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Citation for published version:

Digital Object Identifier (DOI):
10.15252/msb.20145766

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular Systems Biology

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Linked circadian outputs control elongation growth and flowering in response to photoperiod and temperature

Daniel D Seaton, Robert W Smith, Young Hun Song, Dana R MacGregor, Kelly Stewart, Gavin Steel, Julia Foreman, Steven Penfield, Takato Imaizumi, Andrew J Millar & Karen J Halliday

Abstract

Clock-regulated pathways coordinate the response of many developmental processes to changes in photoperiod and temperature. We model two of the best-understood clock output pathways in Arabidopsis, which control key regulators of flowering and elongation growth. In flowering, the model predicted regulatory links from the clock to CYCLING DOF FACTOR 1 (CDF1) and FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) transcription. Physical interaction data support these links, which create threefold feed-forward motifs from two clock components to the floral regulator FT. In hypocotyl growth, the model described clock-regulated transcription of PHYTOCHROME-INTERACTING FACTOR 4 and 5 (PIF4, PIF5), interacting with post-translational regulation of PIF proteins by phytochrome B (phyb) and other light-activated pathways. The model predicted bimodal and end-of-day PIF activity profiles that are observed across hundreds of PIF-regulated target genes. In the response to temperature, warmth-enhanced PIF4 activity explained the observed hypocotyl growth dynamics but additional, temperature-dependent regulators were implicated in the flowering response. Integrating these two pathways with the clock model highlights the molecular mechanisms that coordinate plant development across changing conditions.

Keywords: gene regulatory networks; heat; hypocotyl elongation; photoperiodism; seasonal breeding

Subject Categories: Quantitative Biology & Dynamical Systems; Plant Biology

DOI: 10.15252/msb.20145766 | Received 15 September 2014 | Revised 21 November 2014


Introduction

Plants are exposed to a wide range of light and temperature regimes that alter the molecular mechanisms controlling plant development. Seedling de-etiolation and floral transition represent critical stages in the plant life cycle that directly impact plant productivity. De-etiolation, which is characterised by embryonic leaf (cotyledon) greening and the cessation of embryonic stem (hypocotyl) elongation, is important for seedling establishment, whereas the time taken for the plant to reach the floral transition controls the balance between vegetative biomass and seed production. In the model plant Arabidopsis thaliana, long-day (LD) growth regimes lead to earlier flowering and shorter hypocotyls compared to short-day (SD) regimes (Corbesier et al., 1996; Kunihiro et al., 2011). Higher ambient temperatures promote early flowering and hypocotyl elongation (Mazzella et al., 2000; Halliday et al., 2002; Balasubramanian et al., 2006; Kumar et al., 2012).

The circadian clock is central to the photoperiodic response and provides 24-h timing information at the molecular level. While molecular clock components are not generally conserved across taxa, all circadian clocks include a gene circuit with interconnected negative feedback loops. In Arabidopsis, the circadian clock regulates up to 30% of genes at the transcript level, often intersecting with signalling pathways responsive to the external environment (Harmer et al., 2000; Harmer, 2009; Kinmonth-Schultz et al., 2013). This allows the clock to “gate” environmental responses to specific time windows within the daily cycle (Millar & Kay, 1996). In some cases, including photoperiodic regulation of flowering time and hypocotyl elongation, the gating circuit forms an “external coincidence” detector for time-specific environmental signals (Bünning, 1936; Roden et al., 2002; Yanovsky & Kay, 2002; Nozue et al., 2007).

In Arabidopsis, mathematical modelling has been an important tool for elucidating the architecture of the clock circuit, which can be viewed as an elaborated repressilator (Fig 1, bottom inset;
Pokhilkho et al., 2012). Transcript levels of the key morning genes CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) peak at dawn (Wang & Tobin, 1998). The CCA1 and LHY proteins inhibit transcription of EVENING COMPLEX (EC) components, EARLY FLOWERING 4 (ELF4), ELF3 and LUX ARRTHMO (LUX), delaying their accumulation until dusk (Doyle et al., 2002; Hazen et al., 2005; Dixon et al., 2011; Nusinow et al., 2011; Lu et al., 2012). The EC in turn inhibits transcription of PSEUDO-RESPONSE REGULATOR 9 (PRR9) and TIMING OF CAB EXPRESSION 1 (TOC1/PRR1) at night (Heller et al., 2011; Herrera et al., 2012; Pokhilkho et al., 2012). The family of PRR proteins, including PRR9, PRR7, PRR5 and TOC1, repress transcription of CCA1 and LHY through the day and early night, completing the CCA1/LHY-EC-PRR repressilator (Huang et al., 2012; Nakamichi et al., 2012). In addition to this central loop, GI protein suppresses EC formation and TOC1 accumulation through interactions with ELF3 and ZEITLUPE (ZTL) proteins, respectively (Kim et al., 2007; Yu et al., 2008; Pokhilkho et al., 2012).

In the flowering pathway, GI controls expression of floral activators CONSTANS (CO) and FLOWERING LOCUS T (FT) by forming a blue light-dependent complex with FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) (Imaizumi et al., 2003, 2005; Sawa et al., 2007; Sawa & Kay, 2011). The GI-FKF1 complex degrades CYCLING DOF FACTOR 1 (CDF1) protein that represses CO and FT transcription (Imaizumi et al., 2005; Fornara et al., 2009; Song et al., 2012). Under LDs, activation of FLOWERING LOCUS T (FT) expression is principally controlled by CO protein levels, which are stabilised by FKF1 at the end of the long photoperiod (Suárez-López et al., 2001; Song et al., 2012). Furthermore, CO protein is regulated by light such that CO levels are low in red light, while blue and far-red light stabilise CO (Valverde et al., 2004). This control reinforces the accumulation of CO protein levels in the evening of LDs, leading to an increase in the floral signal. Conceptually, these molecular interactions result in a double external coincidence mechanism involving multiple clock outputs, but the combined effects of this rhythmic mechanism have not been tested quantitatively or incorporated into previous mathematical models (Song et al., 2012).

Similarly, photoperiodic elongation growth is controlled by clock- and light-regulated processes (Nozue et al., 2007; Niwa et al., 2009; Kunihiro et al., 2011). The circadian clock regulates the transcription of morning-expressed genes PHYTOCHROME-INTERACTING FACTORS 4 and 5 (PIF4, PIF5) through repression by the EC (Nozue et al., 2007; Nusinow et al., 2011). The repressing PIF proteins control gene expression by forming homo- and heterodimers that bind to G- and E-box motifs in targeted promoters (Hornitschek et al., 2009, 2012; Zhang et al., 2013). During the day, PIF activity is thought to be compromised due to interactions with phyochrome B (phyB), the key red light photoreceptor, resulting in rapid PIF phosphorylation and degradation (Park et al., 2004, 2012; Al-Sady et al., 2006; Nozue et al., 2007; Jang et al., 2010). This is proposed to restrict PIF activity to the end of the night in SDs, coinciding with the time of maximal hypocotyl growth rate (Nozue et al., 2007, 2011; Michael et al., 2008a), a hypothesis that we re-examine here. Several other light-regulated proteins also repress PIF signalling, including DELLAS, PHY RAPIDLY REGULATED 1 (PAR1), LONG HYPOCOTYL IN FAR-RED 1 (HFR1) and ELONGATED HYPOCOTYL 5 (HY5) (de Lucas et al., 2008; Foreman et al., 2011; Hao et al., 2012; Chen et al., 2013). Among the known targets of PIF4 and PIF5 are INDOLE-3-ACETIC ACID INDUCIBLE 29 (IAA29) and ARABIDOPSIS THALIANA HOMEBOX 2 (ATHB2) (Kunihiro et al., 2011), genes which are involved in auxin signalling. Thus, PIF4 and PIF5 appear to regulate hypocotyl elongation through auxin signalling (Kunihiro et al., 2011; Nozue et al., 2011; Hornitschek et al., 2012). While a number of the key molecular interactions in this pathway have been experimentally characterised, a model of the dynamic regulation of PIF activity in light:dark (L:D) cycles has not previously been developed.

