Antisense suppression of the *Arabidopsis PIF3* gene does not affect circadian rhythms but causes early flowering and increases *FT* expression

Atsushi Oda[a], Sumire Fujiwara[a], Hiroshi Kamada[a], George Coupland[b], Tsuyoshi Mizoguchi[a,*]

[a] *Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*
[b] *Max Plank Institute for Plant Breeding, Carl von Linne Weg 10, D-50829 Cologne, Germany*

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Abstract **Photoperiodic control of flowering is regulated by light and a circadian clock. Feedback regulation of the transcription of clock components is one of the most common and important mechanisms that control clock functions in animals, fungi, and plants. The *Arabidopsis* circadian clock is believed to involve two myb-related proteins, LHY (late elongated hypocotyl) and CCA1 (circadian clock associated 1), which negatively regulate *TOC1* (*timing of cab expression 1*) gene expression through direct binding to the *TOC1* promoter. PIF3 (phytochrome-interacting factor 3), a bHLH transcription factor binds promoter regions of the *LHY* and *CCA1* genes, affecting the light induction of these genes, and interacts with TOC1 protein. Although the positive feedback regulation of clock components in plants has been predicted, and PIF3 has been assumed to be involved, the molecular nature of this process has not been elucidated. Here we demonstrate that the antisense suppression of the *PIF3* gene causes higher levels of mRNA of floral activator genes *CO* (*constans*) and *FT* (*flowering locus T*) and results in early flowering under long days (LD). Neither the circadian rhythms of the clock-controlled genes (CCGs) under constant conditions nor the diurnal rhythms of the CCGs under LD conditions are affected by the reduction in *PIF3* gene expression. These results suggest that PIF3 may play an important role in the control of flowering through clock-independent regulation of *CO* and *FT* gene expression in *Arabidopsis*.**

Key words: Circadian clock; Feedback regulation; Transcriptional regulation; Flowering time; Photoperiod; *Arabidopsis*

1. Introduction

The molecular genetic dissection of flowering time in *Arabidopsis* has identified several of the clock components, photoreceptors, and light signaling proteins that are involved in the photoperiodic control of flowering time [1,2]. Loss-of-function of one of these genes, *constans* (*CO*), causes late flowering under inductive long days (LD) conditions [3], whereas the gain of *CO* function results in early flowering even under non-inductive short days (SD) conditions [4]. CO is a transcriptional activator that accelerates flowering time under LD conditions by, at least in part, increasing the expression of the gene *flowering locus T* (*FT*) [1,5,6]. Changes in the levels of *CO* and *FT* mRNAs are responsible for the alterations in flowering time observed in two late flowering mutants, *gigantea* (*gi*) and *late elongated hypocotyl-1* (*lhy-1*), and in three early flowering mutants, *early flowering 3* (*elf3*) [5], *early flowering 4* (*elf4*) [7], and *timing of cab expression 1* (*toc1*) [1,6], all of which have circadian defects [1,2]. The regulation of flowering time by day length is thought to require the integration of temporal and environmental light information at the molecular level [1,2]. It has been proposed that this integration takes place at the level of CO [1,5]. Recent models suggest that an external coincidence mechanism, based on the circadian control of *CO* mRNA levels, and the modulation of CO function by light may constitute the molecular basis for the regulation of flowering time by day length in *Arabidopsis* [1,5,6].

