Molecular convergence of clock and photosensory pathways through PIF3-TOC1 interaction and co-occupancy of target promoters

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A mechanism for integrating light perception and the endogenous circadian clock is central to a plant’s capacity to coordinate its growth and development with the prevailing daily light/dark cycles. Under short-day (SD) photocycles, hypocotyl elongation is maximal at dawn, being promoted by the collective activity of a quartet of transcription factors, called PIF1, PIF3, PIF4, and PIF5 (Phytochrome (phy)-Interacting Factors). PIF protein abundance in SDs oscillates as a balance between synthesis and photoactivated-phy-imposed degradation, with maximum levels accumulating at the end of the long night. Previous evidence shows that elongation under diurnal conditions (as well as in shade) is also subjected to circadian gating. However, the mechanism underlying these phenomena is incompletely understood. Here, we show that the PIFs and the core-clock component, TOC1, display coincident co-binding to the promoters of pre-dawn-phased, growth-related genes under SD conditions. TOC1 interacts with the PIFs and represses their transcriptional activation activity, antagonizing PIF-induced growth. Given the dynamics of TOC1 abundance (displaying high post-dusk levels that progressively decline during the long night), our data suggest that TOC1 functions to provide a direct output from the core clock that transiently constrains the growth-promoting activity of the accumulating PIFs, early post-dusk, thereby gating growth to pre-dawn, when conditions for cell elongation are optimal. These findings unveil a previously unrecognized mechanism whereby a core-circadian-clock output signal converges immediately with the phy-photosensory pathway to directly co-regulate the activity of the PIF transcription factors, positioned at the apex of a transcriptional network that regulates a diversity of downstream morphogenic responses.

Significance

This study defines a molecular mechanism for how clock- and light-signaling pathways converge in Arabidopsis. The data reveal that TOC1, an essential core component of the central oscillator, binds to and represses PIF transcriptional activators, which are also the direct molecular signaling partners of the phytochrome photosensory receptors. This finding shows that TOC1 functions as a clock output-transducer, directly linking the core oscillator to a pleiotopically-acting transcriptional network, through repression of target genes. Collectively, in the plant, these components comprise a transcriptionally-centered signaling hub that provides clock-imposed gating of PIF-mediated, photosensory-regulated diurnal growth patterns. These results provide a framework for future research aimed at understanding how circadian dynamics are integrated with other plant physiological processes important for optimal plant fitness.

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transcription are maintained constant during the diurnal cycle (8, 11).

Fig. 1. PIF3 and TOC1 display coincident co-binding to dawn-phased genes under diurnal short-day conditions (A) Comparison of TOC1-bound (14) and PIF-bound genes (5) using identical criteria for defining binding. (B) Expression phases in SD of gene sets defined in (A): The 144 "PIF-TOC1" genes (green), the 159 "TOC1 only" genes (blue), and the 2,103 "PIF only" genes (yellow). Phases as defined by PHASER (http://phaser.mocklerlab.org) are indicated on the circumference, and fold-change phase enrichment of genes (count/expected) on the radius. Day: white; Night: gray (C-F) Chromatin immunoprecipitation (ChIP)-qPCR analysis. Samples of 3-day-old, SD-grown, pTOC1::TOC1::YFP (TMG) (23) and pPIF3::YFP::PIF3 (YFP-PIF3) (32) seedlings (see SI) were harvested at the indicated times during the third day, and immunoprecipitated using anti-GFP (C, D, F) or anti-MYC antibodies (E). Data are from two independent ChIP experiments. Error bars indicate SEM. (C) TOC1 and PIF3 binding to the promoters of selected dawn-phased genes at ZT14 and 24 in TMG and YFP-PIF3 seedlings, respectively. WT controls: Col-0 for YFP-PIF3, C24 for TMG. (D-F) TOC1 and PIF3 binding to the PIL1 promoter at ZT8, 14 and 24 in TMG, YFP-PIF3 and TOC1ox::YFP-PIF3 seedlings as indicated. (G) Frequency distribution of the pairwise distance in base pairs (bp) between the TOC1 (14) and PIF (5) binding-sites in each of the 49 dawn-phased co-bound genes. (H) Visualization of PIF3 and TOC1 ChIP-seq data in the genomic region encompassing the A75G02580 locus co-bound by PIF3 and TOC1. The statistically significant binding sites identified are indicated by an asterisk below the ChIP-seq pile-up tracks. G-box and PBE-box motifs in the promoter are indicated.

