chromatin modification (Bastow *et al.*, 2004; Sung and Amasino, 2004).

Genes of the autonomous pathway promote floral transition independently of temperature and daylength, also via the suppression of *FLC*. For example, FVE is a component of a histone deacetylase complex (He *et al.*, 2003), and inhibits *FLC* expression by promoting deacetylation of *FLC* chromatin (Ausin *et al.*, 2004). FLOWERING LOCUS D (FLD) is a homolog of human lysine-specific demethylase 1 (LSD1), but has also been found to affect the histone acetylation state at the *FLC* locus (He *et al.*, 2003; Shi *et al.*, 2004). Additionally, RNA processing appears to play a crucial role in the autonomous pathway. FCA and FPA harbor multiple RNA recognition motifs (RRMs), and FLOWERING LOCUS K (FLK) has three K homology domains implicated in RNA binding (Lim *et al.*, 2004; Macknight *et al.*, 1997; Schomburg *et al.*, 2001). FY encodes a protein with similarity to the yeast polyadenylation factor Psf2p (Simpson *et al.*, 2003). FCA negatively regulates its own expression in concert with FY by promoting the cleavage and polyadenylation at a poly(A) site within its third intron (Quesada *et al.*, 2003; Simpson *et al.*, 2003). This leads to the formation of a truncated transcript, *FCA*-β, limiting the production of fully spliced transcript *FCA*-γ, which encodes the full-length functional protein (Macknight *et al.*, 2002). This auto regulation adjusts the level of active FCA, and thus ultimately determines the *FLC* mRNA accumulation.

Negative autoregulation at the post-transcriptional level is also the hallmark of the *At*GRP7 (*Arabidopsis thaliana* glycine-rich RNA binding protein 7) protein that influences the amplitude of its own circadian transcript oscillations through alternative splicing (Schöning *et al.*, 2007; Staiger *et al.*, 2003b). *At*GRP7 has a single RRM and a glycine-rich stretch. The transcript oscillates with a peak at the end of the daily light phase. Constitutive over expression of *At*GRP7 favors the use of a cryptic intronic 5′ splice site, leading to a short-lived splice form and damping of the At*GRP7* transcript oscillations. This feedback loop is under the control of the Arabidopsis core clockwork, and in turn influences other transcripts including *AtGRP8* encoding a closely related glycine-rich RRM protein (Schöning *et al.*, 2007; Staiger *et al.*, 2003b).

Here, we identify a previously unrecognised task for *At*GRP7 in the network regulating floral transition. An *atgrp7* T-DNA mutant, as well as transgenic plants with reduced *At*GRP7 expression, as a result of RNA interference, have a late-flowering phenotype, whereas transgenic plants ectopically overexpressing *At*GRP7 flower early. Nevertheless, these plants with altered *At*GRP7 levels retain a photoperiodic response. The effect on flowering is in large parts mediated by *FLC*, and the late-flowering phenotype can be overcome by vernalization, implicating *At*GRP7 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 *At*GRP7 plays a role in floral transition. First, we investigated how the loss of *At*GRP7 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 *At*GRP7, 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 *At*GRP7 in *atgrp7-1* had a relatively mild effect on flowering time, we examined in detail the expression of *AtGRP8*, which shows 77% sequence identity at 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 *At*GRP7 and *At*GRP8 proteins, anti peptide antibodies specifically

Table 1 The *atgrp7-1* mutant is late-flowering

	leaf number	*n*	*P*
SD			
Col	54.82 ± 4.65	34	0.000012
grp7-1	61.57 ± 5.92	30	
LD			
Col	12.91 ± 1.54	34	0.000011
grp7-1	15.38 ± 2.30	32	

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 $n \geq 30$ each is shown.