Circadian clocks represent widespread endogenous mechanisms that allow organisms to time biological processes appropriately throughout the day–night cycle. At least three genes are putative central oscillators of a circadian clock in *Arabidopsis* [1,2]. Two of these, *LHY* and *circadian clock associated 1* (*CCA1*), encode closely related transcription factors of the MYB family [2]. The third, *TOC1*, encodes a protein with a sequence related to the receiver domain of two-component signaling [1]. The *TOC1* gene has also been described as *Arabidopsis pseudo-response regulator 1* (*APRR1*) and *Arabidopsis* has four additional homologs (APRR3, 5, 7 and 9) [8]. The reciprocal regulation of clock-associated genes is central to the function of all circadian oscillators [9]. Molecular biochemical analyses and molecular genetics have shown that LHY and CCA1 negatively regulate *TOC1* expression in a direct manner [10,11]. TOC1 has been assumed to function reciprocally as a positive effector of *LHY* and *CCA1* expression; however, because TOC1 lacks DNA-binding domain motifs and because there is no experimental evidence that it directly binds to DNA, TOC1 may require protein partners in order to regulate *LHY* and *CCA1*. Recently two mutations in the *Arabidopsis pseudo-response regulator 7* (*PRR7*) gene have been reported [12]. The *PRR7* gene has been shown to be required for the negative regulation of *LHY* and *CCA1* in etiolated seedlings in response to light pulses. *prr7* also showed a clear defect in the sustained circadian expression pattern of *LHY* and *CCA1* [12].

It has been suggested that the basic helix-loop-helix (bHLH) transcription factor phytochrome-interacting factor

*Corresponding author. Fax: (81)-298-53 7723.
E-mail address: mizoguchi@gene.tsukuba.ac.jp (T. Mizoguchi).

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3 (PIF3) acts as a positive element in *LHY* and *CCA1* expression, at least in etiolated seedlings in response to light pulses [13]. and a protein–protein interaction between TOC1 and PIF3 has been demonstrated [14]. Therefore, PIF3 appears to be a good candidate for a positive regulator of *LHY* and *CCA1* expression. Although the regulation of hypocotyl elongation by PIF3 under various light conditions and the expression of light-inducible genes by PIF3 in etiolated seedlings have been fully studied, and detailed biochemical analyses of the interactions between PIF3 and phytochromes have been conducted [15,16], the possible roles of PIF3 in the control of processes such as flowering and circadian rhythmicity have not been reported to our knowledge.

Here, we describe the first characterization of the antisense suppression of the *PIF3* gene with respect to circadian rhythms, flowering times, and the expression of the floral activator genes *CO* and *FT* in light/dark (L/D) cycles.

2. Materials and methods

2.1. Plant materials and growth conditions

The No-0 ecotype of *Arabidopsis thaliana* was used unless otherwise indicated. The *PIF3* antisense line A22 was described previously [15]. Plants used for the reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis were grown on agar plates in controlled environment rooms under LD (16 h light/8 h dark) conditions for 12 days. For continuous light (LL) experiments, the LD-grown plants were transferred to LL conditions. For the measurement of flowering times, plants were grown on soil under LD (16 h light/8 h dark) and SD (10 h light/14 h dark) conditions.

2.2. Northern blot analysis and analysis of period length

RNA (10 μg) was separated on 1.2% agarose/formaldehyde denaturing gels and transferred to Biodyne B membranes (Nippon Genetics, Tokyo, Japan). Hybridization was done in 0.3 M sodium phosphate buffer (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1 mM ethylenediamine tetraacetic acid (EDTA), and 1% bovine serum albumin overnight at 65°C. The blot was washed with 0.2× standard sodium citrate (SSC) and 0.1% SDS for 30 min at 65°C. Probes were full-length *LHY*, *CCA1*, *TOC1*, and *GI* cDNAs [11]. Images were visualized using a BioImaging Analyzer (BAS 5000; Fuji Photo Film, Tokyo, Japan); signal intensity was quantified with Science Lab 98 Image Gauge software (version 3.1; Fuji Photo Film, Tokyo, Japan). Fourier transforms and period estimates were obtained using the fast Fourier transform-non-linear least squares program (FFT-NLLS) as described [17]. The relative amplitude error, RAE, is the value of the amplitude error estimate divided by the value of the most probable amplitude estimate. RAE can range from a value of 0 for an infinitely well-determined rhythmic component (zero error) to a value of 1, theoretically, for a minimally determined rhythmic component (error in the amplitude equals the amplitude value itself).

2.3. RT-PCR analysis

RT-PCR was performed with 1 μg of total RNA using a SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). *CO* [5], *FT* [18], and *TUB2* [19] primers have been described. The products were separated on 1.5% agarose gels and analyzed as described above.

