the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **83,** 952–961 doi: 10.1111/tpj.12938

The dynamics of *FLOWERING LOCUS T* expression encodes long-day information

Martín Krzymuski[1], Fernando Andrés[2], Juan I. Cagnola[1], Seonghoe Jang[2], Marcelo J. Yanovsky[3], George Coupland[2] and Jorge J. Casal[1,3,*]

[1]*IFEVA, Faculty of Agronomy, University of Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas, Av. San Martín 4453, 1417 Buenos Aires, Argentina,*

[2]*Max Planck Institute for Plant Breeding Research, Carl von Linné Weg 10, D–50829 Cologne Germany, and*

[3]*Fundación Instituto Leloir, IIBBA-CONICET, C1405BWE Buenos Aires, Argentina*

Received 26 October 2014; revised 5 July 2015; accepted 8 July 2015; published online 25 July 2015.
*For correspondence (e-mail casal@ifeva.edu.ar).

SUMMARY

Long days repeatedly enhance the expression of the *FLOWERING LOCUS T* (*FT*) gene during the evening and early night. This signal induces flowering despite low *FT* expression the rest of the day. To investigate whether this temporal behaviour transmits information, plants of *Arabidopsis thaliana* were exposed to different day–night cycles, including combinations that induced *FT* expression out of normal hours. Flowering time best correlated with the integral of *FT* expression over several days, corrected for a higher evening and early night sensitivity to *FT*. We generated a system to induce *FT* expression in a leaf removed 8–12 h later. The expression of flowering genes in the apex and flowering required cycles of induction repeated over several days. Evening and early night *FT* induction was the most effective. The temporal pattern of *FT* expression encodes information that discriminates long days from other inputs.

Keywords: *FLOWERING LOCUS T*, signalling dynamics, flowering, photoperiod, *Arabidopsis thaliana*.

INTRODUCTION

In some cases cells use the dynamics of signalling molecules, rather than the state of these molecules at just a single point in time, to transmit information. This implies that meaningful information is encoded in the frequency, amplitude, duration and other features of the temporal signal (Purvis and Lahav, 2013). In mammals and budding yeast, for instance, transcription factors often exhibit complex dynamics that provide input-specific information (Hansen and O'Shea, 2013). As sessile organisms, plants show strong developmental plasticity to cope with the changing environment (Casal *et al.*, 2004). Signalling dynamics is therefore likely to be crucial to discriminate among diverse environmental stimuli.

Arabidopsis thaliana flowers earlier under long days (LDs) than under short days (SDs). LDs enhance the expression of the *FLOWERING LOCUS T* (*FT*) gene, which shares homology with RAF kinase inhibitor protein genes and strongly promotes flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). The transcription of *FT* is activated by CONSTANS (CO), a nuclear protein containing B-box zinc fingers and a CCT domain (Samach *et al.*, 2000; Suarez-Lopez *et al.*, 2001; Wenkel *et al.*, 2006). CO forms complexes that directly bind to the promoter of the *FT* gene (Song *et al.*, 2012).

The expression of *CO* is controlled by the circadian clock, and under SDs the phase of high *CO* expression occurs mainly in darkness (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002). Under SDs this pattern is reinforced by the repression of *CO* expression, mediated by CYCLING DOF FACTORs (CDFs) during the morning (Fornara *et al.*, 2009) and DAY NEUTRAL FLOWERING (DNF) mainly during the last part of the photoperiod (Morris *et al.*, 2010). Furthermore, under SDs, CO protein is tagged for proteasome degradation by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) during the morning (Lazaro *et al.*, 2012) and by the CONSTITTUTIVE PHOTOMORPHOGENIC 1 (COP1)–SUPRESSOR OF PHYA-105 1 (SPA1)–SPA3–SPA4 complex at night (Liu *et al.*, 2008). Therefore, under SDs, CO is unable to stimulate *FT* expression.

Long days (LDs) enhance the expression of *CO*, and the stability of CO, and hence promote the expression of *FT*.

The afternoon expression of *CO* is enhanced by light activation of the FLAVIN-BINDING, KELCH REPEAT, F-BOX (FKF1) photoreceptor, which forms a complex with GIGANTEA that reduces the stability of the CDF repressors (Sawa *et al.*, 2007). The LD signal enhances CO stability during the afternoon via the cryptochrome 2 and phytochrome A photoreceptors (Valverde *et al.*, 2004), which are predicted to reduce COP1 activity (Song *et al.*, 2013), and via the action of the LOV subdomain of FKF1 (Song *et al.*, 2012).

CO and *FT* are expressed specifically in the vascular bundles of phloem in the leaves (Takada *et al.*, 2003; An *et al.*, 2004; Endo *et al.*, 2007). Therefore, the LD signal that stimulates flowering is perceived in the leaves (Zeevaart, 2006). Then, the FT protein migrates from the leaves to the apex to induce flowering (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007). FLOWERING LOCUS T INTERACTING PROTEIN 1 (FTIP1) is essential for FT movement from companion cells to sieve tube elements (Liu *et al.*, 2012).

In the apex, FT forms a complex with the bZIP transcription factor FD (already expressed at the shoot apex before floral induction), and this complex enhances the expression of FT-dependent floral identity genes following a specific chronology and hierarchy of expression (Abe *et al.*, 2005; Wigge *et al.*, 2005; Torti *et al.*, 2012). These genes include *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*; Onouchi *et al.*, 2000), *APETALA 1* (*AP1*; Abe *et al.*, 2005; Wigge *et al.*, 2005), *FRUTIFUL* (*FUL*; Andrés and Coupland, 2012) and *LEAFY* (*LFY*; Lee *et al.*, 2008).

