# 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.**

PIFs | growth | circadian clock | photoperiod | TOC1

## Introduction

Given the importance of solar energy to plants, they have evolved sophisticated photosensory-response systems to monitor and adapt to the diurnal photoperiod (1). This environmental parameter provides a precise index of the progression of the earth's seasons and the time of the day, and thereby a signal that regulates a spectrum of growth and developmental responses (such as elongation growth, flowering and dormancy) appropriate to the prevailing conditions.

The phytochrome (phy) family of photoreceptors (phyA to E in Arabidopsis) are the primary sensors of this signal (2, 3). These chromoproteins regulate two pathways in parallel that converge to control the morphogenic response: (a) the PIF (phy-Interacting Factor) pathway, whereby the photoactivated phy molecules bind to and induce the degradation of the PIF proteins (notably the PIF1, PIF3, PIF4 and PIF5 quartet, a subfamily of basic helix-loop-helix (bHLH) transcription factors), thereby altering the expression of the PIF direct-target genes and the cognate downstream transcriptional network (4, 5); and (b) the circadian clock, whereby the phys entrain the circadian oscillations of the core clock components by sensing the dark-to-light transition at dawn each day (6). Much has been learned about these two pathways, but the mechanism by which their activities are integrated is not well understood.

A central consequence of light-regulated phy activity, is that PIF protein abundance oscillates diurnally over each 24-h cycle, with low PIF levels during the light hours (when the phys are photoactivated) and progressive accumulation during the long dark period (as the levels of the active Pfr form of the phys declines) (7-9). This PIF protein oscillation controls rhythmic growth under short photoperiods, where they collectively promote increased elongation rates in the pre-dawn hours when they are most abundant (7, 8, 10, 11). In parallel, transcription of *PIF4* and *PIF5* genes are regulated by the circadian clock, most likely in direct fashion by several central clock components (4), which drive an internal rhythm, whose periodicity is also set by the external photoperiodic information. In contrast, *PIF1* and *PIF3*