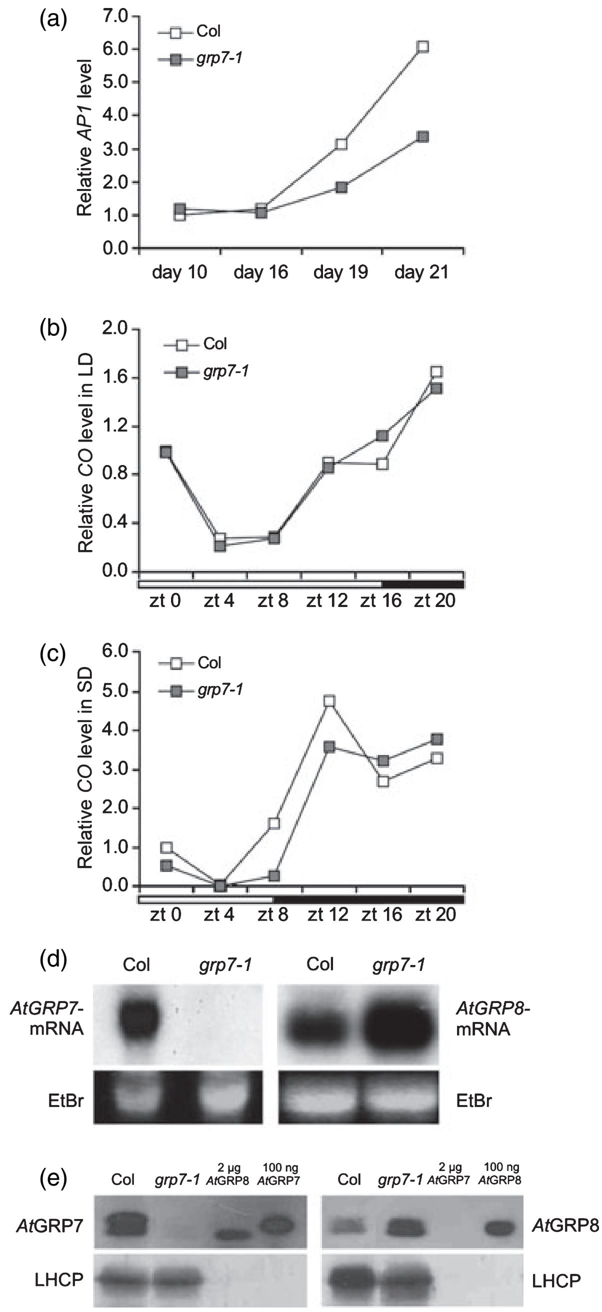

Figure 1. Characterization of the *atgrp7-1* T-DNA insertion line.
(a) Developmental changes in *AP1* levels in long-day (LD) grown Col-0 and *atgrp7-1* plants.
Plants were harvested at days 10, 16, 19 and 21 after the release from stratification. *AP1* levels were assayed with real-time PCR with respect to the *PTB* reference gene. The relative expression in wild-type (WT) plants at day 10 was set to 1.
(b, c) Diurnal *CO* transcript pattern in *atgrp7-1*.
Col-0 WT and *atgrp7-1* mutants were harvested at 4-h intervals on day 12 (LD) (b) and day 19 (short-day grown, SD) (c), respectively. *CO* levels were assayed with real-time PCR with respect to the *PTB* reference gene. The relative expression in WT at zeitgeber time 0 (zt0; lights on) was set to 1.
(d) The *atgrp7-1* mutant and WT plants were harvested at zt12. The RNA gel blot was hybridized with the gene-specific *AtGRP7* probe (left), and with the gene-specific *AtGRP8* probe (right). The ethidium-bromide stained gel confirms equal loading.
(e) The immunoblot with 40 μg of total protein was incubated with the antibodies against *At*GRP7 (left) and *At*GRP8 (right). To demonstrate the preference of the antibodies, recombinant *At*GRP7 and *At*GRP8 proteins were loaded in the quantities indicated. Incubation with an LHCP antibody served as a loading control.

directed against *At*GRP7 or *At*GRP8 were generated (see Experimental procedures). The specificity of the antibodies was tested using recombinant *At*GRP7 and *At*GRP8, respectively (Figure 1e). Weak cross-reactivity was only observed for the *At*GRP7 antibody when as much as 2 μg of pure recombinant *At*GRP8 protein was blotted. Whereas *At*GRP7 protein was absent in *atgrp7-1* plants, the *At*GRP8 protein level was elevated compared with WT (Figures 1e and S1). This indicates that the release from negative regulation in the absence of *At*GRP7 leads to higher *At*GRP8 accumulation.