Our current knowledge of the mechanisms allows for reasonable predictions of the patterns of expression of *FT* and the timing of flowering in responses to photoperiod (Salazar *et al.*, 2009; Song *et al.*, 2012); however, there are still important gaps in our understanding of the quantitative nature of the photoperiodic responses. For instance, in *A. thaliana* plants grown under SDs, several successive LDs may be necessary to trigger the induction of full flowering (Mozley and Thomas, 1995), despite the fact that a single LD is enough to promote *FT* expression (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002). Repeated inductive cycles might be necessary to: reach higher levels of *FT* expression; enhance the sensitivity to *FT*; and/or maintain high levels of *FT* expression over several days. Both LD and shade enhance the expression of *FT* in the vascular bundles (Endo *et al.*, 2005, 2007), but only shade generates a morning peak (Kim *et al.*, 2008; Wollenberg *et al.*, 2008). Selective regulation of flowering in response to either shade or LDs would require the ability to discriminate between *FT* expression peaks occurring at different times. The aim of the present work is to investigate the features of the temporal signature of *FT* expression that quantitatively define flowering time under LDs.

RESULTS

One LD is enough to induce maximum transcriptional responses of flowering genes, but not to induce maximum flowering

Plants of Arabidopsis were grown under SDs (with 8 h of white light/16 h of darkness) for 24 days (approximately 10 leaves per plant), transferred to LDs (16 h of white light/8 h of darkness) for between 0 days (SD control) and 7 days, and then returned to SDs, and the number of rosette leaves was counted at flowering as a measure of flowering time on a biological scale. A single LD was not enough to induce the maximum acceleration of flowering, which approached saturation with four LD cycles (Figure 1a). Repeated inductive LD cycles could accelerate flowering by enhancing flowering gene expression responses to LDs in the leaves. To test this hypothesis we harvested leaves of plants grown under SDs for 24 days, at the end of the first, second, fourth and sixth LD (i.e. 16 h after the beginning of the photoperiod). SD controls were harvested simultaneously with the samples corresponding to LD1 and LD6 (i.e. after 8 h of light and 8 h of darkness on the relevant day). The samples were used for the analysis of expression with ATH1 Affymetrix microarrays. We focused the analysis on a set of genes related to flowering (according to the information available at http://www.arabidopsis.org; Table S1). Several of these genes are involved in the control of *FT* expression (Song *et al.*, 2013). For each gene and harvest time we calculated the ratio between the expression under LDs and the expression under SDs (average expression of both harvest times for SD controls). For each harvest time we plotted the LD/SD expression ratio of each gene against the LD/SD expression ratio of seedlings grown since sowing under SDs or LDs [publicly available data corresponding to expression values after 16 h of white light (LD) or 8 h of white light and 8 h of darkness (SD); Michael *et al.*, 2008;]. Despite their different impact on flowering, the different number of LD cycles induced largely similar gene expression responses (Figure 1b). In particular, *FT* expression, which represents the major leaf transcriptional output leading to flowering, was promoted by the first LD, and additional LD cycles did not enhance this promotion (Figure 1c).

Quantitative relationship between flowering time and *FT* expression patterns

Although *FT* is the major point of integration of the photoperiodic signal in the leaves, and flowering responds quantitatively to the photoperiodic input (e.g. Figure 1a), the heterozygous *ft FT* mutant shows wild-type (WT) flowering time under LDs (Segarra *et al.*, 2010). The latter observation indicates either that *FT* is not a quantitative determinant of photoperiodic flowering or that the dosage of *FT* is not limiting when the LD signal is saturating. To elucidate these possibilities, in order to learn whether the

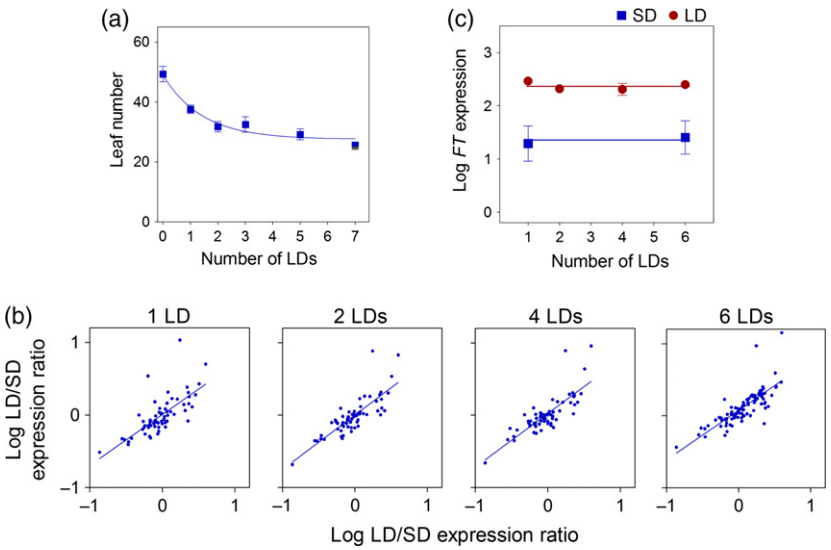

Figure 1. One long-day (LD) cycle is enough to elicit maximum flowering gene expression responses in the leaves, but not maximum flowering time responses.
(a) One LD is not enough to saturate the flowering response: time to flowering (on a biological scale, i.e. the number of rosette leaves at bolting) as affected by the number of inductive LDs. Wild-type (WT; Columbia) plants were grown under short days (SDs) for 24 days (approximately 10 leaves per plant), then exposed to the indicated number of LDs and finally returned to SDs. Data are means and SEs of 10 plants, fitted with a one-phase exponential decay curve (R^2 = 0.87, $P < 0.001$).
(b) One LD is enough to elicit maximum flowering gene expression responses in the leaves: rosette leaves were harvested at the end of LD1, LD2, LD4 and LD 6, and under SD controls simultaneously with LD1 and LD6, and then used for the analysis of gene expression. The log LD/SD expression ratio of flowering genes in the latter experiments is plotted against the log LD/SD expression ratio of the same genes in plants grown under either SDs or LDs since sowing (Michael *et al.*, 2008). Note that the response (linear regression) does not change significantly with the number of LDs. Data are means of two biological replicates.
(c) One LD is enough to elicit the maximum promotion of *FT* expression. Data correspond to the experiment described in (b).

quantitative analysis of the relationship between flowering and *FT* is justified, we tested the heterozygous *ft FT* mutant under a different number of inductive cycles, because the heterozygous *ft FT* mutant shows reduced *FT* expression compared with the WT under LDs (Figure 2, inset). We found that the *ft FT* mutant flowers later than the WT when the number of LDs is limiting, and flowers simultaneously with the WT when the number of LDs is saturating (Figure 2). This observation provides genetic evidence for a quantitative relationship between *FT* and flowering time, opening the way for a detailed molecular analysis.