PIF4 and, to a lesser extent, PIF5 promote hypocotyl elongation in response to warm ambient temperatures (27°C; Koini et al., 2009; Stavang et al., 2009). Increased temperature leads to higher PIF4 transcript and protein levels and longer hypocotyls (Koini et al., 2009; Stavang et al., 2009; Foreman et al., 2011; Nomoto et al., 2012a; Yamashino et al., 2013). Recently, PIF4 has also been implicated in the warm temperature-induced acceleration of flowering in SDs by binding to the FT promoter independently of the Co-FT photoperiodic pathway (Kumar et al., 2012). Other temperature-sensitive regulators of FT have recently been identified that are not thought to be part of the photoperiodic response (Lee et al., 2013; Posé et al., 2013). Once again, while a number of regulators have been identified in this pathway, their combined effects have not previously been described in a mathematical model.

In this study, we have constructed a mathematical model to integrate and reconcile the complex molecular mechanisms in the photoperiodic pathways of flowering and hypocotyl elongation in Arabidopsis. In the flowering pathway, we extended our previous model, which was built to determine how FKF1 protein regulates levels of FT mRNA through CDF1 and CO protein interactions (Song et al., 2012). The updated model was able to match CO and FT mRNA rhythmic expression data in different photoperiods and in mutants of the flowering pathway (e.g. fkl1, gi, cdf1, CO-ox, CO-ox; fkl1, CO-ox; C; Koini et al., 2011; Nomoto et al., 2014). However, this model required FKF1 protein and CDF1 mRNA timeseries data to be input into the model, meaning that simulation of multiple photoperiods and mutants would require the generation of multiple input data sets (Fig 1, top inset; Song et al., 2012). To improve this aspect of the model, we wished to incorporate circadian regulation of CDF1 and FKF1 mRNA, removing data inputs to the model (Fig 1, bottom inset). This modification improved the predictive power of the model and allowed us to investigate how changes in clock dynamics affect components of the flowering pathway in clock mutants and different photoperiods. By postulating and experimentally validating circadian regulators of CDF1 and FKF1 transcription, the model recapitulates the acceleration of flowering in LDs.

In the hypocotyl elongation pathway, we demonstrate that known transcriptional and post-transcriptional regulation of PIF explain phenotypes and PIF target transcript dynamics under a variety of environmental and genetic manipulations. We then use microarray data to identify other transcripts that have similar dynamics and that are therefore likely to be under the control of PIFs in light:dark cycles. Finally, we explore crosstalk between the flowering and hypocotyl pathways by simulating PIF regulation of FT mRNA, in order to test the hypothesis that temperature regulates flowering independently of CO. The results highlight the complexity of the network structure underlying circadian-, light- and temperature-regulated processes.
Results

Refining the photoperiodic flowering model

We determined potential mechanisms by which the circadian clock might regulate _FKF1_ and _CDF1_ mRNA by inspection of published data sets (Mizoguchi et al., 2005; Niwa et al., 2007; Ito et al., 2008; Edwards et al., 2010). From these, we observed that _FKF1_ mRNA peaks at a similar phase to _GI_ transcription across multiple photoperiods, while both respond in a similar manner to perturbations of the circadian clock. Under 10L:14D and 16L:8D cycles, the peak of _FKF1_ expression at ZT9–10 matches that of _GI_ (ZT = zeitgeber time, where dawn in an L:D cycle is at ZT0; note: throughout, we will refer to 8L:16D and 10L:14D as short-day (SD) conditions, and 16L:8D as long-day (LD) conditions). Both _FKF1_ and _GI_ expression have an earlier peak phase in _cca1;lhy_ mutants, while they exhibit only minor phase changes in _prr_ mutants (Imaizumi et al., 2003; Niwa et al., 2007; Ito et al., 2008). Furthermore, _FKF1_ transcription, like that of _GI_, is acutely stimulated by red light (Tepperman et al., 2004; Locke et al., 2005). Based on this evidence, we modelled _FKF1_ transcription similar to _GI_: to be inhibited by CCA1/LHY proteins and the EC, and acutely activated by light (see Computational Methods in Supplementary Information; Figs 1 and 2A). This model is additionally supported by recent work highlighting similarities between the promoter sequences of _FKF1_ and _GI_ (Berns et al., 2014).

In deciphering how _CDF1_ transcription is regulated by the clock, we refer to 8L:16D and 10L:14D as short-day (SD) conditions, and 16L:8D as long-day (LD) conditions. Both _FKF1_ and _GI_ expression have an earlier peak phase in _cca1;lhy_ mutants, while they exhibit only minor phase changes in _prr_ mutants (Imaizumi et al., 2003; Niwa et al., 2007; Ito et al., 2008). Furthermore, _FKF1_ transcription, like that of _GI_, is acutely stimulated by red light (Tepperman et al., 2004; Locke et al., 2005). Based on this evidence, we modelled _FKF1_ transcription similar to _GI_: to be inhibited by CCA1/LHY proteins and the EC, and acutely activated by light (see Computational Methods in Supplementary Information; Figs 1 and 2A). This model is additionally supported by recent work highlighting similarities between the promoter sequences of _FKF1_ and _GI_ (Berns et al., 2014).

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noted that previous reports have shown that CDF1 mRNA levels are strongly regulated by the transcription-repressing PRR protein family (Nakamichi et al., 2007, 2012; Niwa et al., 2007; Ito et al., 2008; Huang et al., 2012). Mutations of the PRRs (e.g. the prr9;7 double mutant in Fig 2E) lead to elevated daytime expression of CDF1 (Nakamichi et al., 2007; Ito et al., 2008). However, if CDF1 mRNA was solely regulated by the PRRs, we would predict an increase in CDF1 expression at dawn in cca1;Ihy double mutants, since PRR levels are low in this mutant (Dixon et al., 2011). Instead, the cca1;Ihy double mutant has an advanced phase of CDF1 expression, with decreased expression at dawn in both SDs and LDs (Fig 2C, Supplementary Figs S1A and S2A; Nakamichi et al., 2007; Niwa et al., 2007). The simplest explanation for this difference between predicted and observed rhythms of CDF1 mRNA in cca1;Ihy is that CCA1/LHY proteins play a role in activating CDF1 expression alongside repression by the PRR proteins. By incorporating both regulatory features, the model qualitatively matched the peak of CDF1 mRNA expression at dawn in the WT. The model can also describe CDF1 transcript profiles in the prr9;7 and cca1;Ihy double mutants (Fig 2E and G; Supplementary Figs S1D and S2D), indicating that

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Modelling the circadian regulation of CDF1 and FKF1 mRNA.

A Schematic of proposed circadian regulators of FKF1 and CDF2 transcription.

B Experimental validation for CCA1 regulation of the flowering pathway. ChIP data showing CCA1 enrichment in regions containing CCA1 elements (CBS or EE; white bars) in GI, FKF1 and CDF1 promoters, compared to regions of their respective exons (dark bars). Locations of primers for GI (GI-a, GI-N), FKF1 (FKF1-a, FKF1-N) and CDF1 (CDF1-a, CDF1-N) are shown in Supplementary Fig S3. Seedlings were grown for 14 days 12L:12D cycles at 22°C and harvested at ZT2. Statistical analysis performed using Welch tests, *P < 0.005. Error bars represent standard error of technical replicates.

C, D CDF1 mRNA in LDs (C) and FKF1 mRNA in SDs (D), from WT (data: black lines, filled squares; simulation: black lines), and the cca1;lhy mutant (data: green lines, open circles; simulation: dashed green lines) (data sets used for parameter optimisation).

E, F CDF1 (E) and FKF1 (F) mRNA data in LDs, from WT (as in C, D) and the prr9;7 mutant (blue-green line, open circles).

G, H CDF1 (G) and FKF1 (H) mRNA simulations in LDs, from WT (as in C, D) and the prr9;7 mutant (dashed blue-green line).

this combination of regulatory mechanisms is sufficient to explain
the observed transcript profiles.

Our model proposes that the morning component CCA1/LHY
regulates both CDF1 and FKF1 transcription. These new hypotheses
were tested experimentally with chromatin immunoprecipitation
(ChIP) experiments using pCCA1:CCA1-HA-YFP (Fig 2B and Supple-
mental Fig S3; Yakir et al, 2009). Promoter sequences containing
the CCA1-binding sites (CBS = AAAATCT; Wang et al, 1997) and
evening elements (EE = AATATCT; Michael & McClung, 2002)
within 3 kb of the transcription start sites of CDF1 and FKF1 were
enriched in the pCCA1:CCA1-HA-YFP ChIP (Fig 2B and Supple-
mental Fig S3; Supplementary Dataset S1). These data, therefore,
suggest that CDF1 and FKF1 are directly regulated by CCA1.