Thus, it remains possible that the elevated *At*GRP8 levels may mask part of the effect of *At*GRP7 on flowering time. To substantiate the floral-promotive function of *At*GRP7 we decided to analyze flowering time in transgenic plants with reduced levels of *At*GRP7 and *At*GRP8 on the one hand, and with constitutively elevated *At*GRP7 levels on the other hand.

Molecular analysis of At*GRP7 and* At*GRP8 RNAi lines*

To obtain transgenic Arabidopsis plants with reduced *At*GRP7 or *At*GRP8 expression, we performed RNA interference using the pKannibal system encoding a Cauliflower moasic 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 *At*GRP7 or *At*GRP8, and long (l) constructs comprising additionally the part encoding the glycine-rich C terminus (Figure 2). The short construct directed against *At*GRP7 could not be stably propagated in *Agrobacterium tumefaciens*, and thus was not used further (not shown).

Transformed plants were selected on phosphinotricin, 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*i and *AtGRP8*i hp constructs would further reduce the protein levels, crosses were performed between selected *AtGRP7*i and *AtGRP8*i lines (Table S1).

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

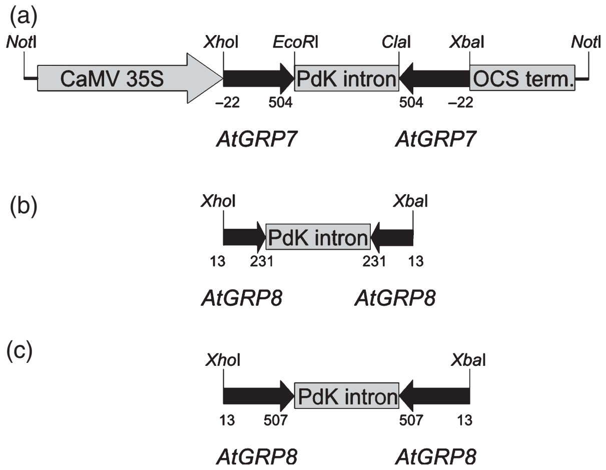

Figure 2. Map of the *AtGRP7* and *AtGRP8* RNAi constructs. Numbers indicate nucleotide positions relative to the ATG start codon.
(a) Construct *AtGRP7i-l* comprises 523 bps, including 22 nucleotides of the 5′ untranslated region (UTR), the RRM and the glycine stretch.
(b) Construct *AtGRP8i-s* comprises 218 bps spanning the RNA recognition motif (RRM).
(c) Construct *AtGRP8*i*-l* comprises 496 bps spanning the RRM and part of the glycine stretch.

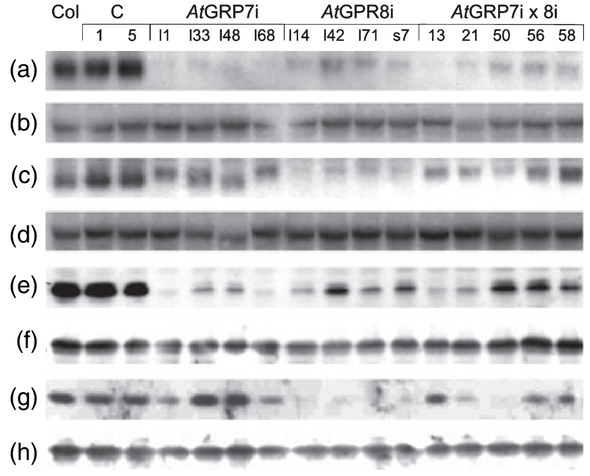

Figure 3. Molecular characterization of the *AtGRP7i* and *AtGRP8i* lines.
Plants harbouring the Cambia (C) vector, the constructs *AtGRP7i-l*, *AtGRP8i-l* or *AtGRP8i-s*, and the offspring of crosses of selected lines (*AtGRP7i×8i*) were grown on phosphinotricin-containing plates along with Col-0 wild-type (WT) plants, and were harvested at zt12.
The RNA gel blot was hybridized with the gene-specific *AtGRP7* probe (a), gene-specific *AtGRP8* probe (c) and *ACTIN* to confirm equal loading (b, d). Immunoblots of the corresponding total protein extract were incubated with antipeptide antibodies against *At*GRP7 (e), *At*GRP8 (g) and an antibody directed against LHCP as the loading control (f, h).