2.4. Measurement of flowering time

Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem. Data are presented as mean ± S.E.M. Measurement of flowering time was done twice in LD and SD with similar results. All differences in flowering times were confirmed as statistically significant using Student's *t*-test ($P < 0.00005$).

3. Results

3.1. Reduction of PIF3 gene expression causes early flowering under LD conditions

PIF3 has been predicted to function as one of the positive regulators of *LHY* and *CCA1* gene expression in the *Arabidopsis* circadian clock [13]. LHY and CCA1 have partially redundant roles in maintaining circadian rhythms. A reduction in the total amount of LHY and CCA1 protein by the loss-of-function of either the *LHY* or *CCA1* gene causes a short-period phenotype under LL and continuous dark

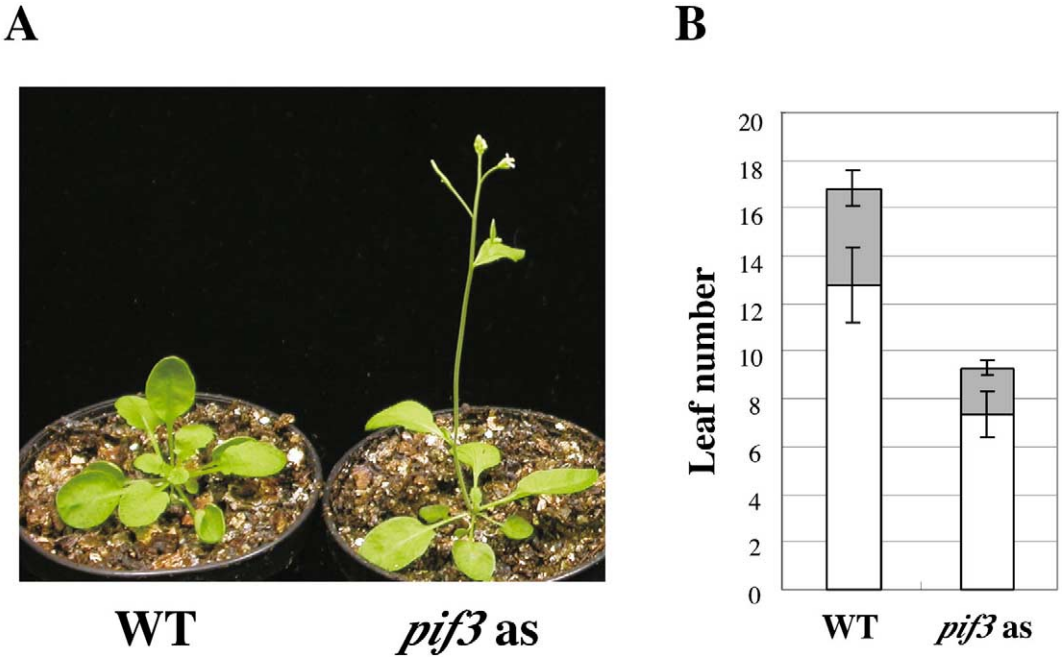

Fig. 1. Early flowering phenotype of the *PIF3* antisense line. No-0 (left) and *pif3*as line (right) plants were grown for 3 weeks under LD conditions (A and B). Means are shown ± standard deviation. Open and filled boxes represent the numbers of rosette leaves and cauline leaves, respectively.

(DD) conditions [11]. If PIF3 is involved in the positive regulation of *LHY* and *CCA1* gene expression and if *Arabidopsis* does not have genes with functions redundant with those of *PIF3*, then circadian rhythms should be affected in a *pif3*as line.

Numerous *Arabidopsis* circadian clock mutants display not only an altered light sensitivity during seedling emergence [1,2], but also a reduction or even an absence of sensitivity to day length [20]. The latter phenotypes are often associated with changes in flowering time [21]. First we tested *pif3*as for flowering time phenotypes. We used the *pif3*as line A22 (Section 2) in which a dramatic reduction of *PIF3* mRNA levels were reported previously. Under SD conditions, the *pif3*as line had a subtle early flowering phenotype, but under LD conditions, *pif3*as plants flowered much earlier than did the wild-type in our experiments (Fig. 1); Student's *t*-test confirmed that these differences were statistically significant (Section 2).