To investigate the quantitative relationship between the *FT* signal and flowering time, plants grown under 17 SDs were transferred to a series of photoperiodic treatments to measure the levels of *FT* expression every 4 h over 4 days, and their flowering times when they were returned to SDs. The conditions included plants transferred from SDs to LDs (16 h light) for 0 (SD control), 1, 2 or 3 days, plants exposed to three 12-h photoperiods (predicted to result in an intermediate degree of induction), plants exposed to a sequence of 1 LD, 1 SD and 1 LD, and plants exposed to 2 days of continuous light (i.e. photoperiods of 24 h). The idea behind these natural and artificial photoperiodic treatments was to investigate the patterns of temporal integration of the *FT* expression signal. For instance, to investigate whether flowering time responds to the maximum values of *FT* or to the integral of *FT* expression.

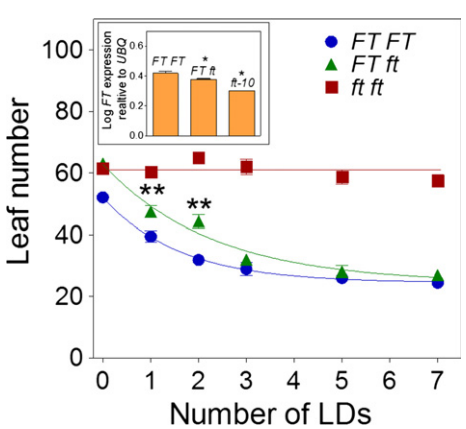

Figure 2. Genetic evidence for a quantitative relationship between *FT* and flowering. Leaf number plotted against the number of successive long days (LDs). Plants of wild type (WT; *FT FT*), the homozygous *ft ft* mutant and the heterozygous *FT ft* mutant were grown under short days (SDs) for 17 days (with approximately eight leaves per plant), exposed to the indicated number of LDs and then returned to SDs. The *ft ft* mutant failed to respond to LDs (linear regression, $P > 0.05$). The other genotypes showed one-phase exponential decay of the number of leaves as a function of the number of inductive cycles (R^2 = 0.79 for *FT FT*, 0.87 for *FT ft*). The inset shows the levels of *FT* expression at the end of the first LD. $^{**}P < 0.01$ between *FT FT* and *FT ft* in Student's *t*-tests. Data are means and SEs of 15 plants or three biological replicates (inset).

The maximum peak of *FT* expression was similar in plants exposed to either 2 LDs (Figure 3e) or 3 LDs (Figure 3c), but plants exposed to 2 LDs flowered significantly

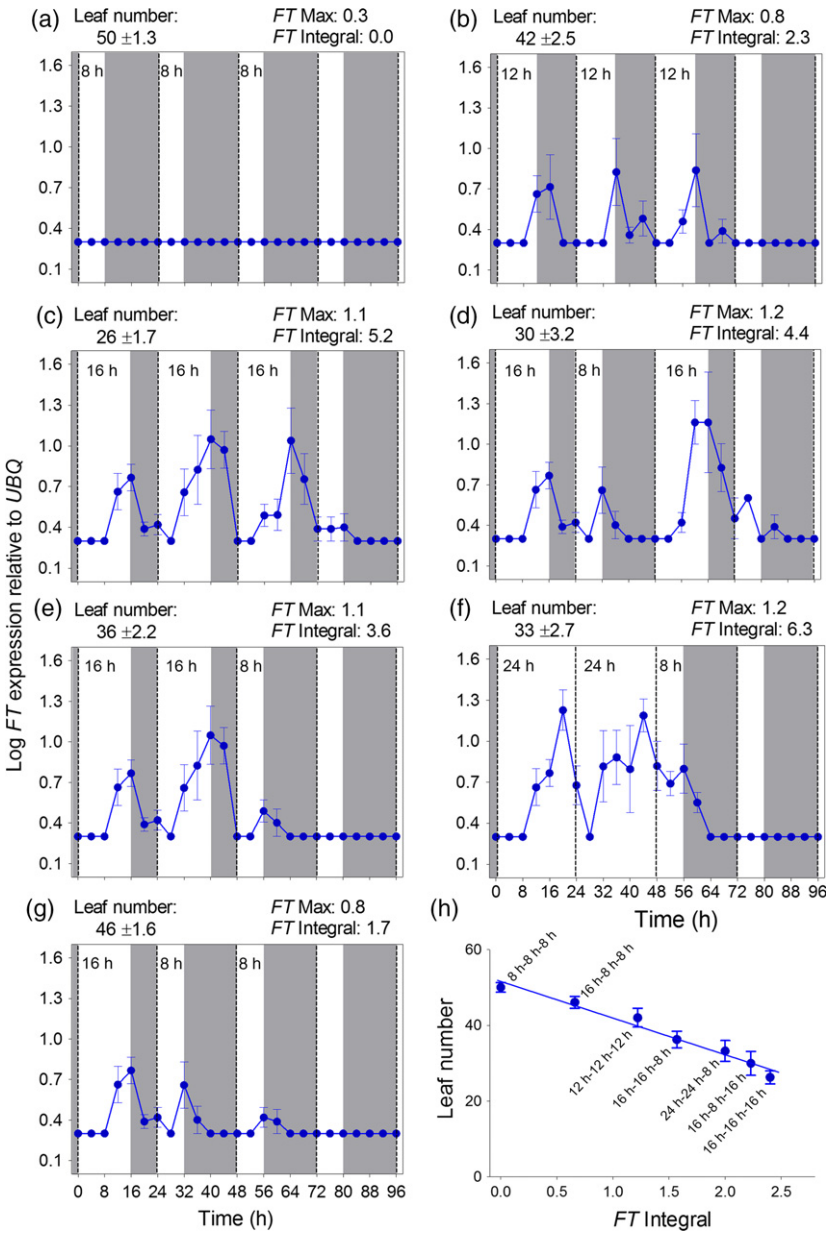

Figure 3. Quantitative relationship between *FT* expression patterns and flowering time.