and S1). In *AtGRP7*i-l lines the endogenous *AtGRP7* transcript was strongly reduced compared with WT plants and Cambia (C) plants harboring the empty vector (Figure 3a). The *AtGRP8* transcript was also weakly affected (Figure 3c). Similarly in *AtGRP8*i-l and *AtGRP8*i-s lines, the endogenous *AtGRP8* transcript was almost undetectable (Figure 3c), and the *AtGRP7* transcript was reduced (Figure 3a). In all *AtGRP7*i-l lines, the *At*GRP7 protein level was strongly reduced or almost undetectable compared with WT and Cambia lines (Figure 3e), and in the *AtGRP8*i lines, the *At*GRP8 protein was almost undetectable (Figures 3g and S1). Again, in the *AtGRP7*i-l lines, the *At*GRP8 protein was only weakly affected, or remained at WT levels, whereas in the *AtGRP8*i lines the *At*GRP7 protein level was clearly lower than in WT or Cambia lines (Figure 3g). Taken together, these data suggest that the *AtGRP7*i or *AtGRP8*i constructs are effective against endogenous *AtGRP7* or *AtGRP8*, respectively, but also show some cross-regulation. The fact that in the *AtGRP8*i plants the *At*GRP7 protein is also strongly affected, whereas in the *AtGRP7*i plants the *At*GRP8 protein remains more or less at WT levels may indicate that the *AtGRP8*i constructs are more effective against *AtGRP7* than vice versa. Alternatively, the effect of the *AtGRP7*i construct on *AtGRP8* may be partially masked by enhanced *AtGRP8* accumulation as a consequence of *At*GRP7 down-regulation. In the crosses containing both hp constructs simultaneously, a further reduction of the *At*GRP7 and *At*GRP8 levels was only observed in line *AtGRP7i×8i-21*, compared with the parental lines (Figure 3e,g).

AtGRP7*i and* AtGRP8*i plants are late flowering*

Lines of all three genotypes *AtGRP7*i-l, *AtGRP8*i-l and *AtGRP8*i-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