3.2. *Antisense suppression of* PIF3 *gene does not affect rhythmicity of expression of clock-controlled genes (CCGs)*

The loss-of-function of either *LHY* or *CCA1* causes a short-period phenotype associated with early flowering under light/dark cycles, especially under SD conditions [11]. Similar correlations between period lengths and flowering times have been reported in *toc1* and *zeitlupe* (*ztl*); *toc1* shortens free running rhythms (FRRs) of CCGs under LL conditions and causes early flowering under SD conditions [22], whereas *ztl* lengthens FRRs and causes late flowering [23]. Therefore, we next tested whether the expression of CCGs was altered in the *pif3*as line under LL conditions. In wild-type plants, *LHY* and

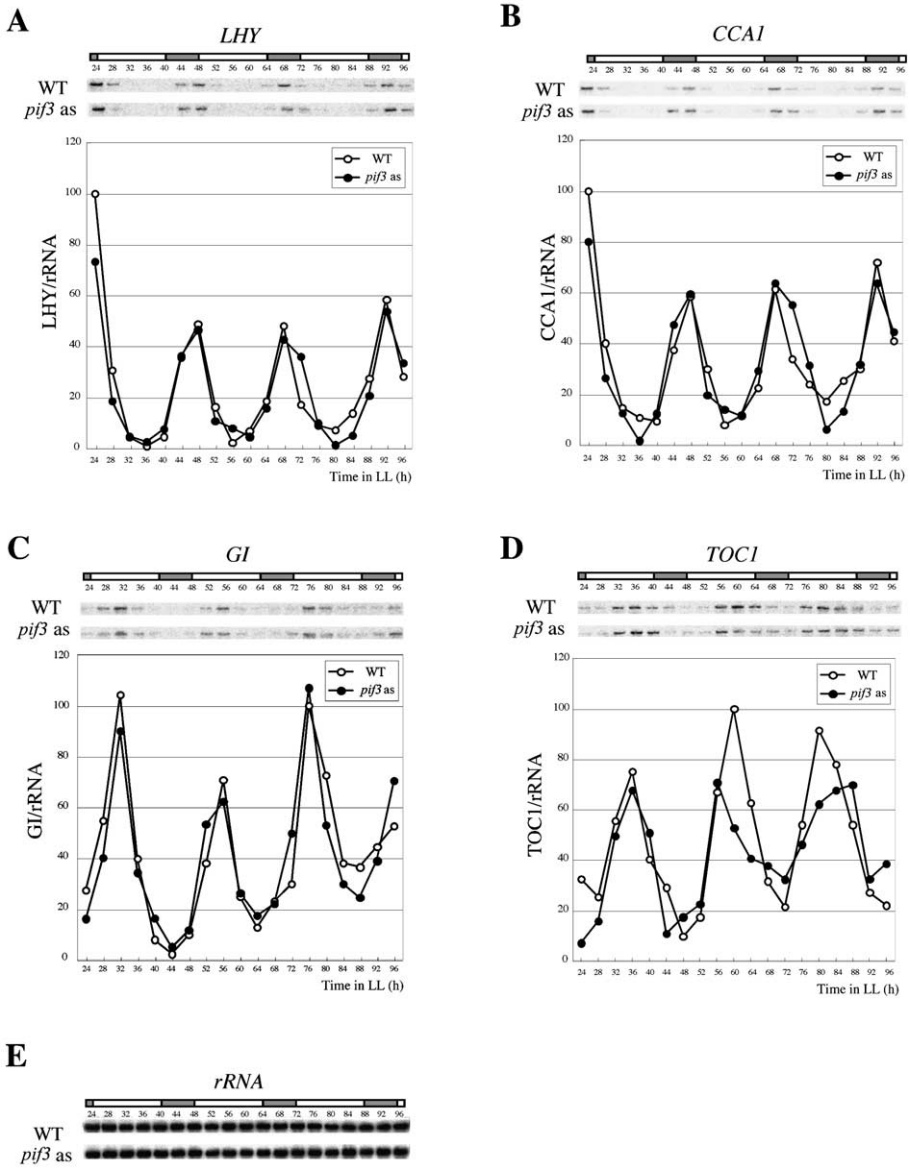

Fig. 2. Antisense suppression of *PIF3* does not affect the expression of CCGs under LL conditions. Shown are the Northern blot analysis of *LHY* (A), *CCA1* (B), *TOC1* (C), and *GI* (D) and the abundance of rRNA (E) in No-0 (open circles) and *pif3*as lines (filled circles). Plants were entrained under LD (16 h light/8 h dark) conditions for 12 days and then released into LL conditions. The analysis is shown from the time 24 h after transferring to LL. Open and filled boxes indicate subjective day and night periods, respectively. Each experiment was performed at least twice with similar results. Quantification was performed with Science Lab 98 Image Gauge software as described in Section 2.

Table 1
Free running period estimates of the expression of CCGs in No-0 and the *PIF3* antisense lines under constant white light

	Experiment 1			Experiment 2		
	Period (h)	S.D.	RAE	Period (h)	S.D.	RAE