(a–g) Diurnal patterns of *FT* expression under the indicated light treatments (the light and dark periods of each cycle are indicated by the white or grey background of that period, respectively). Wild-type (WT; Columbia) plants were grown under short days (SDs) for 17 days (with approximately eight leaves per plant), and during the subsequent 3 days were exposed to SDs (a), photoperiods of 12 h (b), long days (LDs) (c), 1 LD followed by 1 SD and 1 additional LD (d), 2 LDs followed by 1 SD (e); 2 days of continuous light followed by 1 SD (f), or 1 LD followed by 2 SDs (g). All plant groups were then returned to SDs. Leaves were harvested every 4 h during the 3 days of treatment and the subsequent first day back under SDs to determine *FT* expression. Data are means and SEs of four biological replicates, with four pooled plants per replicate, presented as log (*FT*/*UBQ* expression ratio +2). For clarity, data corresponding to conditions shared by more than one experimental protocol are presented for each relevant protocol, but they do not come from different samples. The number of rosette leaves at bolting (means and SEs for 15 plants), the maximum *FT* expression value (*FT* Max) and the *FT* integral are indicated in each case. (h) Linear relationship between rosette leaf number at bolting and the integral of *FT* expression above a threshold excluding *FT* values between ZT20 and ZT04 ($P < 0.05$; $R^2 = 0.97$) (ZT, zeitgeber time or time from dawn). See Figure S1 for the analysis of alternative fits.

later (Figure 3c,e). This observation indicates that it is not the maximum attained level of *FT* that alone determines the time to flower (see also Figure S1a). The simplest alternative hypothesis is to consider that flowering time directly depends on the *FT* integral (area below the *FT* expression curve); however, when flowering time is plotted against the *FT* integral, two conditions deviate significantly from the line that connects most of the cases (Figure S1b). On the one hand, 1 LD did not accelerate flowering to the extent expected on the basis of their *FT* expression integral, suggesting that the *FT* signal is integrated above a threshold of expression. In fact, integrating *FT* expression above a threshold level is enough to include the SD condition within the same line that adjusts most of the other conditions (Figure S1c). On the other hand, 2 days of continuous light also failed to accelerate flowering to the extent expected on the basis of their *FT* expression integral (Figure S1b), and the use of a threshold did not correct the deviation (Figure S1c). The latter indicates that the effectiveness of the *FT* integral is overestimated under these conditions, i.e. that *FT* would not be equally important at any time.

The comparison of the *FT* expression patterns in plants exposed to 2 days of continuous light (Figure 3f) and those exposed to either LD–SD–LD (Figure 3d) or 3 LDs (Figure 3c), i.e. two conditions where the *FT* integral appears to be more effective to accelerate flowering (Figure S1), reveals two major differences that could point to the

temporal windows of differential sensitivity to *FT*. One of the differences is that under LD–SD–LD or 3 LDs the levels of *FT* at the end of the third day are higher than under 2 days of continuous light, and the other is that the plants exposed to 2 days of continuous light have higher expression at the end of the subjective night and beginning of the next photoperiod. Therefore, *FT* levels could be more important during the third day than in previous days (i.e. increased sensitivity to *FT* with increased number of LD cycles) and/or *FT* expression could be less effective during the end of the night and beginning of the day (indicating the occurrence of a diurnal rhythm of sensitivity towards *FT*). Several models were tested in which the *FT* expression values during days 1 and 2 were multiplied by coefficients lower than 1 in order to increase the impact of third-day *FT* values in the equation; however, the best two adjustments still show severe deviation from the linear regression line (Figure S1d,e). Hence, the levels of expression of *FT* during the third day appear important but not more important than that on previous days. Conversely, the exclusion of end-of-night and dawn (ZT, zeitgeber time or time from dawn, ZT20–ZT04) *FT* expression values from the integral resulted in a linear adjustment for all treatments (Figure 3h). Therefore, we propose a model where plants integrate the *FT* signal above a threshold over several days, but excluding the hours corresponding to late night and early morning. Subsequent experiments were designed to test this model.

Accelerated flowering requires high *FT* expression over several days

One of the hypotheses that derive from the proposed model is that plants integrate the *FT* signal over several days, and it is the magnitude of this integral and not just the maximum values of *FT* expression that determines the time to flowering. To test this idea we generated an inducible system, where the expression of *FT* was promoted in plants by the application of 17-β-estradiol to the leaves. Transgenic plants bearing the inducible *FT* gene were grown under SDs during the whole experiment. When they had 25 leaves, *FT* expression was induced from 0 (control) to 8 days. For the induction, a single leaf per plant was painted with estradiol every 4 h for either 8 h (two paintings) or 12 h (three paintings), starting at ZT12, and at the end of the induction period the leaf was surgically removed (Figure 4a). In plants exposed to multiple induction cycles the treated leaf was also removed and a different leaf was used the following day (Figure 4a). A mock treatment followed by defoliation was applied for the plants that were not exposed to the inductive estradiol treatment to avoid distortions caused by differential handling and defoliation. The levels of *FT* expression were higher in leaves exposed to 12 h of induction than in leaves exposed to 8 h of induction (Figure 4b), but these