To further validate the models of CDF1 and FKF1 mRNA regula-
tion, we compared simulations of CDF1 and FKF1 transcription to
data sets that were not used for model optimisation (Fig 2E-H). Figure 2G shows that the mean level of simulated CDF1 mRNA is
increased in prr9;7 double mutants in LDs, while FKF1 mRNA has a
lower amplitude and delayed phase (Fig 2H), both of which qualita-
tively match the data (Figs 2E and F). CDF1 mis-regulation ends
earlier in the night in the data but persists to the late night in the
model, suggesting that additional modes of CDF1 regulation may
exist. As the models were constructed and parameterised using data
from WT and cca1;lhy lines (see Computational Methods in Supple-
mental Information; Fig 2C and D), the qualitative match to the
prr9;7 double mutant validates our simple assumptions for circadian
regulation of CDF1 and FKF1 mRNA. Thus, the model captures the
main features of regulation of CDF1 and FKF1 by the circadian clock,
and their model-predicted regulation by CCA1/LHY was experimen-
tally supported. We next examined the downstream regulation of
the CDF1 and FKF1 target genes CO and FT.

Model of transcriptional regulation of CO and FT mRNA suggests
novel roles for circadian clock components

Previous studies have highlighted that CDF1 protein turnover is
regulated by the blue light-dependent GI-FKF1 protein complex
(Imaizumi et al, 2005; Sawa et al, 2007). Consistent with this
notion, when the fkf1 mutation is introduced into lines expressing
CDF1 transcript, either constitutively or under the control of the
CDF1 promoter, CDF1 protein is degraded at a slower rate than WT,
resulting in rhythmic CDF1 with reduced amplitude and higher
mean levels (Fig 3B; Supplementary Fig S4A; Imaizumi et al, 2005).
However, CDF1 protein levels are still rhythmic in the CDF1-ox;fkf1
plants, suggesting that CDF1 turnover is also regulated by the circa-
dian clock independently of FKF1. As GI acts in protein complexes
with other members of the FKF1 protein family, notably ZTL in the
circadian clock system (Kim et al, 2007), we wanted to determine
how the absence of GI function altered CDF1 levels. To study the
effect of the gi mutation on the post-transcriptional regulation of
CDF1 protein, we used a constitutive CDF1 overexpressor line that
carries the gi-2 mutation (35S:HA-CDF1-gi-2). CDF1-ox and CDF1-ox;
<gi-2 plants were grown for 13 days in 16L:8D cycles and harvested
at 3-h intervals, and CDF1 protein levels were measured by immu-
noblotting. Our data show that the gi-2 mutant had high mean levels
of CDF1 protein, similar to fkf1 mutants, but in gi, the level did not
vary significantly among time points (Fig 3C; Supplementary Data-
set S2; Imaizumi et al, 2005; Sawa et al, 2007). However, in the

case of the fkf1 mutant, a residual shallow rhythm in CDF1 protein
levels was observed (Fig 3B), suggesting that GI may play an addi-
tional role in regulating CDF1. As CDF1 is a key negative regulator
of CO mRNA, high mean level of CDF1 protein in the gi and fkf1
mutants leads to low expression levels of CO mRNA in these
mutants (Fig 3D; Suárez-López et al, 2001; Sawa et al, 2007;
Fornara et al, 2009). However, CO transcript is lower in the gi
mutant than in the fkf1 mutant (Fig 3D), providing further support
for an FKF1-independent role for GI in the regulation of CDF1
protein. The inclusion of FKF1-dependent and FKF1-independent
effects of GI on CDF1 protein stability in the model are sufficient to
explain the lower CO transcript levels observed in the gi mutant,
as compared to the fkf1 mutant (Fig 3D), and the low FT levels were
seen in LDs in both mutants (Supplementary Figs S5 and S6).

Having connected the clock model to a model of CO/FT regula-
tion, we then compared model simulations and data for CO and FT
mRNA from plants with mutations in clock genes. In the case of
the prr9;prr7 and cca1-ox mutants, model simulations matched
data showing reduced levels of CO and FT mRNA throughout the
day (Fig 3F and G; Supplementary Fig S7; data not used for
parameter optimisation). In both cases, the simulated high level
of CDF1 mRNA and low level of FKF1 mRNA (e.g. Fig 2E-H for the
case of the prr9;prr7 mutant) result in low levels of CO and FT
mRNA throughout the day, matching experimental data (Fig 3F
and G; Supplementary Fig S7). In the case of the elf3 mutant,
model simulations matched the increase in FT expression in both
SDs and LDs despite overestimating the increase in CO mRNA
during the day (Lu et al, 2012; Supplementary Fig S7; data not
used for parameter optimisation). In this case, this is explained by
the simulated low level of CDF1 mRNA and high level of FKF1
mRNA in this mutant.

In contrast to the above cases, the model is unable to fully
describe the dynamics of CO and FT mRNA in the cca1;lhy double
mutant [measured in the same experiments (Nakamichi et al,
2007)]. Simulations of CDF1 and FKF1 mRNA match the data for
the double mutant, as described above. However, the predicted tran-
script profiles of CO and FT depart qualitatively from the data at
ZT12-16 (Fig 3H and I; Supplementary Figs S1 and S2). The simula-
tions correctly show a 3- to 6-h advanced phase of CO and FT
expression, and the increase in FT levels with respect to the WT
is sufficient to explain the early-flowering phenotype of the cca1;lhy
mutant in SDs. However, the model underestimates the peak levels
of CO and FT mRNA observed at ZT12-16, especially in LDs (Fig 3H
and I, Supplementary Figs S1 and S2). Thus, CCA1 and LHY may
also regulate CO and FT transcription by another mechanism in
parallel to or downstream of CDF1 mRNA (see Model Behaviour in
Supplementary Information). This regulation might be direct
or might result from a phase shift in the expression of other clock-
regulated components.

Description of flowering mutants is maintained with new
connections to the circadian clock

Our previous flowering time model was able to qualitatively
describe several mutants specific to the flowering pathway. With
the new circadian regulation of CDF1 and FKF1 mRNA and CDF1
protein, our extended model also matches FT mRNA in fkf1 mutants
as well as in CO-ox;fkf1 and CO-ox;CDF1-ox lines (Supplementary
Furthermore, the model retains the important feature of the previous model showing that the FKF1 protein has a larger effect on FT mRNA through its regulation of CO protein than through degradation of CDF1 protein (Supplementary Fig S8B; Song et al, 2012). Thus, the present model is consistent with past results as well as additional molecular and genetic data, providing a suitable basis for further extension. In particular, we extended the model to consider the combined circadian and light regulation of PIF4 and PIF5 activity, allowing us to investigate the regulation of rhythmic growth by PIF4 and PIF5 and to understand crosstalk between PIF4, PIF5 and CO in the regulation of FT.