LHY						
WT	22.70	±1.64	0.58	22.77	±1.17	0.36
*pif3*as	23.07	±0.70	0.36	22.56	±1.88	0.64
CCA1						
WT	23.01	±1.15	0.77	22.71	±1.38	0.42
*pif3*as	23.35	±0.88	0.32	23.94	±1.96	0.48
GI						
WT	22.16	±1.26	0.50	22.25	±0.97	0.31
*pif3*as	22.59	±0.96	0.37	22.39	±3.43	0.69
TOC1						
WT	22.87	±1.20	0.34	21.58	±1.48	0.45
*pif3*as	22.73	±1.58	0.57	21.98	±1.42	0.41

The gene expression data in Fig. 2 (Experiment 1) and an independent experiment (Experiment 2) were Fourier transformed, and period estimates were derived using FFT-NLLS [17]. The periods are given as the variance-weighted means (period) of the estimate with variance-weighted standard deviations (S.D.). The RAE is shown in Section 2. WT is wild-type.

CCA1 gene expression peaked around subjective dawn at Zeitgeber time (ZT) 24, ZT 48, ZT 72, and ZT 96 (Fig. 2A and B), as reported previously [11]. Surprisingly, the antisense suppression of the *PIF3* did not affect FRRs or the amplitude of expression of the *LHY* or *CCA1* gene expression (Fig. 2A and B). Similar results were obtained for the CCG, *GI* and *TOC1*, which normally peak in expression in the evening (Fig. 2C and D). There was no statistical difference in the rhythmicity of the expression of CCGs between *pif3*as and wild-type plants (Table 1). To confirm the strength of rhythms, the RAE (see Section 2) were calculated (Table 1). All of the rhythms were statistically significant (RAE < 1).

3.3. *The early flowering phenotype of* pif3*as correlates with a dramatic increase in the* FT *mRNA level under LD conditions*

Consistent with the early flowering phenotype (Fig. 1), the expression level of the floral marker gene *FT* increased dra-