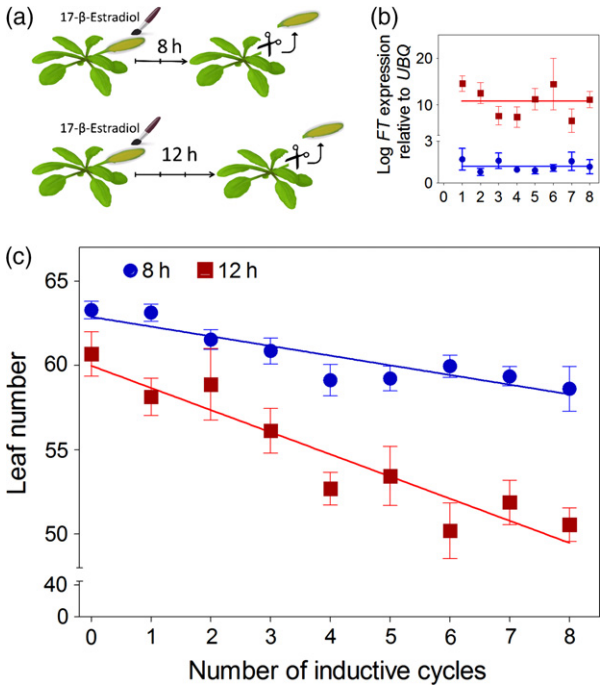

Figure 4. Flowering time responds to repeated cycles of *FT* induction.
(a) Protocol: transgenic plants (Columbia) bearing the *pER8:FT* transgene were grown under short days (SDs) for 35 days (approximately 25 leaves). For the 8-h induction treatment, 17-β-estradiol was applied to one leaf at ZT12 (ZT, zeitgeber time or time from dawn) and ZT16, and the leaf was harvested 4 h later. For the 12-h induction treatment 17-β-estradiol was applied to one leaf at ZT12, 16 and 20, and the leaf was harvested 4 h later. In the case of multiple cycles, this procedure was repeated daily by using different leaves. Mock treatments and surgical leaf harvest was applied for those plants receiving no induction in any particular day.
(b) *FT* expression in the harvested leaves at the end of the inductive cycle. Data are means and SEs of four biological replicates, 4 leaves per replicates, expressed as log(*FT*/*UBQ* expression ratio +2). Linear regressions were not significant.
(c) Number of rosette leaves at bolting, as affected by the number of inductive cycles. Data are means and SEs for 15 plants. Lines depict linear regression ($y = -0.57x + 63$; $P < 0.0001$ for 8-h treatment; $y = -1.3x + 61$; $P < 0.0001$ for 12-h treatment). The slopes are significantly different ($P < 0.0001$).

levels were unaffected by estradiol treatments on previous cycles (Figure 4b). Induction of *FT* expression accelerated flowering under SDs. This response increased linearly with the number of inductive cycles and the slope was steeper for the longer inductive cycles (12 h compared with 8 h, Figure 4c). The observed pattern of response confirms that repeated LD cycles are required to accelerate flowering because they provide high expression of *FT* for several days, and not because they build-up a maximum level of *FT* expression or because they gradually increase the sensitivity to *FT*. In fact, we observed accelerated flowering with repeated inductive estradiol cycles in the absence of changes in *FT* expression with the number of cycles, in the absence of LD treatments and in the absence of changes in the slope of the flowering response indicative of differential sensitivity (note linear fits).

Diurnal rhythm of sensitivity to *FT*

Another hypothesis that derives from the proposed model is that plants have a diurnal rhythm of sensitivity to *FT*. To test this idea, transgenic plants bearing the inducible *FT* gene were grown under SDs, and when they reached leaf 25 they were treated with estradiol for 8 h for five consecutive days (as described in Figure 4a). The 8 h of induction started every day at ZT0, 4, 8 12, 16 or 20 (Figure 5a), and caused a gradual increase in *FT* expression (Figure S2). We included a positive control where the induced leaves were not removed from the plant. We harvested untreated leaves from this positive control, to maintain the same number of leaves under all conditions and to investigate whether the induction of *FT* in selected leaves elevates *FT* expression in untreated leaves. The similar levels of *FT* expression in the negative and positive control rule out this possibility (Figure 5c).

Compared with the non-induced control, flowering was significantly promoted in the plants where the induced leaf was present between ZT12 and ZT20, and in the positive control (Figure 5b). *FT* expression levels were not higher when the induction began at ZT12 (Figure 5c). This indicates that the induction starting at ZT12 is more effective not because it yields more *FT* but because plants are more sensitive to *FT* at that time of the day. A similar experiment focused around the time of maximum effectiveness confirmed and extended these findings by showing that the time window between ZT12 and ZT20 is narrow (Figure S3). In contrast to 8 or 12 h of induction (Figures 4 and 5), 4 h of daily induction (which yields lower levels of *FT*, Figure S2) did not accelerate flowering compared with SD controls (Figure S4), confirming that *FT* expression has to be elevated above a threshold.

We also investigated the daily pattern of expression of *FTIP1*, which is important for phloem transport of FT (Liu *et al.*, 2012). Compared with the non-induced controls, a small but statistically significant decrease of *FTIP1* was observed for the seedlings induced at ZT12 (Figure 5d), i.e. under these experimental conditions there is a negative correlation between *FTIP1* expression and sensitivity to *FT* levels.

Meristem gene expression in response to *FT* in the absence of LDs

External examination of flowering time is consistent with the need of repeated cycles of *FT* induction to reach maximum acceleration of flowering. We investigated the early changes taking place in the apical tissues. The analysis by real-time PCR indicates that one cycle of *FT* induction was not enough to cause detectable changes in the expression of *SOC1*, *FUL*, *AP1*, *LYF*, *TFL1*, *FLOR1* or *FTM1* in apical tissues (Figure 6a); however, *SOC1*, *FUL* and *AP1* increased their expression in response to six inductive cycles. The analysis by *in situ* hybridization revealed some induction of *AP1*, *FUL* and *LFY* expression in response to one *FT* inductive cycle, and a stronger activation in response to six inductive cycles (Figure 6b). Taken together, these observations indicate that the induction of *FT* expression in the leaves is enough to promote the expression of flowering genes in apical tissues if sustained over several days.