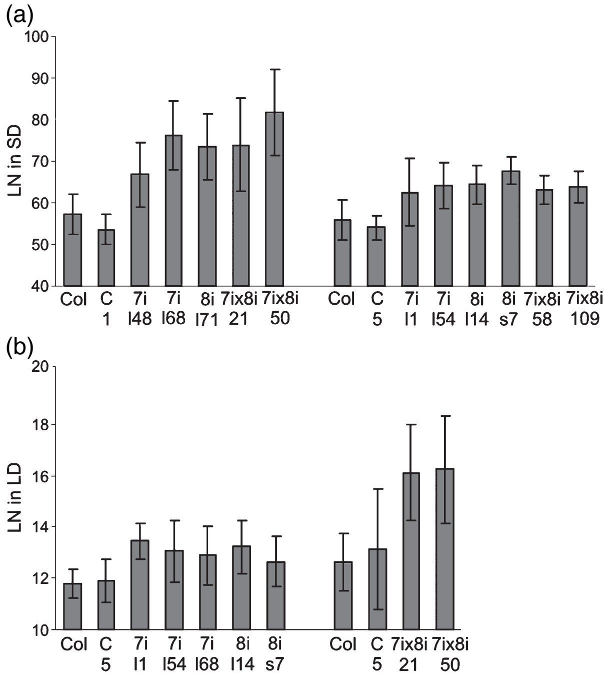

Figure 4. Flowering time of *AtGRP7*i and *AtGRP8*i lines grown in short-day (SD) and long-day (LD) conditions.
(a) Wild type (WT), Cambia-1 (C-1), *AtGRP7*i-l48 and *AtGRP7*i-l68, *AtGRP8*i-l71, *AtGRP7*ix8i-21 and *AtGRP7*ix8i-50 (left), and WT, C-5, *AtGRP7*i-l1 and *AtGRP7*i-l54, *AtGRP8*i-l14, *AtGRP8*i-s7, *AtGRP7*ix8i-58 and *AtGRP7*ix8i-109 (right), respectively, were grown in SDs.
(b) WT, C-5, *AtGRP7i*-l1, *AtGRP7*i-l54 and *AtGRP7*i-l68, *AtGRP8*i-l14 and *AtGRP8*i-s7 (left) and WT, C-5, *AtGRP7*i×8i-21 and *AtGRP7*i×8i-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).

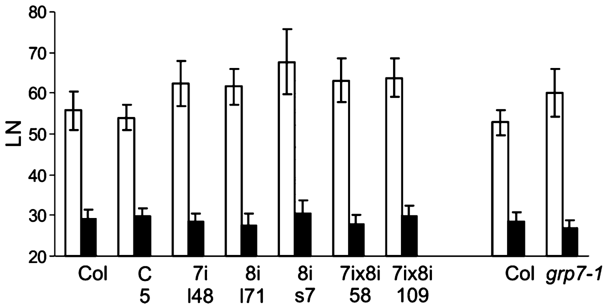

Figure 5. Effect of vernalization on flowering time of wild-type (WT), Cambia-5 (C-5), *AtGRP7i* and *AtGRP8i* lines and *atgrp7-1*.
Flowering time was measured as the number of rosette leaves (LN) produced at bolting in non-vernalized plants (white bar) and vernalized plants (black bar). Values represent the means ± SD (see Table S3).

number compared with WT. Thus, vernalization completely overcomes the late-flowering phenotype of the RNAi lines and *atgrp7-1* mutant.

Influence of At*GRP7 on* FLC

The autonomous pathway acts in parallel with vernalization to constitutively repress *FLC* (Michaels and Amasino, 2001). Because vernalization bypasses the requirement of *At*GRP7 in the RNAi lines and the *atgrp7-1* mutant, we investigated whether the floral promotive effect of *At*GRP7 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 *At*GRP7 levels or complete loss of *At*GRP7 remain fully responsive to vernalization, indicating that *FLC* down-regulation in response to cold treatment does not require *At*GRP7.

If the late flowering effect seen in plants with reduced *At*GRP7 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 *AtGRP7*ix8i-21 and *AtGRP7*ix8i-50 with *flc-3* mutants lacking active FLC because of a 107-bp deletion around the ATG (Michaels and Amasino, 1999). In the F_2 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 F_3 generation. Again, *AtGRP7*ix8i-21 and *AtGRP7*ix8i-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 *AtGRP7*ix8i *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 *At*GRP7 does not manifest itself in the absence of FLC.

Constitutive overexpression of At*GRP7 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 *At*GRP7. Therefore, an effect of ectopic *At*GRP7 overexpression on flowering time was investigated in a complementary approach. The *At*GRP7-ox lines D and G express highly elevated levels of *At*GRP7 protein, resulting in strongly reduced *At*GRP8 protein levels (Figure 7a). In SDs, these plants formed about 10 leaves less than WT at bolting (Figure 7b). This clearly shows that *At*GRP7 by itself is able to promote flowering. In the *At*-