Of particular biological relevance to phy and circadian clock integration, is circadian gating of light signaling, whereby the circadian clock limits the timing of maximum responsiveness to light to specific times of day (6). Elongation growth is subject to permissive gating during shade avoidance (12), and diurnal growth (7, 10, 13), and there is evidence that this behavior is founded on phasing of downstream effector transcript abundance through interaction of the light and circadian clock signaling networks (13). However, despite the importance of temporal gating in the control of the elongation activity in plants, a fundamental understanding of the underlying mechanism is still incomplete.

Here, we provide evidence that the core clock oscillator component, TOC1, directly represses the transcriptional-activator activity of the PIF protein, when TOC1 is most abundant in the circadian cycle. Specifically, we show that, in short days TOC1 constrains PIF growth-promoting activity in early post-dusk darkness, despite rising PIF levels, thereby reducing the extent of the PIF-induced growth that would otherwise have accrued.

Fig. 2. PIF3 and TOC1 interact and co-localize in the nucleus in planta (A) Bimolecular fluorescence complementation (BiFC) assay of PIF3 and TOC1 fusions to N- and C-terminal fragments of YFP, respectively, in transfected onion cells. CyYFP was used as control. (Left) YFP fluorescence image. (Middle) Bright-field image. (Right) Merge of YFP fluorescence and bright-field image. (B) Co-immunoprecipitation of TOC1-MYC and YFP-PIF3 proteins from 3-day SD-grown Arabidopsis seedlings. Samples were harvested under green safelight at ZT14, and extracts were immunoprecipitated with anti-GFP antibody and detected by western blot using anti-GFP and anti-MYC antibodies. (C) TOC1 binds to target promoters in the absence of PIF3. ChIP-qPCR analysis, as in Fig. 1 shows TOC1 binding to the PIL1 and HFR1 promoters at ZT24 in 3-day-old SD-grown TOC1ox::YFP-PIF3 and TOC1ox::PIF3 seedlings. Data from two independent ChIP experiments. Error bars indicate SEM.

Results

PIF3 and TOC1 display coincident co-binding to dawn-phased genes under short-day diurnal conditions. Genome-wide re-analysis of ChIP-seq data for PIF- (5) and TOC1- (14) associated loci, using identical criteria for defining both (see SI Supplementary Text), revealed an overlap of 144 shared genes, representing 48% and 7% of the re-defined TOC1- and PIF-bound loci ("PIF-TOC1" gene set), respectively (Fig. 1A). Although the two ChIP-seq analyses were performed under different conditions (5, 14), the overlap that emerges suggests that the PIFs and TOC1 might bind a common set of genes in conditions where their combined function is concomitantly relevant. Because both light and the clock regulate responses in diurnal light/dark cycles, and the PIFs have been shown to accumulate progressively during the long nights of short-day photoperiods (SD, 8h light:16h dark) (7-9), we hypothesized that these genes might be directly targeted by both TOC1 and PIFs under SD. Consistent
Fig. 3. TOC1 and PIF3 antagonistically regulate dawn-phased growth-related genes in controlling early growth in diurnal SD conditions (A) Average fold-change (FC) expression of the 49 "dawn-specific PIF-TOC1" genes under short-day (SD) compared to free-running (LL) conditions. Expression data for each gene were obtained from http://diurnal.mocklerlab.org. Boxes: Distribution of data for all genes under SD. Error bars denote SEM of three independent studies with at least 25 seedlings each. In (C) and (D), data are from three independent biological replicates. Error bars indicate SEM of three independent studies with at least 25 seedlings each. In (C) and (D), different letters denote statistically significant differences among means by Tukey's test. In (H), the asterisk indicates statistically significant differences between mean values by Student's t test.