**Modelling the circadian regulation of PIF4 and PIF5 mRNA**

Hypocotyl elongation, like flowering time, is subject to photoperiodic regulation. In contrast to the FKF1-CO-FT pathway, which is active in LDs, PIF4-induced and PIF5-induced hypocotyl extension is observed in SDs (Niwa et al, 2009; Kunihiro et al, 2011). Here,
we describe the development of a model describing the photoperiodic induction of hypocotyl elongation through PIF4 and PIF5. As a first step, we constructed a model of PIF4 and PIF5 transcription, which is known to be controlled by the circadian clock (Yamashino et al., 2003). This regulation has been shown to involve direct inhibition of transcription by the EC (Fig 4A; Nozue et al., 2007; Nusinow et al., 2011). In order to test whether this regulation is sufficient to explain observed patterns of PIF4 and PIF5 expression, we started by constructing a model in which the EC is the sole regulator of PIF4 and PIF5 transcription. This model of PIF4 and PIF5 regulation captures important properties of mRNA profiles from WT, elf3 and prr9;7;5 backgrounds (Fig 4B and E; Supplementary Figs S9A and D and S10A and D). In an elf3 background, the level of PIF4 transcripts is increased throughout the night (Nomoto et al., 2012b); this behaviour is matched by simulations (Supplementary Fig S9A and D). Similar behaviour is seen in the case of the prr9;7;5 mutant in data and simulations (Supplementary Fig S10A and D; Nomoto et al., 2012b). Additionally, it should be noted that model simulations do not reproduce the steady increase in PIF4 expression observed during

This model of PIF4 and PIF5 regulation captures important properties of mRNA profiles from WT, elf3 and prr9;7;5 backgrounds (Fig 4B and E; Supplementary Figs S9A and D and S10A and D). In an elf3 background, the level of PIF4 transcripts is increased throughout the night (Nomoto et al., 2012b); this behaviour is matched by simulations (Supplementary Fig S9A and D). Similar behaviour is seen in the case of the prr9;7;5 mutant in data and simulations (Supplementary Fig S10A and D; Nomoto et al., 2012b). Additionally, it should be noted that model simulations do not reproduce the steady increase in PIF4 expression observed during

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**Figure 4. Photoperiodic regulation of PIF activity.**

A Schematic of transcriptional and post-transcriptional regulation of PIF activity.
B-D PIF4, IAA29 and ATHB2 mRNA levels in SDs in WT (data: black lines, filled squares; simulation: black lines).
E-G As in (B–D), for LDs. Data from Nomoto et al. (2012b).
H ATHB2 mRNA at dawn (ZT 0) across a range of photoperiods. Data from Kunihiro et al. (2011).
I–L Comparison of model simulations with microarray time course data from the two largest clusters of PIF-induced targets (Cluster 1: 107 genes, Cluster 2: 84 genes), in SDs (I, K) and LDs (J, L) [data from Michael et al. (2008b)].

Data information: Error bars represent standard deviation.
the night in both the elf3 and prr9;7;5 mutants (Supplementary Figs S9A and B and S10A and B). Rather, model simulations in both cases predict a constant high level of PIF4 transcript. Residual rhythms of PIF4 and PIF5 in the elf3 mutant suggested a role for additional circadian regulators of PIF4 and PIF5 transcription. In particular, a small daytime peak in expression of PIF4 and PIF5 in the elf3 mutant has been observed in multiple experiments (Lu et al., 2012; Nomoto et al., 2012b), and EE motifs are present in the PIF4 and PIF5 promoters. This suggested a possible role for CCA1 and LHY in activating PIF4 and PIF5 expression. However, our CCA1 ChIP experiments did not detect binding of CCA1 to the PIF4 and PIF5 promoters (Supplementary Fig S3). We also note that the observed dynamics of PIF4 and PIF5 mRNA required the simulated activity of the EC to be advanced by ~2 h, providing a closer match to previously published data sets of EC dynamics (see Computational Methods and Model Behaviour in Supplementary Information; Nusinow et al., 2011). The need for further information on the EC was previously highlighted during the development of the circadian clock model (Pokhilko et al., 2012).

Complex dynamics of PIF proteins predict bimodal control of target genes

Our next aim was to extend the model to incorporate post-transcriptional PIF regulation and the PIF target genes ATTHB2 and IAA29, whose expression correlates with hypocotyl elongation (Kunihito et al., 2011; Nomoto et al., 2012a,b). We therefore introduced the regulation of PIF protein levels by active phyB, allowing us to simulate PIF degradation during the day (Supplementary Fig S11). In addition, we modelled inhibition of PIF activity by an “Interactor” class of proteins, representing PIF-binding proteins that are stimulated by light and that have been shown to inhibit PIF activity. This large and varied group includes DELLAs, PAR1, HFR1, H5, PIL1 and phyB (Fig 4A; Osterlund et al., 2000; Achard et al., 2007; de Lucas et al., 2008; Hornitschek et al., 2009; Foreman et al., 2011; Hao et al., 2012; Bai et al., 2012; Oh et al., 2012; Chen et al., 2013; Luo et al., 2014). Active PIF proteins promote the expression of ATTHB2 and IAA29, while the “Interactor” proteins inhibit PIF activity for these targets. Due to increased synthesis of the “Interactor” proteins during the light period, the inhibition of PIF proteins acts in tandem with phyB-dependent degradation to suppress PIF activity during the day (Supplementary Fig S11A–C).

Combining the regulation of PIF protein activity by light with circadian regulation of PIF4 and PIF5 transcription was sufficient to describe the observed photoperiodic response of ATTHB2 and IAA29 mRNA accumulation (Fig 4C, D and F–H). In SDs, a high peak in expression at dawn is observed in both targets in both data and simulations. Additionally, the model matched the increase in IAA29 transcript levels throughout the night in SDs (Fig 4C). In the model, this behaviour is the result of high PIF4 and PIF5 transcript levels coinciding with darkness at ZT8-12 in SDs, resulting in an SD-specific increase in PIF activity at this time and a peak in target gene expression at ZT14. Analysis of model dynamics under random parameter perturbations confirmed that this behaviour is observed across a broad range of parameter values (Supplementary Fig S12; see Model Behaviour in Supplementary Information). The physiological significance of this secondary peak is suggested by the observation that 8 out of 11 putative PIF target genes inspected in Nomoto et al. (2012b) displayed this post-dusk increase in a SD-specific manner and that a similar secondary peak in the rate of hypocotyl elongation has been observed in SDs (Nozue et al., 2007, 2011).

Recent experiments with dark-grown seedlings have identified an expansive transcriptional network downstream of the PIFs (Zhang et al., 2013), with 699 genes identified by RNA-Seq as having decreased transcript levels in pif1;4;5 mutants, while 755 genes were identified as having increased levels in pif1;4;5 mutants. We refer to these sets as PIF-induced and PIF-repressed, respectively. In order to evaluate whether our model of PIF activity could describe the dynamics of PIF targets other than ATTHB2 and IAA29 in light-dark cycles, we used microarray timeseries data available from the DIURNAL database (Mockler et al., 2007). This database includes microarray data sampled across 2 days at 4-h time resolution in diverse conditions and has previously been used to assess interactions between circadian and light signalling (Dalchau et al., 2010). Clustering of transcript dynamics for genes identified as PIF-induced revealed two large and coherent clusters of genes; genes within each cluster shared condition-specific transcript dynamics across 6 conditions, including SDs and LDs (see Supplementary Information for details of analysis; Frey & Dueck, 2007). These genes comprised 191 of the 699 PIF-induced genes, including the known examples of ATTHB2 and IAA29, and showed significant overlap with PIF4-bound and PIF5-bound genes, relative to all PIF-induced genes (P < 10−8, Supplementary Fig S13A). Two additional PIF target species were introduced into the model to represent these two clusters. Their dynamics could be matched in a straightforward way by fitting only five PIF target-specific parameters, as shown in Fig 4L–J, for the comparison of SDs to LDs (microarray data from Michael et al., 2008b). Many of these genes also showed the SD-specific bimodal profile.

The generality of this model of PIF target regulation was further tested by considering genetic perturbations. Two classes of genetic perturbation are of particular interest—mutants with a defective EC (i.e. with the clock regulator of PIF transcription removed) and mutants with a defective circadian clock. In all cases, the model matched available data sets. In the first class are elf3 and lux mutants (Nusinow et al., 2011) where qPCR time course data are available for PIF4, ATTHB2 and IAA29 transcripts in an elf3 mutant (Nomoto et al., 2012b; Supplementary Fig S9A–C) and microarray time course data are available in a lux mutant (Michael et al., 2008a; Supplementary Fig S9G–J). In the second class are the prr9;prr7;prr5 triple mutant and LHY overexpression [LHYox, also referred to as lhy1 (Schaffer et al., 1998)]. For this class, qPCR time course data are available for PIF4, ATTHB2 and IAA29 transcripts in a prr9;prr7; prr5 mutant (Nomoto et al., 2012b; Supplementary Fig S10A–C), and microarray time course data are available in the LHYox mutant (Michael et al., 2008a; Supplementary Fig S10D–G). The consistency of model simulations with experimental data for the identified PIF-induced transcripts under diverse perturbations suggests that PIF4 and PIF5 are the dominant regulators of these transcripts in light-dark cycles.