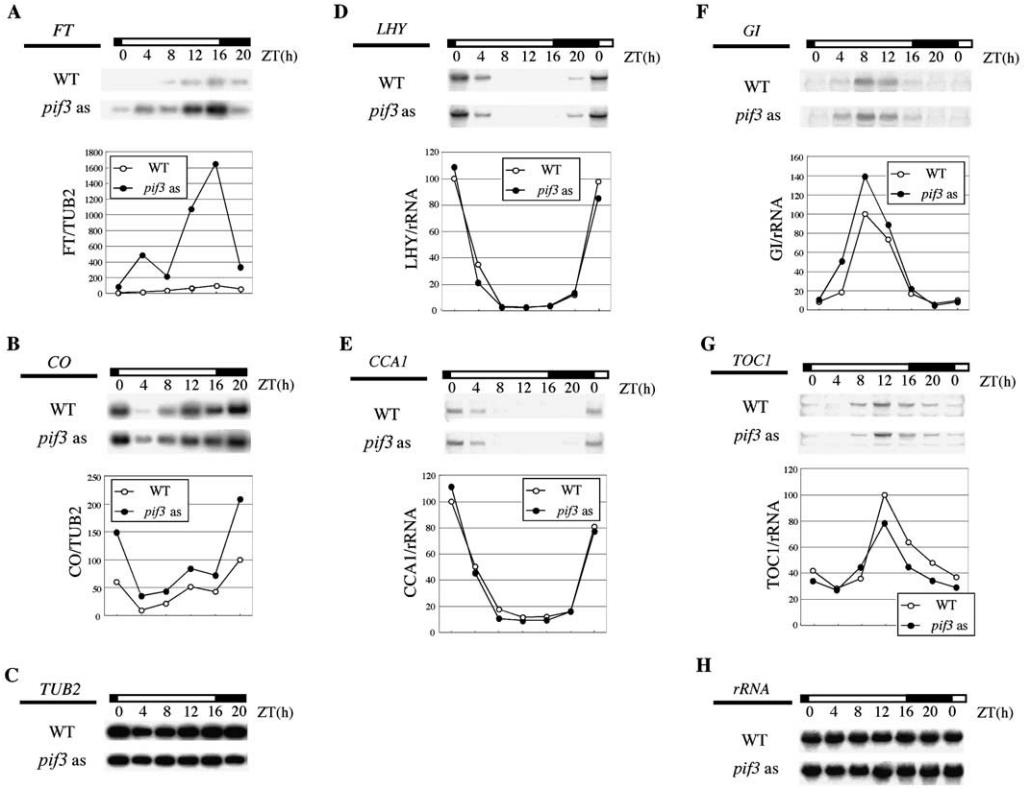

Fig. 3. Reduction of PIF3 increases mRNA levels of the two floral regulator genes *FT* and *CO* but does not alter those of CCGs, *LHY*, *CCA1*, *TOC1* and *GI* under LD conditions. Shown are the RT-PCR analyses of *FT* (A), *CO* (B), and *TUB2* (C) expression and the Northern blot analyses of *LHY* (D), *CCA1* (E), *GI* (F), and *TOC1* (G) expression and the abundance of rRNA (H) in No-0 and *pif3*as lines under LD conditions. Tissue was harvested from 12-day-old seedlings entrained in a LD cycle. Expression levels were normalized against *TUB2* (A and B) and rRNA (D–G). The periods of light and dark are indicated as open and filled boxes, respectively. ZT 0 is the time point just before lights on. Each experiment was performed at least twice with similar results. Quantification was performed as described in Fig. 2.

matically in *pif3*as under LD conditions (Fig. 3A). *CO* mRNA levels were also significantly higher in *pif3*as at all time points (Fig. 3B). Under LD conditions, the expression patterns of *LHY* and *CCA1* were not affected (Fig. 3D and E), and *TOC1* mRNA levels were similar in *pif3*as and the wild-type (Fig. 3G). These results indicated that the early flowering defect of *pif3*as was not due to a generalized alteration in the expression of clock-regulated, flowering time genes. In contrast, we noted a substantially higher level of *GI* expression in *pif3*as than in the wild-type twice in our triplicate experiments under LD conditions (Fig. 3F; Section 2), suggesting that *GI* might function in the regulation of *FT* [24]. Under LL conditions, almost no difference was detected in the maximum levels of *GI* expression between the wild-type and *pif3*as (Fig. 2C).