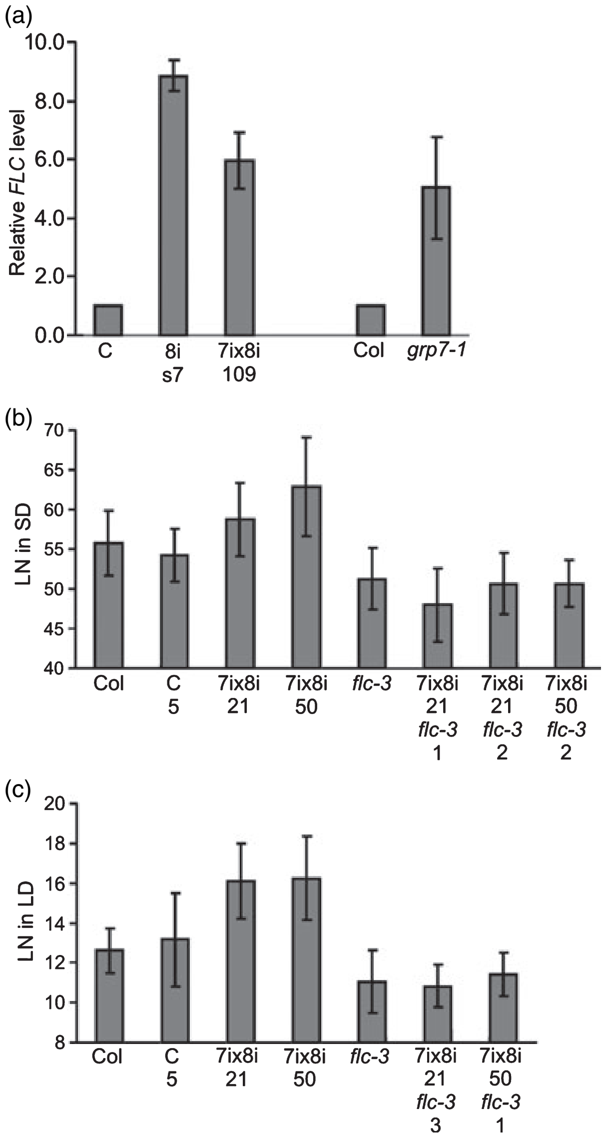

Figure 6. The effect of *At*GRP7 on flowering time is largely mediated by *FLC*.
(a) *FLC* expression in 6-week-old short-day (SD) grown Cambia-5 (C-5), *AtGRP8*i-s7 and *AtGRP7*ix-109 lines (left), and wild type (WT) and *atgrp7-1* (right).
Five plants were pooled for RNA isolation and *FLC* levels were determined by real-time PCR in duplicates for two biological replicates. Expression levels were normalized to *PPR*, and the relative *FLC* level in WT was set to 1.
(b, c) The *flc-3* null allele eliminates the late-flowering phenotype of *AtGRP7i×8i*.
WT, C-5 and the parental lines *AtGRP7i×8i-21*, *AtGRP7i×8i-50* and *flc-3* were grown in parallel with *AtGRP7i×AtGRP8i-21 flc-3* and *AtGRP7i×8i-50 flc-3* in SDs (b) or LDs (c). The numbers 1, 2 and 3 represent F_3 plants of independent crosses. The leaf number (LN) at bolting ± SD is shown. This experiment was performed twice with similar results.