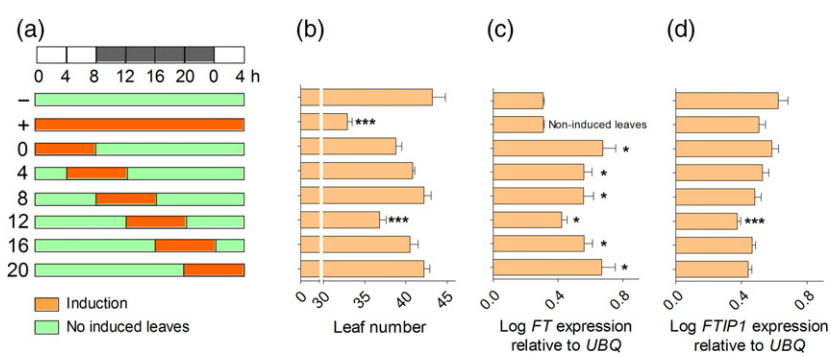

Figure 5. Diurnal rhythm of sensitivity to *FT*.
(a) Protocol: transgenic plants (Columbia) bearing the *pER8:FT* transgene were grown under short days (SDs) for 35 days (with approximately 25 leaves). *FT* expression was induced by applying 17-β-estradiol at the beginning of the 8-h inductive cycle and 4 h later, and the leaf was then harvested after a further 4 h. The ZT (zeitgeber time or time from dawn) at which the 8-h induction period was initiated is indicated. The induction was applied for five consecutive days by treating and harvesting different leaves. A mock treatment was applied to the control (–) at ZT0. The positive control (+) included plants treated at ZT0 where the treated leaf remained attached. The upper part of the diagram shows the photoperiod (white, light; black, darkness) to depict the overlap with the different induction periods.
(b) Number of rosette leaves at bolting as affected by the ZT of the initiation of the 8-h induction period. Data are means and SEs for 15 plants. ***$P < 0.001$ compared with the control.
(c, d) *FT* and *FTIP1* expression at the end of the 8-h inductive period, as affected by the ZT of initiation of the induction period. Data are means and SEs of four biological replicates [four leaves pooled per replicate, log(*FT* or *FTIP1*/*UBQ* expression ratio +2)]. ***$P < 0.001$ compared with the control. Non-induced leaves were used for + and – controls.