Consistent with this pattern, time-course analysis of TOC1 and PIF3 binding to the promoters of three of these dawn-phased genes (PIL1, HFR1, and AT5G02580), through the night (ZT8, ZT14, and ZT24) showed maximum enrichment of TOC1 at ZT14, and of PIF3 at ZT14 and ZT24 (Fig. 1D and Fig. S2C). Using double transgenic lines, that constitutively overexpress constant levels of TOC1-MYC in the YFP-PIF3 background (TOC1ox/YFP-PIF3) throughout the night (Fig. S3A; (14)), we found a significant enrichment of promoter binding at ZT24, similar to the levels at ZT14 (Fig. 1E, Fig. S3B), in contrast to the TMG lines, where TOC1 levels are down by ZT24. This result affirms that TOC1 binding to its target promoters is dictated by its protein abundance (14). The overexpression of TOC1 did not significantly affect the abundance of YFP-PIF3 (Fig. S3C), or the promoter binding of PIF3 at ZT24 (Fig. 1F, Fig. S3D), indicating that TOC1 and PIF3 binding to these promoters is likely simultaneous rather than competitive.

To gain insight into the topology of DNA occupancy by TOC1 and PIF3, we examined the binding distance between the PIFs and TOC1 on the promoters of their co-bound "pre-dawn-specific PIF-TOC1" genes, using the available ChIP-seq data (5, 14) (see SI). The data show that the PIF and TOC1 binding sites lie within 120 bp for 74% of the co-bound genes, and within 400 bp for 40% of them (Fig. 1G). These distances are consistent with concurrent, closely coincident DNA binding of the PIF and TOC1 proteins. A visual example of the high spatially-coincident binding peaks for PIF3 and TOC1 is shown for AT5G02580 in Fig. 1H.

PIF3 and TOC1 interact and co-localize in the nucleus in planta. A previous study showed PIF3 and TOC1 can interact in yeast (15). To determine if the two proteins directly interact in planta, we performed bimolecular fluorescence complementation (BiFC) assays. The data show direct PIF3-TOC1 interaction in the nucleus (Fig. 2A). Furthermore, we observed co-immunoprecipitation of PIF3 and TOC1 from extracts of transgenic TOC1ox/YFP-PIF3 seedlings (Fig. 2B). Together, these results indicate that PIF3 and TOC1 can directly interact with each other in the nucleus under SD conditions. Binding-domain mapping shows that the C-terminal half of PIF3 is predominantly necessary for TOC1 binding (Fig. S4; See SI Expanded Results).

It has been reported that TOC1 can associate with DNA both directly through its CCT domain (16), and indirectly through interaction with DNA-binding factors (17). We examined the possibility that PIF3 might be necessary to recruit TOC1 to the DNA, using TOC1-MYC overexpressing seedlings in a pifi3 background (TOC1ox/pifi3) compared to TOC1ox/YFP-PIF3 seedlings (also in a pifi3 background). The data (Fig. 2C, S3D, S3E) suggest that TOC1 likely binds DNA independently of PIF3 but, the possibility that TOC1 binds through a different PIF-quartet member cannot be discarded. Conversely, as described above for PIF3 promoter-binding (Fig. 1F and S3D), the data suggest that the interaction...
Under SD photoperiods, pre-dawn-phased growth-related genes.