### 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.**

**Reserved for Publication Footnotes**

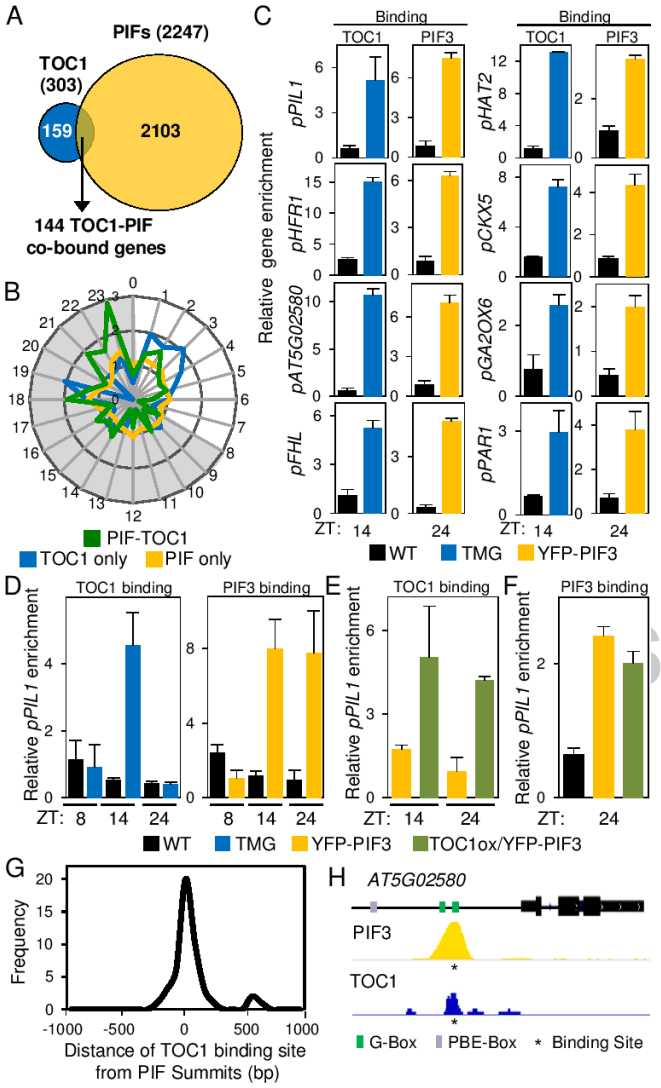


**Fig. 1. PIF3 and TOC1 display coincident co-binding to dawn-phased genes under diurnal short-day conditions**(A) Comparison of TOC1-bound (14) and PIF-bound genes (5) using identical criteria for defining binding.(B) Expression phases in SD of gene sets defined in (A): The 144 "PIF-TOC1" genes (green), 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 *AT5G02580* 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.

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

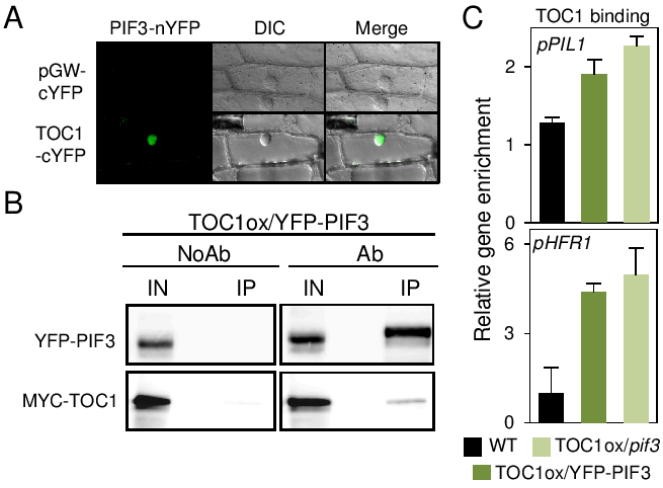


**Fig. 2. PIF3 and TOC1 interact and co-localize in the nucleus *in planta***(A) Bimolecular fluorescence complementation (BiFC) assay of PIF3 and TOC1 fusions to N- and C-terminal fragments of YFP, respectively, in transfected onion cells. cYFP was used as control. (Left) YFP fluorescence image. (Middle) 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.

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.