We next considered how transcripts identified as being repressed by PIFs might be regulated in light-dark cycles. Therefore, we clustered these transcripts according to the similarity of their dynamics in the microarray data, identifying four clusters with consistent dynamics across six conditions, including a total of...
209 of the 755 PIF-repressed genes (Supplementary Fig S13B; see Supplementary Information for details of analysis). The dynamics of these transcripts revealed the expected photoperiod effect, with high levels during the light period and reduced levels during the dark period (Supplementary Fig S14). As with the PIF-induced genes, the model captured the change in dynamics between SDs and LDs. However, in contrast to the case for PIF-induced genes, the model was not able to match some changes in transcript dynamics under genetic perturbations, especially in the case of the LHY国内外 mutant (Supplementary Fig S15). This might reflect a role for other factors in the mechanism by which PIFs repress transcription.

In summary, we have constructed a model of PIF activity that is able to describe the dynamics of PIF-induced transcripts across photoperiods and in clock mutants. Analysis of microarray data allowed identification of 191 PIF-induced and 209 PIF-repressed transcripts with dynamics that are consistent with a model of PIF regulation across multiple conditions (Supplementary Tables S1 and S2). Thus, the modelled PIF dynamics are sufficient to coordinate the photoperiod response of plants far beyond the particular target genes considered previously.

**Modelling illustrates that PIF activity is not confined to the end of night**

The model outlined above was created to describe the regulation of *ATHB2* and *IAA29* mRNA by PIF proteins. *ATHB2* and *IAA29* transcript levels were previously shown to rise towards the end of night in SDs, suggesting that PIF activity is highest at this time (Nomoto et al., 2012b). Model simulations not only suggested that PIF4 and PIF5 protein levels rise during the night in SDs, as described above, but also predicted significant amounts (~50% of the peak level in SDs) of PIF protein during the day in all photoperiods (Supplementary Fig S11C).

In the model, the simulated daytime PIF protein levels result from the increase in *PIF4* and *PIF5* transcript during the day (peaking at ~ZT2-6), which counteracts phyB-mediated PIF protein degradation during the light period. This model prediction was somewhat unexpected, as constitutively expressed PIF protein is strongly depleted by phyB (Nouze et al., 2007; Kumar et al., 2012; Lee & Thomashow, 2012). However, recent analysis of PIF4 protein levels under the control of its native promoter support this possibility (Yamashino et al., 2013; Bernardo-García et al., 2014). This suggests that in light:dark cycles, the turnover of PIF proteins by active phyB is not required for the observed diurnal dynamics of PIF targets. Indeed, it is known that other light-activated pathways act redundantly to repress PIF activity during the day, as represented in the model by the “Interactor” class of proteins.

While it is clear that PIF activity is strongly repressed during the day, reductions in PIF-induced target expression have been observed in *pix* mutants during the day and in constant light (Nomoto et al., 2012a,b; Koini et al., 2009; Sun et al., 2012). This suggests that PIF proteins may not be completely degraded in the light and therefore may retain some residual activity. In order to test this idea further, we inspected the dynamics of the clusters of PIF targets (as identified previously, see above; Supplementary Information) in constant light (LL) conditions under clock-entraining temperature cycles. Clear rhythms of PIF targets in these conditions are observable, with PIF-induced and PIF-repressed transcripts in phase and antiphase, respectively, with the phase of the *PIF4* and *PIF5* transcript rhythms (Supplementary Fig S16).

If the degradation of PIF proteins during the day is not required for the observed dynamics of PIF activity in light:dark cycles, then removal of phyB should not affect the dynamics of PIF targets in these conditions. We assessed this possibility by inspecting the dynamics of the clusters of PIF targets (as identified previously, see above) in the phyB mutant (Supplementary Fig S17). The qualitative dynamics of both PIF-induced and PIF-repressed targets are unchanged in the phyB mutant, with a rapid increase and decrease in PIF-induced and PIF-repressed transcripts during the day, respectively. This is consistent with previous observations of several canonical PIF-induced transcripts, including *ATHB2* and *IAA29* (Nomoto et al., 2012a,b; Yamashino et al., 2013), demonstrating that phyB acts redundantly with other light-signalling components to repress PIF activity in these conditions.

Interestingly, a significant increase in PIF-induced transcript levels is observed during the night in the phyB mutant (Supplementary Fig S17A). This may explain the long hypocotyl phenotype of this mutant in light:dark cycles despite phyB’s apparent redundancy in repressing PIF activity during the day and suggests that phyB regulates PIF activity during the night through a separate mechanism.

In conclusion, the model reconciled apparently conflicting observations of PIF activity during the day in wild-type plants, with the rapid, phyB-dependent degradation of constitutively expressed PIF proteins. The presence of PIF proteins during the day suggests that they might play a regulatory role during this time, a possibility which is further highlighted by our observations of PIF-dependent *FT* expression (see below).

**Linking molecular regulation to flowering time and hypocotyl elongation**

*ATHB2* expression provides a molecular correlate of hypocotyl elongation across multiple conditions (Kunihiro et al., 2011), in a similar manner to *FT* expression in the flowering pathway (Salazar et al., 2009). The model simulates the nonlinear changes in average *ATHB2* mRNA and *FT* mRNA levels across photoperiods (Fig 5A and B). The absolute values of hypocotyl length and flowering time vary among laboratories, so we used simple mathematical functions to relate *FT* and *ATHB2* mRNA levels to flowering and hypocotyl elongation. These represent the complex developmental mechanisms of the vegetative-to-inflorescence transition and the biophysics of elongation growth and can readily be recalibrated for the conditions of particular studies (as described in Supplementary Information). The full model can thereby simulate photoperiod responses for these two phenotypes in wild-type plants (Fig 5C and D; Corbesier et al., 1996; Niwa et al., 2009; Kunihiro et al., 2011). As the model fully couples the circadian clock to both output pathways, it also simulates both phenotypes in clock-mutant lines. The dynamic regulation described above, for example, naturally matched the delayed flowering and long hypocotyls in *prr9*;7 mutants (Supplementary Table S3). However, the changing seasons are also accompanied by changes in ambient temperature, which modifies the expression of both phenotypes.
PIF-mediated temperature control of hypocotyl elongation

Warmer temperatures result in earlier flowering and longer hypocotyls (Gray et al., 1998; Balasubramanian et al., 2006). It has been suggested that PIF4 plays a role in the temperature sensitivity of hypocotyl growth through stimulation of ATHB2, IAA29 and other hormone-related genes (Koini et al., 2009; Nomoto et al., 2012a) and in the temperature sensitivity of flowering through stimulation of FT (Kumar et al., 2012). The present model allowed us to assess how PIF4 may achieve this combined regulation (Fig 6). Recently, it has been shown that EC repression of PIF4 expression is relieved at higher temperatures (e.g. 28°C versus 22°C), leading to higher PIF4 levels during the night in these conditions. The model reproduced these observations through mild temperature modulation of the affinity of the EC for the PIF4 promoter, resulting in less EC repression of PIF4 expression at the higher temperature (Fig 6B–E). Altered affinity is sufficient to prevent full repression of PIF4 mRNA in the early night and to allow a 2–3 h earlier rise of PIF4 before dawn at 28°C; thus, warm temperature in LDs leads to night-time levels of PIF4 mRNA that are similar to the level in SDs at 22°C. In both model and data, this results in 3- to 4-fold higher accumulation of ATHB2 at dawn, qualitatively consistent with the increased hypocotyl elongation observed at the higher temperature (Nomoto et al., 2012b).