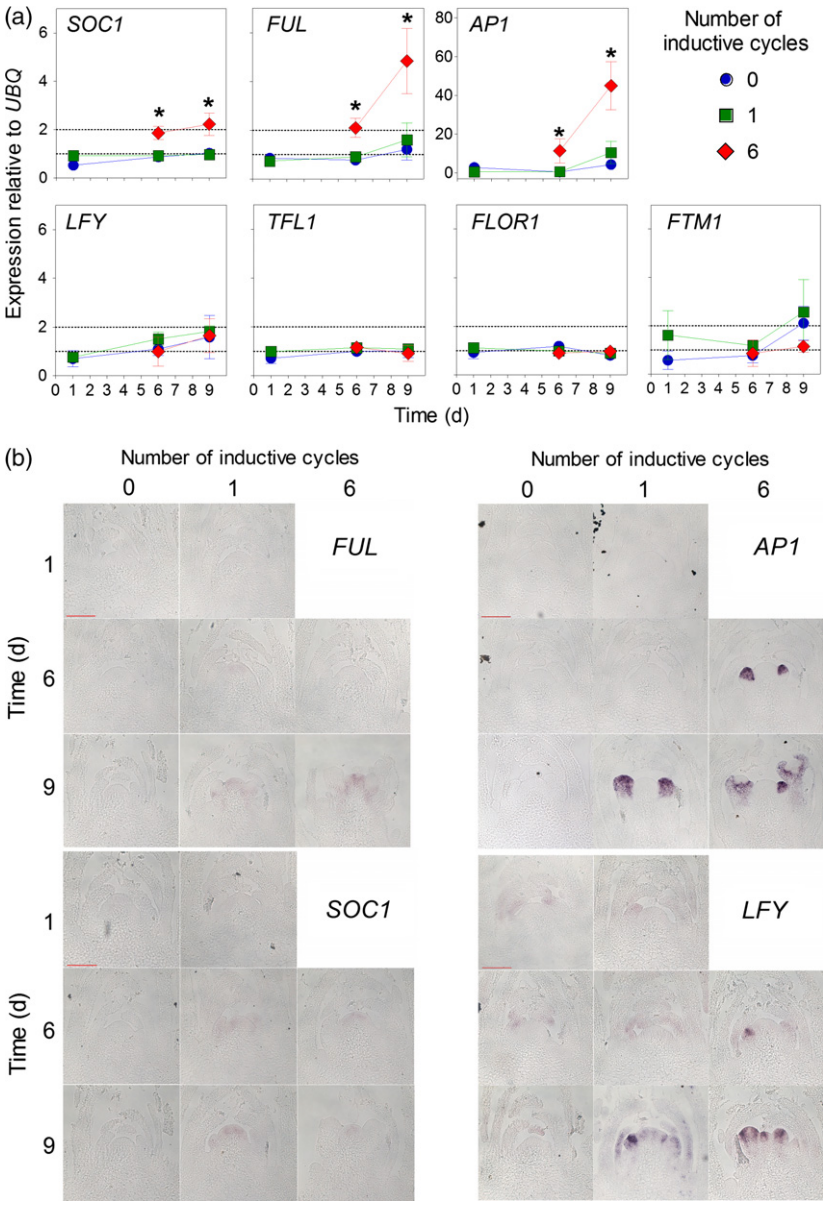

Figure 6. Enhanced expression of floral identity genes in the apical meristem requires multiple cycles of *FT* induction. Transgenic plants (Columbia) bearing the *pER8:FT* transgene were grown under short days (SDs) for 35 days (approximately 25 leaves). *FT* expression was induced by applying 17-β-estradiol (12-h inductive cycles starting at ZT12 (ZT, zeitgeber time or time from dawn), *FT* levels are shown in Figure S5). Samples were harvested at ZT6 of days 1, 6 and 9 after the beginning of the induction cycles. The number of leaves at bolting was 61 ± 1, 58 ± 1 and 50 ± 1.8 (mean and SEs of 15 plants).
(a) Real-time PCR. Data are means and SEs from four biological replicates (four leaves per replicate); $*P < 0.05$.
(b) *In situ* hybridization. Scale bars: 50 μm.

DISCUSSION

The transition to the reproductive phase is a key developmental decision because it sets a major change in the allocation of resources, and is the end of the cycle in many annual species. The control of flowering time by important exogenous and endogenous cues, such as photoperiod, temperature, canopy density, hormonal status and age, is integrated at the level of expression of *FT*. Signal integration is predicted to be advantageous because it provides the means to optimise flowering time under natural conditions, which provide highly variable combinations of these cues in different years and locations; however, signal integration also poses a functional challenge because it requires the ability of *FT* to respond to different signals and the ability of the system to precisely decode the *FT* signature. Our understanding of the latter is relatively poor. Here we show that the temporal behaviour of *FT* expression encodes long-day information.

The results presented here argue against the model where flowering responds to the maximum attained level of *FT* expression, which would build up under repeated LDs. First, under our conditions we did not observe a significant promotion of the expression of flowering-related genes, in particular of *FT*, in response to repeated LDs (Figures 1b,c and 3); the maximum response was already observed during the first or second LD. Second, when the seedlings were exposed to complex photoperiodic treatments, some conditions departed from the overall correla-

tion between flowering time and maximum *FT* levels (Figure S1a). Conversely, the data support the alternative hypothesis that plants respond to the integral of *FT* expression above a threshold level. First, we observed a tight correlation between flowering time and the *FT* integral above a threshold corrected by diurnal sensitivity (Figure 3h and Figure S1). Second, we produced an *FT* system inducible by estradiol to establish periods of high *FT* expression without using LDs. A single leaf per plant was induced during 8 or 12 h, and then removed to terminate the *FT* signal in the leaves, and the process was repeated with different leaves on successive days (Figure 4a), leading to the establishment of similar levels of *FT* expression (Figure 4b). Flowering increased linearly with the number of days of *FT* induction (Figure 4c), confirming that maximal flowering requires the integration of high *FT* expression levels over several days.

There are several reports describing the changes in gene expression experienced at the shoot apex when Arabidopsis plants are transferred from SDs to LDs (Alonso *et al.*, 2003; Torti *et al.*, 2012). Here, we show that repeated cycles of *FT* induction in the leaves are enough to promote the expression of flowering genes such as *LFY*, *AP1*, *SOC1* or *FUL* in the apex, in the absence of LDs (Figure 6). In contrast to repeated cycles, a single cycle of *FT* induction in the leaves did not cause a detectable promotion of gene expression in apex samples investigated by real-time PCR (Figure 6a and Figure S5), confirming that the signal has to be integrated over several cycles. A locally restricted promotion of *AP1* and *LFY* expression by a single cycle of *FT* induction was detected by *in situ* hybridization, but several days after the inductive pulse and not immediately after it (Figure 6b and Figure S5). This suggests that the *FT* signal could be integrated by the gene network that triggers flowering in the apex itself. Reduced persistence of the *FT* signal over a time window would result in the insufficient promotion of flowering genes.

The analysis of the relationship between the diurnal patterns of *FT* and flowering under different photoperiodic treatments revealed changes in diurnal sensitivity to *FT* (Figure 3). The latter idea was confirmed by experiments involving the *FT*-inducible system, where *FT* expression was enhanced at selected times of the daily cycle in plants grown under SDs (Figure 5b). Noteworthy, *FT* was most effective between ZT12 and ZT20, a period when *FT* expression is normally enhanced by LD compared with SD conditions. The inducible system produced slightly lower levels of *FT* mRNA at that time of the daily cycle (Figure 5c), suggesting that mRNA stability might be reduced. This indicates that some steps(s) beyond *FT* mRNA in the leaves occur(s) more effectively at that time of the day. The expression of *FTIP1*, which is required for the translocation of FT from the leaves to the apex (Liu *et al.*, 2012), was also reduced at that time of the daily cycle (Figure 5d). There is an optimum level of *FTIP1* expression because flowering is delayed both by loss-of-function mutations and by overexpression, which affect normal FT translocation from the leaves to the apex (Liu *et al.*, 2012). Therefore, the stronger effect of induction of *FT* in the leaves between ZT12 and ZT20 might be caused by *FTIP1* levels eventually closer to the optimum for translocation at that time of the day.

Under natural conditions, the occurrence of a single LD provides an unequivocal seasonal signal because subsequent days will have a photoperiod differing only by a few minutes. It is therefore reasonable to question the reasons why the photoperiodic induction has to be repeated over several cycles to maximise flowering. The results presented here indicate that repeated LDs are necessary to maintain high levels of *FT* expression during the evening and early night over several days. In contrast to a single LD, a single burst of *FT* expression is not an unequivocal seasonal signal because other environmental fluctuations, such as shade (Kim *et al.*, 2008; Wollenberg *et al.*, 2008) and high ambient temperatures (Blázquez *et al.*, 2003), can promote the expression of *FT*. The ability of *FT* to respond to different stimuli is a requisite of its function as signal integrator. To specifically induce flowering in response to photoperiod the system decodes LD-specific features of the *FT* temporal signature, such as the presence of high levels of *FT* expression during several days and beyond ZT12.