## 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

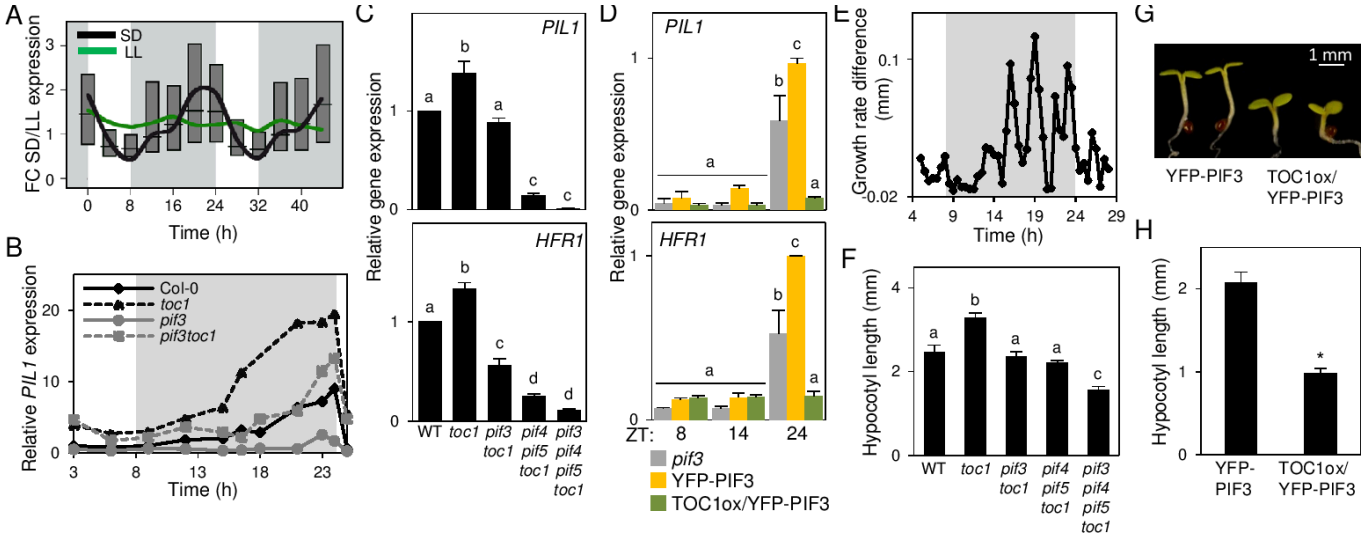


**Fig. 3. TOC1 and PIF3 antagonistically regulate dawn-phased growth-related genes in controlling early growth in diurnal SD conditions**(A) Average fold-change (FC) expression of the 49 "dawn-specific PIF-TOC1" genes under short-day (SD) compared to free-running (LL) conditions. Expression data for each gene were obtained from http://diurnal.mocklerlab.org. Boxes: Distribution of data for all genes under SD.(B-D) Seedlings grown for 2 days in SD conditions were harvested during the third day at the indicated times. Expression was analyzed by qRT-PCR and values were normalized to *PP2A*. In (C) and (D), data are from three independent biological replicates. Error bars indicate SEM.(B) *PIL1* expression in 3-day-old SD-grown WT (Col-0) and mutant seedlings. Data are the average of three technical replicates of one representative biological experiment.(C) *PIL1* and *HFR1* expression in 3 day-old, SD-grown WT and mutant seedlings at ZT23. (D) *PIL1* and *HFR1* expression in 3-day-old, SD-grown *pif3*, YFP-PIF3, and TOC1ox/YFP-PIF3 seedlings. (E) Hypocotyl elongation-rate difference between *toc1* and WT under SD conditions. Seedling growth was monitored by infrared imaging (n=7) from 2 day onwards every 30 min. Growth rate per 30 min of WT seedlings was subtracted from the growth rate of *toc1* seedlings at each time point. (F) Hypocotyl length of 3-day-old SD-grown WT, and mutant seedlings. (G) Visible phenotype of 3-day-old SD-grown YFP-PIF3 and TOC1ox/YFP-PIF3 seedlings. (H) Hypocotyl length of seedlings shown in (G). (F and H) Error bars indicate SEM of three independent studies with at least 25 seedlings each.In (C), (D), and (F), different letters denote statistically significant differences among means by Tukey-b's test. In (H), the asterisk indicates statistically significant differences between mean values by Student's *t* test.



with this possibility, time-of-day-expression enrichment-analysis of these genes, using the available data at the PHASER website (http://phaser.mocklerlab.org/) (see SI), showed that the 144 co-bound "PIF-TOC" genes displayed an overrepresented phase of expression, under SD photocycles, at the end of the dark period (Fig. 1B), with 49 of these genes phased between 18 and 23 h ("pre-dawn-specific PIF-TOC1" set), when PIF abundance is maximum. Notably, this phase-overrepresentation pattern was absent from the 159 "TOC1 only" and the 2,103 "PIF only" genes (Fig. 1A,B), and was specific for SD versus LD (Fig. S1). These data suggest that the "pre-dawn-specific PIF-TOC1" genes might be directly targeted by both TOC1 and PIFs to drive a SD-specific expression pattern. Chromatin immunoprecipitation (ChIP)-qPCR assays confirmed the direct binding of TOC1 and PIF3 to the promoters of selected "pre-dawn-specific PIF-TOC1" genes, at post dusk (ZT14) and dawn (ZT24), respectively (Fig. 1C), when each protein is most abundant in the SD diurnal cycle, respectively (Fig. S2A,B) (5, 14) (see SI Expanded Results for details).