PIF-dependent control of the flowering regulators

Extending PIF-dependent regulation to FT in the model highlighted two areas that contrast with the relatively simple link from EC activity to hypocotyl elongation. We first tested whether FT displayed a PIF-dependent change in dynamics, similar to that seen in IAA29 and ATHB2 at the higher temperature in LDs. Wild-type and pif4;pif5 double mutant plants were grown for 13 days in 16L:8D cycles at 22 and 27°C and harvested at 4-h intervals (with additional time points around dawn and dusk), and CO and FT RNA levels were measured by qRT–PCR. The CO mRNA profile was unaffected by the pif mutations (Fig 6F). Moreover, no PIF-dependent peak in FT expression at dawn was observed at either 22 or 27°C in LDs (Fig 6H). Instead, PIF4 and PIF5 stimulated FT expression in the wild-type plants at ZT8-12. Interestingly, the double mutants showed lower expression at both temperatures. The absence of FT induction at dawn is consistent with results from the elf3 mutant, in which PIF4 and PIF5

Figure 5. Model describing the photoperiod response of flowering and hypocotyl elongation in WT plants.
A, B Simulated levels of FT and ATHB2 mRNA (as calculated by taking the area under the curve) across multiple photoperiods.
C Using FTAREA values, the number of days to flower was calculated and compared to data from Corbesier et al (1996) (see Supplementary Information). Error bars represent standard deviation. Simulated flowering: solid black line, filled squares; data: dashed black lines, empty squares.
D Same as in (C) using ATHB2AREA to calculate hypocotyl length. Data taken from Kunihiro et al (2011) (error bars were unavailable).
transcript levels during the night are also increased (Nusinow et al., 2011; Lu et al., 2012; Nomoto et al., 2012b) without a corresponding increase in FT expression at this time (Kim et al., 2005; Lu et al., 2012). This contrasts with the dynamics of the canonical PIF targets such as ATHB2 and suggests that PIF activity at FT is temporally modulated by other factors. Since changes in PIF activity during the night do not affect FT expression at either temperature (Fig 6H), co-activation of FT by PIFs and a light-dependent factor is one possible mechanism for this modulation. A candidate for such a light-dependent factor is CO, as ChIP analysis has shown that PIF4 and CO bind to the FT promoter in overlapping regions (compare locations of FT-c1/c/15 from Kumar et al., 2012 with amplicons 12 to 14 from Song et al., 2012). If PIF4 and CO assemble at the FT promoter, then they are likely to interact when both are present. Alternatively, other light-dependent activators of FT have been also identified [e.g. the CRY2-interacting bHLH (CIB) family of transcription factors (Liu et al., 2013)]. Light-dependent regulation of PIF activity at the FT promoter was sufficient for the model to recapitulate the observed, phase-dependent effect of the PIFs on FT at the reference temperature, 22°C (Fig 6I).
The contribution of PIFs to temperature-induced FT expression

Having accounted for the temporal distinction between the effects of PIFs on FT and on canonical targets, we next tested whether increased PIF protein levels at 27°C would explain the temperature response of FT, as it had for the canonical target genes. Our transcript data showed a 2- to 3-fold increase in FT expression levels at 27°C (Fig 6H), consistent with the early flowering of wild-type plants at this temperature. The strongest effects were at ZT8-16. The pif4; pif5 mutant clearly retained temperature sensitivity of FT expression in LDs (Fig 6H; Supplementary Dataset S5). Our data collected in SDs also showed temperature sensitivity of FT expression in the pif4 mutant (Supplementary Fig S18; Supplementary Dataset S6). This is consistent with previous reports (Kumar et al., 2012; Thines et al., 2014), though we did not detect a reduction in peak FT levels in the pif4 mutant. This suggests that additional, temperature-sensitive regulators of FT play a role in these conditions.

As the time of the greatest temperature response in FT (ZT8-16) coincides with when CO protein is active, we reasoned that temperature regulation of CO activity might explain these effects. Measured CO transcript levels did not change with temperature (Fig 6F; Supplementary Dataset S3) [as observed previously, (Kumar et al., 2012; Thines et al., 2014)], so we measured HA-tagged CO protein in transgenic lines that expressed this transgene from the CO promoter. No difference in CO protein levels was observed between the two temperatures (Fig 6G; Supplementary Dataset S4).

Recently, it has been shown that FLOWERING LOCUS M (FLM) and SHORT VEGETATIVE PHASE (SVP) are involved in mediating the temperature sensitivity of FT expression in the temperature range 5–27°C, and in both SDs and LDs (Lee et al., 2013; Pose et al., 2013). However, no single, dominant component was identified, as mutants in each of these factors retain some temperature sensitivity (Lee et al., 2013). The action of these regulators can be modelled by introducing a uniform activation of FT expression at 27°C, leaving the model behaviour at 22°C unchanged. With this simple assumption, the model is able to reproduce the observed change in FT dynamics at 27°C (Fig 6I).

Thus, PIF4 plays qualitatively distinct roles in the transcriptional regulation of ATHB2 and FT. An external coincidence model successfully describes the response of ATHB2 to photoperiods and accommodates known transcriptional regulation of PIF4 to describe its response to temperature. In contrast, the effects of PIF4 on FT are limited to the daytime and are mediated by a mechanism that is apparently independent of transcriptional regulation of PIF4.

Discussion

Linking the circadian clock model to two well-characterised output pathways has accomplished three goals. First, in the photoperiodic CO-FT pathway, we proposed circadian mechanisms to regulate central components CDF1 and FKF1 (Figs 1 and 2A), with experimental validation (Fig 2B). This model refinement removed the need for data inputs present in previous models of flowering time that limited their utility (Salazar et al., 2009; Song et al., 2012). Second, to create the primary model for photoperiodic control of hypocotyl elongation, we linked light-dependent regulation of PIF proteins to circadian regulation of PIF transcription (Figs 1 and 4A). Third, to examine crosstalk between these two pathways, we tested PIF-dependent regulation of FT expression (Figs 1 and 6A). Consolidating these diverse experimental data within a mathematical model extended our understanding of this system in several ways.

Refinement of the photoperiodic CO-FT pathway

The new model links CDF1 and FKF1 mRNA to the clock, allowing multiple photoperiods and genetic perturbations to be simulated. CDF1 transcription was known to be controlled by the PRR proteins, while the regulation of FKF1 mRNA appeared similar to GI (Nakamichi et al., 2007, 2012; Niwa et al., 2007; Ito et al., 2008). A good qualitative match to CDF1 and FKF1 mRNA data in WT, cca1;ltby and prr9;7 was achieved with CDF1 and FKF1 under the dual regulation of CCA1/LHY and of PRRs or the EC, respectively (Fig 2 and Supplementary Figs S1 and S2). Subsequent ChIP assays showed significant enrichment of CCA1 at CBS/EE motifs in the CDF1 and FKF1 promoters (Fig 1B and Supplementary Fig S3). Thus, our data validated the model prediction that CCA1 is a regulator of CDF1 and FKF1 transcription. The refinement of our model to incorporate clock control of CDF1 and FKF1 transcription allowed us to evaluate how the clock coordinates the timing of flowering with the photoperiod and demonstrated a striking coordination in the regulation of FT by clock components (Fig 7). At each step of the flowering pathway, the EC acts to inhibit FT mRNA accumulation and prevent flowering (Fig 7A), while the PRR proteins act to promote the floral transition by increasing the rate of FT transcription (Fig 7B). Thus, these network motifs form part of a family of coherent feed-forward loops within the flowering system (Mangan & Alon, 2003).

Post-translational regulation of the flowering pathway

The role of GI-FKF1 in controlling CO and CDF1 stability to control FT expression is well established. However, two lines of evidence suggest a further role for GI in the regulation of CDF1 stability: first, our measurements of CDF1 protein in a CDF1ox/ox line suggest that CDF1 is more stable in the absence of gi than in the absence of fKF1 and second, the decrease in CO expression in the gi mutant as compared to the fKF1 mutant (Fig 3D and E). The independent regulation of CDF1 by GI may be direct, for example with GI acting in complex with ZTL, and/or LKP2 (Ito et al., 2012), or indirect, as a result of GI’s widespread regulation of other pathways. For example, GI is known to act antagonistically with ELF3 (Yu et al., 2008; Pokhilko et al., 2012).

The model highlights PIF protein activity through a diurnal cycle

In line with published data, our model simulated a rise in PIF activity towards the end of the night in SDs (Fig 4; Kunihiro et al., 2011; Nomoto et al., 2012b). The consistency of model predictions across a range of conditions then allowed us to use clustering analysis to identify putative targets of PIFs which display dynamics consistent with the model in light:dark cycles. Interestingly, the model was able to describe the essential differences between the clusters of PIF-induced genes, including the pattern of sensitivity to changes in conditions. In particular, transcripts with sharp peaks at dawn in SD (i.e. Cluster 1) are predicted to display enhanced sensitivity to the
lux and LHYox mutations, in line with observations (Supplementary Fig S21). Together, these results demonstrate the widespread nature of transcriptional regulation undertaken by PIF4 and PIF5 in light:dark cycles.