Before the FRp (B-FRp). In (B) and (F), different letters denote statistically followed by 8 h of darkness (A-FRp; Fig. S10C), compared to samples collected 15 min FRp, given at CT8, CT14, CT18, and CT24 to Col-0 and toc1

PCR and values were normalized to (B-FRp) (black and gray bars) and after (A-FRp) (red and pink bars) the FRp-specified in Fig. S11A. Values are shown relative to Col-0 B-FRp at CT 7 set at FRp) (black lines), or after (A-FRp) (red lines) the FRp-plus-dark treatment, as followed by 15 min of darkness. Samples were collected either before (B-FRp) (Fig. S5).

Previous evidence indicates that TOC1 can act as a transcriptional repressor (14, 16). To begin to assess potential TOC1 repression of PIF activity under SD, we examined whether TOC1 levels affect the diurnal pattern of dawn-phased, rising expression of their co-bound target genes in these conditions. The transcript levels of these genes begins rising at ZT14–ZT16 in the TOC1-deficient toc1-101 mutant (18), several hours earlier than in Col-0 (10–13 h), and continues to increase at this elevated level throughout the night, peaking at dawn (Fig. 3B and Fig. S6). This window of early expression in toc1 coincides with the time of highest TOC1 protein abundance in WT (Fig. S2B). In contrast to the clock-output gene, CAB2, this pattern cannot be attributed to toc1 being a short-period mutant (19) (Fig. S7A). Together, these data indicate that TOC1 prevents early, post-dusk, PIF-induced expression of pre-dawn-phased, direct-target genes, when PIF3 first begins to accumulate in the middle of the dark period in SD (ZT12–ZT16). In strong support of this suggestion, we found that the early (ZT12–ZT16) PIF1 expression in toc1 compared to WT was suppressed in a pif3toc1 mutant (Fig. 3B). Also, PIF4 and PIF5 removal in the pif4pif5toc1 and pif5pif5toc1 mutants partially suppressed the expression of PILL1 and HFR1 (Fig. 3C and Fig. S8A). Although potentially complicated by higher PIF4 and PIF5 levels in toc1 (Fig. S9A; 14), this result suggests that TOC1 represses PIF4 and PIF5 activity, as well as PIF3. It is also notable that TOC1 repression of PILL1 and HFR1 expression also occurred under LD as well as SD conditions (SI and Fig. S8A), and that, conversely to toc1, constitutive overexpression of high levels of TOC1 throughout the night completely suppressed dark-induced expression of PIF3 target genes, not only at ZT14 but also at ZT24 (Fig. 3D) (see SI Expanded Results for discussion). Because PIF3 transcript and protein levels are not affected in toc1 (Fig. S9A), this data indicate that TOC1 acts at low levels as a transcriptional repressor of PIF3, which itself acts intrinsically as a transcriptional activator (4), and thus that PIF3 and TOC1 act antagonistically in regulating the expression of their co-target genes.

Under SD conditions, hypocotyl elongation is rhythmic and peaks at the end of the night (7, 8, 20). To determine whether the apparent antagonistic activities of the PIFs and TOC1 affect this phenotype, we initially compared the growth rates of WT and the toc1 mutant in SD under our conditions. The data show that toc1 elongates more rapidly through the middle of the night than WT (Fig. 3E, Fig. S9E) and is therefore taller after than WT (Fig. 3F) in agreement with previous reports (7). This tall phenotype persists under T21 conditions (Fig. 3B, C), consistent with the conclusion that it is not a consequence of toc1 being a short-period mutant. The phenotype is, however, strongly suppressed in the pif3toc1 double mutant (Fig. 3F), indicating that PIF3 is necessary for the long toc1 hypocotyls, and that PIF3 and TOC1 act antagonistically in regulating growth under diurnal conditions. Similarly, the pif4pif5toc1 triple mutant partially suppresses the tall toc1 phenotype, and PIF3 removal in

of TOC1 with PIF3 does not significantly affect PIF3 binding to DNA (See SI Expanded Results).