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 40 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 *pif3* background (TOC1ox/*pif3*) compared to TOC1ox/YFP-PIF3 seedlings (also in a *pif3* 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

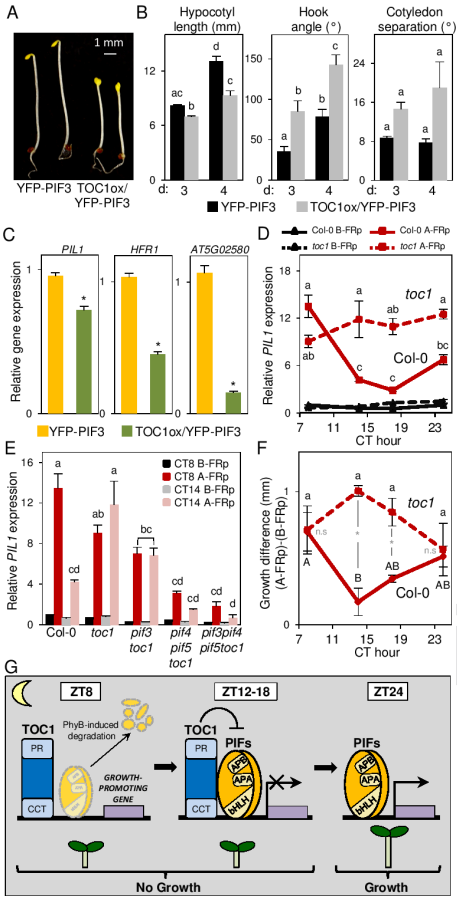


**Fig. 4. The transcriptional activity of PIF3 is repressed by TOC1.** (A) Visible phenotype of 3-day-old dark-grown YFP-PIF3 and TOC1ox/PIF3-YFP seedlings. (B) Quantification of hypocotyl length, hook angle and cotyledon separation in YFP-PIF3 and TOC1ox/PIF3-YFP seedlings. d: days in dark. Error bars indicate SEM. (C) Geneexpression in 3-day-old dark-grown YFP-PIF3 and TOC1ox/PIF3-YFP seedlings. (D) *PIL1* expression in Col-0 and *toc1* seedlings grown for 2 days in SD and released into continuous white light, until exposure to a 15-min far-red light pulse (FRp) at CT8, CT14, CT18, and CT24, followed by 15 min of darkness. Samples were collected either before (B-FRp)(black lines), or after (A-FRp)(red lines) the FRp-plus-dark treatment, as specified in Fig. S11A. Values are shown relative to Col-0 B-FRp at CT 7 set at 1. (E) *PIL1* expression at CT8 and CT14 in Col-0 and mutant seedlings before (B-FRp) (black and gray bars) and after (A-FRp) (red and pink bars) the FRp-plus-dark treatment described in (D). Expression in (C-E) was analyzed by qRT-PCR and values were normalized to *PP2A*. Data are for three independent experiments. Error bars indicate SEM. (F) Growth difference induced by a 15 min FRp, given at CT8, CT14, CT18, and CT24 to Col-0 and *toc1* seedlings, followed by 8 h of darkness (A-FRp; Fig. S10C), compared to samples collected before the FRp (B-FRp). In (B-E) and (F), different letters denote statistically significant differences among means by Tukey-b's test. In (F), Col-0 (upper case) and *toc1* (lower case) data were processed independently. In (C) and (F), asterisks indicate statistically significant differences between mean values by Student's *t* test. n.s., not significant. (G) Model of the proposed role of TOC1 as a repressor of PIF transcriptional regulatory activity in gating growth to the pre-dawn hours. (Left) TOC1 binds directly or indirectly to the promoters of growth-promoting genes as it accumulates during the post-dusk hours. (Middle) PIFs progressively accumulate during the night and bind to the same promoters. TOC1 directly interacts with PIFs and represses their transcriptional activity. (Right) As night proceeds, TOC1 abundance declines while PIFs accumulate. At pre-dawn, TOC1 is no longer present, repression is relieved, and PIFs induce growth-promoting gene expression.

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" 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).