The increase in PIF activity at the end of the night in SDs coincides with a high rate of hypocotyl growth (Nozue et al., 2007) and has been associated with transcriptional activation of phytohormone signalling pathways at this time (Michael et al., 2008a; Nomoto et al., 2012b). Our modelling and data analysis explained two further aspects of the regulation of PIF activity in light:dark cycles. We highlighted a SD-specific increase in PIF activity at the beginning of the night. Modelling suggested that the SD-specific increase in PIF-induced targets at the beginning of the night results from the coincidence of PIF4 and PIF5 transcript with darkness at this time in these conditions.

In addition, we reassessed the role of protein turnover in the diurnal regulation of PIF activity. Light-stimulated degradation of PIF protein by active phyB plays an important role in the de-etiolation response of dark-grown seedlings (Al-Sady et al., 2006), and measurements of PIF4 and PIF5 protein levels in constitutive overexpressors have demonstrated that this regulation also occurs in light:dark cycles (Nozue et al., 2007; Niwa et al., 2009; Kumar

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**Figure 7. Coherent feed-forward networks coordinate the regulation of FT by the circadian clock.**

As highlighted in the discussion, the flowering system is based on a combination of coherent feed-forward pathways, allowing a single component to play multiple reinforcing roles in the system (Mangan & Alon, 2003). This is highlighted here in the case of the EC and the PRRs.

A The EC can repress FT expression through at least three partially redundant pathways, involving coordinated control of CDF1, FKF1 and GI levels.

B Similarly, the PRRs can activate FT expression through at least three pathways. Rectangles denote protein species, and parallelograms denote transcript species. Solid and dashed lines indicate direct and indirect forms of regulation, respectively.

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et al., 2012; Lee & Thomashow, 2012). However, our model suggested that the counteracting effect of increased levels of PIF4 and PIF5 transcript during the day may lead to significant levels of PIF protein at this time. Assessment of PIF target dynamics in microarray timeseries data supported this hypothesis, in line with recent data showing significant PIF4 protein levels during the daytime (Yamashino et al., 2013; Bernardo-García et al., 2014). In addition, the presence of PIF proteins during the day suggested that PIFs may play a regulatory role during this time. This was further highlighted by our analysis of PIF regulation of FT expression (see below).

Temperature regulation of hypocotyl elongation and flowering time

At higher temperatures, hypocotyl growth is increased and flowering time is reduced. This response is mediated, in part, by components that are also involved in the photoperiod responses of both pathways. In the case of hypocotyl elongation, the increase in hypocotyl growth correlates with increases in the PIF targets ATHB2 and IAA29 (Nomoto et al., 2012a). This response appears to be mediated in part by an alleviation of EC repression of PIF4 expression during the night at higher temperatures, with a resultant increase in PIF4 transcript at this time (Mizuno et al., 2014). The model demonstrated that this mechanism was sufficient to understand the response of hypocotyl elongation to temperature.

In the case of flowering time, the reduction in flowering time at higher temperatures correlates with increases in FT expression (Kumar et al., 2012; Lee et al., 2013; Posé et al., 2013), and FT is required for the response of flowering to high temperature (Balasubramanian et al., 2006; Kumar et al., 2012). Recently, this sensitivity to temperature has been shown to be mediated in part by increased activation of FT by PIF4 (Kumar et al., 2012) and a reduction in SVP/FLM-dependent suppression of FT (Lee et al., 2013; Posé et al., 2013). A combination of modelling and experiments allowed us to examine how these elements are integrated with the regulation of FT expression by CO. This provided a mechanistic basis for the observation that the transcriptional response of PIF4 to temperature during the night is not required for PIF4-dependent activation of FT (Kumar et al., 2012). Finally, model simulations demonstrated that changes in the activity of constitutive repressors of FT such as SVP and FLM with temperature are sufficient to explain the observed changes in FT expression in the range 22–27°C.

While we have focussed here on the effects of temperature on FT, we note that in some cases, changes in FT expression are much more subtle than the accompanying changes in flowering time. This is especially the case in the pif mutants, where large changes in flowering time can be accompanied by small changes in FT expression across a light:dark cycle (Thines et al., 2014). While the sensitivity of flowering time to changes in temperature requires FT (Balasubramanian et al., 2006; Kumar et al., 2012), it seems likely that additional temperature-sensitive stimuli are required downstream of FT in the floral induction pathway.

Interactions between circadian- and light-regulated components provide a generalised mechanism for external coincidence

Taken together, our results demonstrate the importance of external coincidence as a mechanism for photoperiod sensing in plants. This mechanism requires the combined regulation of pathways by both the circadian clock and light. In several cases, a single component plays multiple roles at different points of the pathway. The extensive interconnectivity of these pathways requires that quantitative approaches be taken to disentangle the various regulations and identify gaps in our existing knowledge. This is especially the case in scenarios where the complex dynamics of the circadian clock are altered.

The coordination of plant development and physiology by the circadian clock is not limited to flowering and hypocotyl elongation, but extends to processes as diverse as metabolism (Graf et al., 2010), cold tolerance (Fowler et al., 2005) and stomatal opening (Dodd et al., 2005). As our understanding of the circadian clock and its role in these pathways is further refined, it may be possible to develop an understanding of the role of the clock through multiple, interacting output pathways across the whole plant life cycle. Such a holistic approach may provide fresh insights into the contributions of the clock to plant fitness (Dodd et al., 2005) and suggest approaches to engineer aspects of plant physiology for improved growth in both existing and new environments.

Materials and Methods

Experimental methods

Growth conditions for RNA analysis

For the measurements of CO and FT transcript in LDs and SDs (Fig 5F and H and Supplementary Fig S18), seeds of WT (Col-4, Columbia accession) and pif4;5 plants were surface-sterilised, then 30–40 seedlings were sown on 55-mm-diameter plates containing half-strength MS media (Melford, Ipswich, UK), pH 5.8 and 1.2% agar without added sucrose. For the LD experiments (Fig 5F and H), the seeds were stratified at 4°C for 3 days and then grown for 13 days in 16-h light:8-h dark cycles (100 μmol m⁻² s⁻¹ from cool white fluorescent tubes) at 22 and 27°C. Seedlings were harvested from triplicate samples at ZT0, 1, 2, 4, 8, 12, 15, 16, 17, 20 and 24 (ZT = zeitgeber time, ZT0 = lights on) into RNAlater solution (Sigma-Aldrich, Gillingham, UK). For the SD experiments (Supplementary Fig S18), the seeds were stratified at 4°C for 3 days and then grown for 7 days in 8-h light:16-h dark cycles (100 μmol m⁻² s⁻¹ from cool white fluorescent tubes) at 22°C. Seedlings were then transferred to soil and grown at either 22 or 27°C for a further 21 days. At 27°C, nine whole rosettes were singularly harvested at ZT16 and twelve whole rosettes were singularly harvested at ZT8 (ZT = zeitgeber time, ZT0 = lights on) into RNAlater solution. Triplicate rosettes were harvested at 22°C. In both experiments, the plants were left overnight at 4°C in the RNAlater solution to allow full penetration into the tissue (Locke et al., 2005). The generation and growth of CO:HA-CO constructs have been previously described (Song et al., 2012).

RNA extraction

For the LD experiment (Fig 5F and H), RNA was extracted from the plant tissue using the Illustra RNAspin 96 RNA isolation kit (GE Healthcare, Chalfont St. Giles, UK) manually, as described (Salvo-Chirnside et al., 2011). For the SD experiment (Supplementary Fig S18), RNA was extracted from the plant tissue using the
qPCRs were set up using a liquid-handling robot (freedom Evo, TECAN, Reading, UK) and run in a Lightcycler 480 system (Roche, Burgess Hill, UK) using 480 SYBR green master mix (Roche, Burgess Hill, UK) (for the CO measurements, Fig S5) or KAPA SYBR FAST qPCR kit (Kapa Biosystems, Massachusetts, USA) (for the FT measurements). Data were analysed with Roche Lightcycler 480SW 1.5 using relative quantification based on the 2\(^{-\Delta\Delta CT}\) derivative maximum method. Each cDNA sample was assayed in triplicate. The primers used for ACT7 were 5′-CAGTCTGATCTGG AGGAT-3′ and 5′-TGAACTCGTGAACGTGA-3′; for CO were 5′-TAACGTACAACTCAGTCC-3′ and 5′-CCTGAAGCATACCT TATTGTGC-3′; and for FT were 5′-CATTTATGATCTAGGTAACGA ACGGTG-3′ and 5′-CCTCTCATTTTTCCTCCCCCTCTC-3′. Transcript levels were normalised to ACT7 expression (Hong et al., 2010). Expression analysis of CO and FT transcription in 35S:3HA-CO constructs has been previously described (Song et al., 2012).

Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation was performed following the protocol in Nelson et al. (2006) with modifications. Wild-type seedlings from the Col-0 (Columbia) accession were grown on ½ MS agar plates at 22°C for 14 days with 12-h white light:12-h dark cycles and harvested at ZT2. The chromatin was sheared to between 100 and 1,000 bp in a Bioruptor UCD 200 (Diagenode, Liege, Belgium) at high intensity for 10 min (cycles of 30 s on/30 s off) at 4°C after Lau et al. (2011). An aliquot of the chromatin was reserved at this point as the input chromatin. Immunoprecipitation used equilibrated Dynabeads Protein A (Invitrogen/Life Technologies, Paisley, UK). The pre-cleared chromatin was transferred away from the beads and incubated with rotation overnight at 4°C with a 1:1,000 dilution of anti-AFP (Abcam ab290; Abcam, Cambridge, UK). A new aliquot of equilibrated beads was then added and incubated with the chromatin solution for 2 h at 4°C with rotation and then washed with low salt, high salt and lithium chloride washes. The immunocomplexes were recovered from the beads by boiling for 10 min in the presence of 10% Chelex resin (Bio-Rad, Hemel Hempstead, UK) and the proteins removed using Protease K Solution (Invitrogen/Life Technologies, Paisley, UK) at 50°C. The reserved input chromatin was also processed in parallel with Chelex and Proteinase K and then purified using QIAquick PCR purification kit (Qiagen, Manchester, UK). qPCR on the ChIP and input DNA was performed in triplicate using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent, Wokingham, UK) on a Mx3005P machine. The results were calculated so that percent input was equal to 100 × (primer efficiency\(^{\Delta CT}\)) where \(\Delta CT\) is the difference between the adjusted input cT and the ChIP sample cT. The input cT was adjusted to account for the dilution factor of the input chromatin. The primer efficiency was unique to each primer pair and is equal to 10\(^{-1}\) (\(\text{slope} + \text{intercept}\)). The primers used are listed in Supplementary Table S5.

Immunoblot analysis and protein quantification

To detect CDF1 protein in 35S:HA-CDF1 (Imaizumi et al., 2005) and 35S:HA-CDF1/gi-2 (Sawa et al., 2007) and CO protein in CO:HA-CO transgenic lines, plants from Col-0 (Columbia) accession were grown on Linsmaier and Skoog (LS) media (Caisson, Rexburg, Idaho, USA) containing 3% sucrose at 22 or 27°C with a fluence rate of 60 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) in long-day (16-h light/8-h dark) and short-day (8-h light/16-h dark) conditions for 10 days. Seedlings were harvested at each time point on day 10 and were ground in liquid nitrogen for protein extraction. Whole proteins including the nuclear fraction were extracted with buffer containing 50 mM Tris, pH 7.4, 100 mM KCl, 10% glycerol, 5 mM EDTA, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 \(\mu\text{M}\) MG-132 and Complete protease inhibitor cocktail tablets (Roche, Indianapolis, Indiana, USA). Approximately 50\(\mu\text{g}\) of extracted proteins was resolved in 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Whatman, GE Healthcare, USA). HA-CDF1 and HA-CO protein was detected using anti-HA HRP conjugates (3F10, Roche, Indianapolis, Indiana, USA) and visualised with SuperSignal West Femo Maximum Sensitivity Substrate (Thermo Scientific, USA). For quantification of HA-CDF1 and HA-CO protein, non-specific binding of anti-HA around 25 kDa was used as a loading control. The method for protein quantification was described previously (Song et al., 2012).

Data analysis

Data used in this study came from new experiments (see Materials and Methods, above) or from published sources (see Supplementary Table S4). While transcript data characterising the dynamics of the flowering and hypocotyl pathways are remarkably consistent across experiments and laboratories, care must be taken in interpreting results. In particular, since measurements were taken relative to different internal standards and with different normalisation across experiments in the literature, quantitative comparisons were only made within a particular experiment. For each transcript in each experiment, the unknown absolute scale of the measurements means that these data needed to be rescaled to be compared to model simulations, as detailed below.

As with previous models of flowering time, data for CO and FT mRNA taken from Imaizumi et al. (2003) were normalised such that in LD conditions, FT peaked with relative WT expression level equal to 1 (at ZT16) and in 8L:16D CO, mRNA was normalised similarly (Salazar et al., 2009; Song et al., 2012). New data for FT mRNA in the WT and pfts4.5 backgrounds were similarly normalised. In order to obtain comparable relative expression levels of CDF1 mRNA, raw data taken from Nakamichi et al. (2007) and Niwa et al. (2007) were normalised to the maximum expression of FT mRNA in LD. Due to the lack of experimental data in 8L:16D or 16L:8D cycles, FKF1 mRNA data taken from SD = 10L:14D cycles were normalised such that relative WT expression levels peaked at 1 (Niwa et al., 2007). Since there is a lack of raw FKF1 mRNA data across multiple photoperiods, we have refrained from making any direct comparisons of expression levels between FKF1 mRNA and other components of the model. Relative protein levels for CDF1 and FKF1 were normalised such that the WT FKF1 protein peaked with relative expression level equal to 1 in LD, as in Song et al. (2012). Supplementary Fig S4 shows the model simulations matched to the data.
sets described here that were used for optimisation for the components of the flowering pathway.

*Pif4* mRNA rhythmic data in WT, elf3, and prr9;pr7; prr5 were taken from Nomoto et al. (2012b) (Fig 4 and Supplementary Figs S9 and S10). *Pif4* and *Pif5* data in WT were taken from Nuscinov et al. (2011) in 8L:16D, 12L:12D and 16L:8D diurnal cycles and used for parameter optimisation (data not shown). Data were normalised, so the peak of relative WT expression was 1 in all photoperiods since the peak expression level of *Pif4* mRNA has been observed not to change greatly with photoperiod (Nomoto et al., 2012a). Data for the PIF transcriptional targets, *IAA29* and *ATHB2*, were taken from Nomoto et al. (2012b) in 8L:16D and 16L:8D conditions and normalised to peak with a relative expression level of 1 in 8L:16D cycles (Fig 4 and Supplementary Figs S9 and S10).

**Resources**

Computational methods are described in detail in the Supplementary Information. The model is provided as Supplementary File S1 and will be available from the PlaSMo repository upon publication (http://plasmo.ed.ac.uk; identifier PLM_1010). Literature data used to parameterise and test the model are provided as Supplementary File S2. Data generated in this study are provided as Supplementary Datasets S1, S2, S3, S4, S5 and S6. In addition to the Supplementary data files, numerical data are available from the BioDare repository (http://biodare.ed.ac.uk; identifiers are given in Supplementary Table S7).

**Supplementary information** for this article is available online: http://msb.embopress.org

**Acknowledgements**

RWS, DDS, JF, KS and GS are supported by UK Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/F005237/1 (ROBuST project) awarded to KJH. RWS is also supported by graduate student training award BB/F59011/1 awarded to KJH. DDS is further supported by European Commission FP7 Collaborative Project 245143 (TiMet project) and BBSRC award D019621 (SynthSys, Centre for Integrative and Systems Biology) to AJM. DRM is supported by BB/F005237/1 (ROBuST project) awarded to SP, TI and YHS are supported by the National Institutes of Health grant GM079712. YHS is also supported by the Next-Generation BioGreen 21 Program grant (SSAC, Pj009495).

**Author contributions**

DDS and RWS constructed and analysed the mathematical model and co-wrote the paper. KS, GS, JF, DRM and YHS conducted experiments to obtain new data in this study. DRM contributed to the writing. TI, SP, AJM and KJH supervised the study, contributed to and supervised the writing.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Molecular Systems Biology** 11: 776 | 2015 17


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