EXPERIMENTAL PROCEDURES

Experiments involving photoperiodic treatments

Seeds of *A. thaliana* accession Col-0 (Columbia) were sown in clear boxes containing 0.8% agar-water, stratified for 4 days at 4°C in darkness, transferred to 22°C and irradiated with red light for 3 h (5 μmol m^{-2} sec^{-1}), followed by 21 h of darkness. Then, the boxes were transferred to the growth chamber providing SDs, i.e. 8 h of white light (Phillips 40W fluorescent tubes, PAR 150 μmol m^{-2} sec^{-1}) and 16 h of darkness, at 22°C. Three days later, the seedlings were transplanted to cylindrical plastic pots (70 mm in height, 35 mm in diameter; Colombraro, http://www.colombraro.com.ar) containing perlite (Perlome), peat moss (Finca Don Calvino) and vermiculite (Intersum) (2 : 2 : 1), and then watered twice a week using a solution containing 1 g L^{-1} Hakaphos R (Compos) (all obtained from Agroquimica Larrocca, http://www.agroquimicalarrocca.com.ar).

After growth under SDs for the time indicated for each experiment, plants were exposed to LDs (16 h of white light and 8 h of darkness), intermediate photoperiods (12 h of white light and 12 h of darkness) or continuous white light for a variable number of cycles, and then returned to SDs. Flowering time was scored as the number of rosette leaves when the bolting shoot had reached approximately 2 cm in height.

The *ft-10* mutant (Hanzawa *et al.*, 2005) and the *FT ft-10* heterozygous mutant were included in some experiments. The genotypes were confirmed by PCR using the primers indicated in Table S1, with 39 cycles, and hybridization temperatures that varied according to the DNA melting points of each amplicon.

FT-inducible system

FT cDNA was amplified using the primers *Xho*I-FT-F (5′-GCG GCCTCGAGATGTCTATAAATATAAGAGACCCTC-3′) and SpeI-FT-R (5′-GGCGACTAGTCTAAAGTCTTCTTCCTCCGCAGCCAC-3′). The *FT* cDNA sequence was verified by sequencing and cloned into the MCS of the pER8 vector (Zuo *et al.*, 2000) at the *Xho*I/*Spe*I restriction sites to originate the *pER8::FT* plasmid. Plants were transformed with *Agrobacterium tumefaciens* strain GV3101 pMP90 RK by floral dipping (Clough and Bent, 1998). The pER8 vector was kindly provided by Prof. Nam-Hai Chua (Rockefeller University, NY, USA).

Transgenic *pER8:FT* plants were grown under SDs as described for Col-0. *FT* induction was achieved by soaking just one leaf per plant for 10 sec in an aqueous solution containing 10 μM 17-β-estradiol (E8875; Sigma-Aldrich, http://www.sigmaaldrich.com) and 0.1 μl per ml Tween 20 (H5152; Promega, http://www.promega.com). The leaf was submerged into a 50-ml plastic tube with the assistance of a thin brush. The solution was regularly stirred in order to keep it foamy and to encourage adhesion to both leaf surfaces. This procedure was repeated every 4 h during the induction period. At the end of the induction period the leaf was removed by cutting its petiole with a pair of surgical scissors, wrapped in aluminium foil, submerged in liquid nitrogen and stored at −80°C until further processing.

Real-time PCR

Between 50 and 100 mg of leaf tissue was pulverized in liquid nitrogen and extracted with Trizol (Invitrogen, now Life Technologies, http://www.lifetechnologies.com) following the manufacturer's specifications. For apex RNA, Spectrum Plant Total RNA Kit (Sigma-Aldrich) was used, following the manufacturer's specifications. The dried pellet was re-suspended in 40 μl of distilled water and RNA concentration was quantified by using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, now GE Healthcare Life Sciences, http://www.gelifesciences.com). For cDNA synthesis, 2 μg of total RNA was incubated with DNAse/RNase-Free (Promega) for 30 min and reverse transcription was performed with SuperScript III (Invitrogen), following the manufacturer's specifications. Synthesized cDNA was diluted to a final volume of 40 μl with distilled water. The control for genomic DNA degradation included the same steps but omitted the addition of reverse transcriptase. For real-time PCR, 2 μl of cDNA was diluted to a final reaction volume of 10 μl [5 μl FastStart SYBR Green PCR MIX (Roche, http://www.roche.com), 2 μl of distilled water plus 1 μl of 5 mM primer mix]. Each sample was analysed twice using a 7500 Real Time PCR System (Applied Biosystems, now Life Technologies, http://www.lifetechnologies.com). Results were processed using the 7500 System SDS software (Applied Biosystems). The primers are detailed in Table S2.

In situ hybridization

The methods used for digoxigenin labelling of mRNA probes, tissue preparation and *in situ* hybridization were as described previously (Torti *et al.*, 2012). A different set of primers was used for *AP1* (Table S2).

ACKNOWLEDGEMENTS

We thank Maida Romera-Branchat for providing the *AP1* probe. We thank Elizabeth Karayekov for her excellent technical assistance. This work was supported by Agencia Nacional de Promoción Cienífica y Tecnologica (grant PICT 2006-1913 to JJC), Universidad de Buenos Aires (grant 20020100100437 to JJC) and a Marie Curie Intra-European Fellowship for Career Development (Project Intra-European Grant Agreement-2009-251839 to FA).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Quantitative analysis of the relationship between flowering time and *FT* expression features.

Figure S2. Time course of estradiol induction of *FT* expression in treated leaves.

Figure S3. Diurnal rhythm of sensitivity to *FT*.

Figure S4. Four hours of daily induction of *FT* expression are not enough to promote flowering.

Figure S5. *FT* expression, as affected by the number of daily estradiol induction cycles.

Table S1. List of flowering-related genes used in Figure 1.

Table S2. Primers used for real-time PCR, PCR and *in situ* hybridization.

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