TOC1 represses PIF3 transcriptional activity in regulating pre-dawn-phased growth-related genes. Under SD photoperiods, PIFs directly promote a progressive increase in expression of genes like PIL1 and HFR1 during the second half of the night to peak at dawn (7, 8, 10, 11). Consistent with this pattern, the average expression of the “dawn-specific” PIF-TOC1 target gene set under SD shows such an oscillatory pattern, with maximum expression at the end of the night (Fig. 3A), suggesting that the PIFs directly target these genes to promote their expression at dawn. Strikingly, by contrast, under free-running conditions, the average expression of this gene set is almost constant (Fig. 3A), a pattern that is not a classical clock-output pattern. We confirmed directly here that the dawn-specific PIF-TOC1 genes PIL1, HFR1, and AT5G02580 lose rhythmicity and are maintained at low levels across the day and subjective night, in seedlings grown for 2 days under SD and then released into constant light, in contrast to the oscillation of clock outputs like CAB2 (Fig S5).

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pf3pfpfpfStoc1 further suppresses the hypocotyl elongation of
pf3pfStoc1 (Fig. 3F, Fig S8C). This effect was stronger in SD
than LD (Fig. S8B,C). Overall, these results mirror the PIF-
direct-target-gene expression data presented above. Conversely,
TOC1 overexpression in TOC1oxYFP-PF3 lines resulted in a
strong inhibition of hypocotyl length (Fig. 3G,H), also consistent
with the repression of ‘pre-dawn-specific PIF-TOC1’ genes when
TOC1 is overexpressed (Fig. 3D). Consistent with a role of these
genes in growth, gene ontology (GO) analysis shows enrichment
for genes responsive to the growth-regulating hormones, auxin,
brassinosteroids, cytokinin and gibberellin (Fig. S10; SI Expanded
Results).

TOC1 can repress PIF activity during skotomorphogene-
sis. PIFs accumulate to maximum levels in post-germinative
seedlings in the dark, thereby promoting skotomorphogenesis, a
developmental stage where TOC1 levels are low and constant
(18). Comparison of dark-grown YFP-PF3 and TOC1oxYFP-
PF3 seedlings, shows that TOC1 overexpression induces partial
photomorphogenenic development in darkness (hypocotyl-length
inhibition, open hooks and partially separated cotyledons) (Fig.4A,B), suggestive of TOC1 repression of PIF activity, under these
conditions (21). Indeed, expression analysis confirms that TOC1
overexpression suppresses full PIF3-target-gene expression (Fig.
4C).

TOC1 gates shade-stimulated PIF activity. The above data
suggest that growth rate is determined by the balance between
PIF and TOC1 abundance. We reasoned that this concept might
provide mechanistic insight into the permissive gating of growth
by the clock, previously reported under seasonal and shade-
avoidance conditions (12, 13). To test this, we artificially induced
accumulation of PIFs at different time points during a subjec-
tive night in SD-grown seedlings released into continuous light
(Fig. S11A,B). Under these conditions, TOC1 oscillations persist
in TOC1 mutants (Fig. 4D), confirming that TOC1 is essential to
avoidance conditions (12, 13). To test this, we artificially induced
accumulation of PIFs at different time points during a subjec-
tive night at CT8 and CT14 (time points with low and high TOC1
levels, respectively) in WT) shows that repression was absent
after the FRp given during a subjective night at CT8, CT14, CT18,
and CT24 (Fig. S11C), and measured the hypocotyl elongation
that took place during this time. The difference in hypocotyl
length before and after the FRp plus 8h of darkness was low in
the WT at CT14 and CT18, when TOC1 levels are high, and was
significantly greater at CT8 and CT24 (the beginning and end of
the subjective night, respectively), when WT levels of TOC1 are
low (Fig. 4F). By contrast, the repression of growth at CT14 and
CT18 was absent in the toc1 mutant (Fig. 4F). This pattern mirrors
the marker gene expression data (Fig. 4D,E), strongly supporting
the conclusion that the transcriptional repressor activity of TOC1
toward the PIFs mediates the gating of PIF-promoted growth
by the clock. Together, these data support our hypothesis and
provide a direct mechanism explaining the permissive gating of
growth by TOC1 to precisely time maximum PIF3-promoted
hypocotyl elongation to the pre-dawn period.