4. Discussion

Recently, PIL1 (PIF3-like 1) was identified as a TOC1/APRR1-interacting protein [14]. The *PIL1* gene encodes a putative bHLH transcription factor with an amino acid sequence that is highly similar to that of PIF3 [14]. It has also been reported recently that, as seen with *PIF3* (Fig. 2 and Table 1), the loss-of-function of *PIL1* (*pil1-1*; T-DNA insertion mutant of PIL1) does not affect the rhythmicity of *LHY* or *CCA1* gene expression under DD or light/dark cycles [25]. In fact, *Arabidopsis* has at least five additional genes (*PIL2*, *PIL5*, *PIL6*, *PIF4*/*SRL2*, and *HFR1*) encoding bHLH proteins that are highly similar to both PIF3 and PIL1, and all PIF3 family members, except HFR1, interact with TOC1/APRR1 in yeast two-hybrid analyses [25]. Because closely related genes often have redundant functions, as has been demonstrated for the two closely related genes *LHY* and *CCA1*, it is possible that the loss-of-function of one redundant gene would not cause a severe phenotype, but would result in only a subtle defect. It may be that the defects in *pif3* are completely or partially compensated for by *PIF3*-related genes, even though PIF3 might play a vital role in clock functions. PIF4 and PIL6 have recently been shown to be clock-controlled genes and have been proposed to play important roles in the control of circadian rhythms [25], supporting this explanation. Alternatively, PIF3 might not be involved in maintaining a circadian clock.

The antisense suppression of *PIF3* accelerated flowering (Fig. 1) without affecting the expression of CCGs under LD conditions (Fig. 2), therefore we investigated the role(s) of PIF3 in the regulation of key genes that affect flowering time. *FT* integrates several flowering time pathways [1,19,26], and its expression is lower in *phyA* (*phytochrome A*) and *cry2* (*cryptochrome 2*) mutants under conditions in which these mutants are late flowering [1]. The early flowering phenotype of *pif3*as was seen under LD conditions (Fig. 1) and was not as severe under SD conditions in our experiments (data not shown). Under LD conditions, we observed a large increase in *FT* mRNA levels in *pif3*as (10- and 20-fold wild-type levels; Fig. 3). PIF3 interacts with Pfr forms of phyB [16]. The similarities in phenotypes in terms of (i) long hypocotyls under red light, (ii) a reduction in gene expression induced by red light (e.g. *CAB* and *RBCS*), (iii) lower chlorophyll content, and (iv) long petiole length strongly suggest that PIF3 plays a role in phyB signaling [13,15]. Consistent with this idea, the loss of *phyB* function accelerates flowering under both LD and SD conditions [27], and significant increases in both *CO* and *FT* expression similar to those observed in *pif3*as were seen in *phyB* under LD conditions (Fig. 3A–C) [27].

The loss-of-function of *TOC1* (*toc1-2* and TOC1 RNAi lines) results in a reduced sensitivity to red light and far-red light in the control of hypocotyl elongation [20]; this phenotype is similar to that of *pif3*as [15]. The short-period mutation *toc1* causes early flowering under SD conditions. This is associated with a phase advance of *CO* expression, which leads to relatively high levels of *CO* mRNA during the illuminated part of the day at dusk and the upregulation of the floral activator gene *FT* [1]. Although flowering time is not affected, substantial increases in *FT* and *CO* expression in *toc1* are seen during the day time under LD conditions [1,6]. The increase in *FT* expression in *toc1* is less than that in *pif3*as under LD conditions (approximately 2-fold vs. 10- to 20-fold; Fig. 3A) which might explain in part the early flowering in *pif3*as but not in *toc1* under LD conditions. We think it is likely that the early flowering of *pif3*as is caused by the upregulation of the floral activator gene *FT* through a substantial increase in *CO* expression. One alternative possibility is that the increase in *FT* expression might be caused by a post-translational modification of the CO protein induced by light signaling [1,5]. Light might activate or stabilize the CO protein directly or indirectly to increase *FT* expression.

Although several phenotypes of *pif3*as and *toc1* are common, functional cooperation in vivo between PIF3 and TOC1 is still unclear. Investigations using *pif3*/*toc1* double mutants and multiple mutants of the *PIF3* family members would provide new insights into the positive feedback loop of the *Arabidopsis* clock and the connections between circadian rhythms and light inputs in the photoperiodic control of flowering.

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