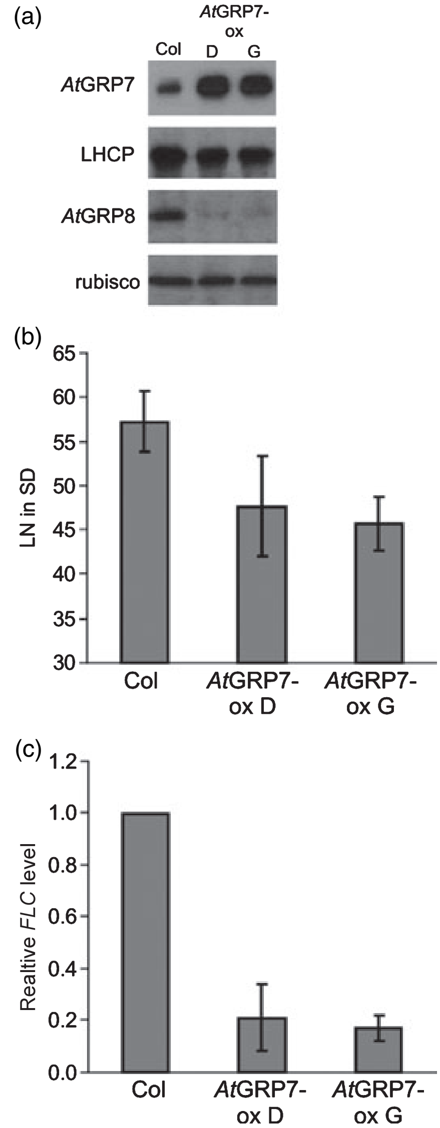

Figure 7. Influence of *At*GRP7 overexpression on flowering time.
(a) *At*GRP7 and *At*GRP8 protein levels were analyzed in short-day (SD) grown *At*GRP7-ox and wild-type (WT) plants using specific antibodies. The reaction with an LHCP antibody or the unspecific reaction of the *At*GRP8 antibody with the small subunit of Rubisco served as the loading control.
(b) *At*GRP7 overexpression causes early flowering in SDs. Independent *At*GRP7-ox lines were grown along with WT plants.
The leaf number (LN) at the onset of bolting is shown ± SD.
(c) *FLC* levels are lower in *At*GRP7-ox plants. *FLC* levels were determined by real-time PCR, as described in the legend to Fig. 6a, and were normalized to *PTB*. The level in WT was set to 1.

GRP7-ox lines, *FLC* was downregulated to 10–20% of the level in WT (Figure 7c). After vernalization, *At*GRP7-ox and WT plants formed about the same number of leaves at bolting (Table S5). In LDs, the *At*GRP7-ox lines flowered only slightly earlier than WT plants (Table S5), indicating that the promotive effect of *At*GRP7 has a stronger impact under non-inductive photoperiods.

To address the question of whether *At*GRP7 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 *At*GRP7 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 *At*GRP7 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 *At*GRP7-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 LUMINIDEPENDENS (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 *At*GRP7-ox lines (Figure S3e).

Discussion

*The RNA-binding protein At*GRP7 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 *At*GRP7 delays the transition to flowering, whereas the gain of function through ectopic overexpression of *At*GRP7 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 *At*GRP7 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, *At*GRP7 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 *At*GRP7 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 *At*GRP8, the closest homolog of *At*GRP7 that shares much of its regulatory properties, may act redundantly. In fact, by taking advantage of specific antipeptide antibodies, we uncovered an elevated *At*GRP8 protein level in the *atgrp7-1* mutant compared with WT. Also, the *AtGRP8* transcript level is elevated in the absence of *At*GRP7 protein. This is consistent with our previous observation that *At*GRP7 negatively regulates *AtGRP8* oscillations (Schöning *et al.*, 2007; Staiger *et al.*, 2003b). Thus, it remains possible that the elevated *At*GRP8 level in *atgrp7-1* may partly obscure the loss of *At*GRP7 as a result of overlapping functions. Therefore, we aimed to generate plants with reduced *At*GRP7 and *At*GRP8 levels through RNA interference, as well as plants ectopically overexpressing *At*GRP7.

Using hp constructs targeted against the respective RRMs, or the entire coding region, we obtained a series of transgenic plants in which *At*GRP7 and *At*GRP8 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 *At*GRP7 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 *At*GRP7 by itself promotes flowering, as *At*GRP8 is almost completely downregulated in *At*GRP7-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 (Schöning *et al.*, 2007).

Nevertheless, it remains possible that *At*GRP8 also promotes floral transition to some degree. For example, the flowering of *AtGRP7*i-I48, which displays WT levels of *At*GRP8 but almost no *At*GRP7, is only weakly retarded, and *AtGRP7*ix8i-50, with a weak reduction of *At*GRP7, but strong reduction of *At*GRP8, flowers very late. So plants with selective reduction of *At*GRP8 or strong ectopic overexpression of *At*GRP8 will be needed to unequivocally resolve this issue.

*At*GRP7 is a novel autonomous pathway component

Because the lack of *At*GRP7 does not affect the photoperiodic response, we investigated whether *At*GRP7 may share other features with components of the autonomous pathway. Three lines of evidence indeed place *At*GRP7 in the autonomous pathway.

(i) The late flowering of *At*GRP7 loss-of-function plants in SDs correlates with an elevated *FLC* level, whereas the early flowering of *At*GRP7 gain-of-function plants correlates with a reduction in *FLC*.