Discussion
We show that TOC1 directly interacts with, and acts to repress
the transcriptional-activation activity of PIF3 (and by extension likely
the other PIFs (see SI Discussion)) on the promoters of their co-
targeted genes. Given the different dynamics of TOC1 and PIF3
protein levels during short-day photocycles, we propose a model
whereby TOC1 binds, directly or indirectly, to the promoters of pre-dawn-phased, PIF- and TOC1-co-target genes during the
early post-dusk hours (Fig. 4G). Then, as the PIFs accumulate
during the night, they are initially subjected to the transcriptional-
repression action of TOC1, a repression that is lifted toward the
end of the dark period, when TOC1 levels decline, coincident
with maximum PIF levels. The co-targeted genes include growth-
related and hormone-associated genes (8, 13, 20), which are PIF-
induced, pre-dawn, thereby promoting an increase in hypocotyl
elongation rates (Fig. 4G).

These data indicate that the net transcriptional activation
activity of the PIFs is determined by a dynamic balance in relative
abundance of the PIF and TOC1 proteins. We propose that
this antagonistic interaction is potentially operative throughout
the life cycle. In fully dark-grown, etiolated seedlings, the PIFs
are at high levels that appear to be saturating for promotion of
skotomorphogenesis, because the absence of any single member
of the quartet in monogenic pf mutants has little or no effect on the
phenotype (22). Under these conditions, the absence of native
levels of TOC1 in the toc1 mutant has a minimal, albeit promotive,
effect (23); J. Soy and E. Monte, unpublished). Exposure to light
induces a precipitous reduction in PIF abundance through
degradation to levels that become susceptible to significant re-
pression by TOC1. We suggest that this repression explains the
gene expression patterns observed in de-etiolated seedlings under
two different conditions. First, during the early night of diurnal
photocycles as shown here (Fig. 3B), and second, during the light
period in seedlings exposed to vegetative shade (Fig. 4D; (12)).
The latter conclusion was suggested by the report of Salter et al.
(12) that rapid shade-induced increases in PAIL1 expression are
gated in circadianly-entrained seedlings released into constant
light (LL) conditions.

Although previous evidence has established TOC1 (also
known as PRR1) as a general transcriptional repressor (14,
16), our identification of the PIF transcriptional activators as
direct molecular targets of TOC1 repression, reveals a molecular
mechanism by which that activity is exerted. Moreover, given the
evidence that other members of the PRR-protein family, PRR5,
PRR7 and PRR9, impose transcriptional repression on target
genes by recruiting the co-repressor TOPLESS (TPL) (24), we
speculate that TOC1 may invoke a similar mechanism to repress
PIF activity, albeit using a different co-repressor, as Wang et al.
(24) failed to detect any direct interaction of TPL with TOC1.
The question of the topology of PIF-TOC1 co-occupancy of target
promoters remains open. The recruitment of TOC1 to G-box-
containing promoter regions ((14, 16); Fig. 1G,H) is consistent
with either direct or indirect interaction with these genomic sites.
The interaction could be the result of binding to DNA-bound,
PIFs only, or indirectly to the pervasive TGGT DNA motifs, as
reported by Gendron et al. (16), accompanied by interaction with
neighboring PIFs (Fig. 4G).

One consequence of this general mechanism of TOC1 as a
repressor of PIF transcriptional activation activity, is that, while
core-clock generated oscillations in TOC1 abundance have the
potential to generate sustained, circadianly-entrained oscillations
in direct-target-gene transcription in subsequent constant dark-
ness (DD), where PIF levels are high, they lose this capacity
in constant light (LL), where PIF levels are too low to activate

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