(ii) Whereas *FLC* is also a target of the vernalization pathway, in *At*GRP7 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 *At*GRP7, 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 *At*GRP7 influences the floral transition through *FLC*, as the plants flower with a similar number of leaves as *flc-3* mutants. However, *At*GRP7 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 *At*GRP7-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 *At*GRP7-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 *At*GRP7 on floral transition. To further define the position of *At*GRP7 within the autonomous pathway, however, we must analyze whether a lack of *At*GRP7, and/or *At*GRP8, 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 *FLM*1/*MAF*1 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 *At*GRP7, 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.

*At*GRP7 was originally identified on the basis of its clock regulation and cold responsiveness (Carpenter *et al.*, 1994; Heintzen *et al.*, 1994). The *At*GRP7 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, *At*GRP7 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 *At*GRP7 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 *At*GRP7 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 *At*GRP7 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 *flc-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 *At*GRP7 and *At*GRP8

Fragments of *AtGRP7* and *AtGRP8* cDNAs were amplified using forward primers, while simultaneously introducing *Xho*I and *Xba*I sites, and reverse primers, introducing *Cla*I and *Eco*RI sites. The amplification products were inserted into pTOPOII (Invitrogen, http://www.invitrogen.com), verified by sequencing and moved to pKannibal in the antisense orientation after *Xho*I-*Eco*RI restriction, and in the sense orientation after *Cla*I-*Xba*I restriction (Wesley *et al.*, 2001). The entire casette was cut out using *Not*I, blunted by Klenow fill-in and subcloned into *Sma*I-cut pCAMBIA3300.

The following primers were used: for construct *AtGRP7*i-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′-CTCGAGTCTAGATCTTCTTTT-3′) and p7i-523R (5′-ATCGATGAATTCCGTAACCTCC-3′); for constructs *AtGRP8*i-s, comprising the RRM (positions 13–231 relative to the ATG), p8i-F (5′-TTTCTAGACTCGAGTACCGG-3′) and p8i-218R (5′-TTGAATTCATCGATGACACG-3′); for constructs *AtGRP8*i-l, comprising the RRM and the glycine-rich part (positions 13–507 relative to the ATG) p8i-F and p8i-496R (5′-TTATCGATGAATTCCAGCCGCC-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} phosphinotricin.

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.clf.de). The flowering time was determind 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 *t*-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 ^{32}P-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 68°C. Threshold cycle (C_T) 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 the translation initiation factor eIF-4A-1 (At3g13920), PTB (At3g01150) and PPR (At5g55840) (Czechowski *et al.*, 2005). The absence of amplification products from genomic DNA was confirmed in a non-retrotranscribed control.

For semiquantitative RT-PCR, retrotranscribed RNA was amplified with Taq Polymerase. To determine the linear range of amplification for each primer pair, samples were withdrawn after 24, 26, 28, 30, 32 and 34 cycles. PCR products were separated on agarose gels, or polyacrylamide gel in the case of *FY*, and were visualized by ethidium-bromide staining.

Generation of antibodies against *At*GRP7 and *At*GRP8, and protein analysis

Antibodies were raised against synthetic polypeptides spanning amino acids 22–31 of *At*GRP7 and amino acids 20–29 of *At*GRP8, respectively, which are divergent between the two proteins (Pineda Antikörper Service, http://pineda-abservice.com). The specificity of the antibodies for *At*GRP7 and *At*GRP8 was monitored by immunoblots against recombinant glutathione-*S*-transferase fusion proteins after the release of the *At*GRP7 and *At*GRP8 moiety by PreScission protease (GE Healthcare, http://www.gehealthcare.com) cleavage.

Protein extraction and immunoblots 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 *At*GRP7 and *At*GRP8 levels on autonomous pathway components.

Table S1. Crosses between *AtGRP7*i and *AtGRP8*i lines.

Table S2. Flowering time of *AtGRP7*i and *AtGRP8*i 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, *AtGRP7*i and *AtGRP8*i lines and *atgrp7*-1.

Table S4. Flowering time in crosses between *flc-3* and *AtGRP7*ix8i lines.

Table S5. Flowering time in *At*GRP7-ox lines.

Table S6. Primers used for PCR.

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