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 (WT), 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) *PIL1* expression in *toc1* compared to WT was suppressed in a *pif3toc1* mutant (Fig. 3B). Also, PIF4 and PIF5 removal in the *pif4pif5toc1* and *pif3pif4pif5toc1* mutants partially suppressed the expression of *PIL1* 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 *PIL1* 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. S9B-D), the data indicate that TOC1 acts directly 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 than WT (Fig. 3F) in agreement with previous reports (7). This tall phenotype persists under T21 conditions (Fig. S7B,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 *toc1pif3* 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

*pif3pif4pif5toc1* further suppresses the hypocotyl elongation of *pif4pif5toc1* (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-PIF3 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 skotomorphogenesis.** 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-PIF3 and TOC1ox/YFP-PIF3 seedlings, shows that TOC1 overexpression induces partial photomorphogenic 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 subjective night in SD-grown seedlings released into continuous light (Fig. S11A,B). Under these conditions, TOC1 oscillations persist (www.diurnal.mocklerlab.org), but PIF3 levels remain low due to phy-imposed degradation (8, 11). By giving a far-red light pulse (FRp) followed by darkness at different time points during the subjective night (CT8, CT14, CT18, and CT24), we induced rapid PIF3 accumulation that was able to induce rapid *PIL1* expression (detected within 15 min) at the beginning and at the end of the subjective night (CT8 and CT24) (when TOC1 levels are low), but only to much lower levels at CT14 and CT18 (when TOC1 levels are high) (Fig. 4D). This result strongly suggests that PIF3-induced expression of target genes is indeed gated by high TOC1 levels. Consistent with this suggestion, this repression was absent in the *toc1* mutant (Fig. 4D), confirming that TOC1 is essential to gate PIF-dependent growth promoting activity. In addition, *PIL1* expression in *toc1* and *piftoc1* mutants at CT8 and CT14 (time points with low and high TOC1 levels, respectively, in WT) shows that PIF removal suppresses expression in *toc1* after a FRp both at CT14 and CT18 (Fig. 4E). To test whether the TOC1-imposed permissive or restrictive gene expression pattern correlates with growth, we submitted WT and *toc1* seedlings to 8 h of darkness 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 *pif* 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 repression 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 *PIL1* 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 TGTG 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 darkness (DD), where PIF levels are high, they lose this capacity in constant light (LL), where PIF levels are too low to activate

those genes (Fig. S12). The initially surprising lack of sustained oscillations in LL for the "pre-dawn-specific PIF-TOC1" genes in Figures 3A and S5 support the generality of this notion.

An additional ramification of the present data is that the functionally antagonistic interaction between the PIF and TOC1 proteins provides insight into the mechanism underlying the anticipated convergence of the light- and clock-regulated pathways in controlling common facets of plant morphogenesis ((4, 7, 12, 13, 25); Fig. S12). In addition to implementing this specific convergence, evidence continues to accumulate that the PIFs function to integrate the activities of an increasing number of other signaling pathways, including the gibberellin, ethylene and brassinosteroid hormones, sugar and temperature (4, 25, 26). Many of the outputs from these pathways, in addition to diurnal growth, such as cellular metabolism and responses to temperature and biotic and abiotic stress (25), are subjected to permissive gating by the clock. At the transcriptome level, a striking feature of circadian activity is the large number of expressed genes that are regulated by the clock (27). Our present findings indicate that a significant fraction of this regulation is channeled through modulation of the PIF transcriptional network, known to control a broad range of biological processes, from seed germination and seedling development, through vegetative-shade avoidance and temperature responsiveness, to flowering. Thus, more generally, our data provide evidence that a core-clock component functions as an output transducer that directly links the plant central oscillator to the regulatory machinery of a transcriptionally-centered signaling hub that pleiotropically controls a diversity of plant growth and developmental responses to multiple inputs throughout the life cycle.

## Materials and Methods

Available online tools were used to analyze and visualize the ChIP-seq data. *Arabidopsis thaliana* lines were in Columbia and C24 ecotypes. See SI Material and Methods for transgenic and mutant line references, seedling growth conditions, and hypocotyl measurements. Gene expression analysis: RNA extraction, cDNA synthesis and qRT-PCR were done as described (28). *PP2A* was used for normalization. Primer details can be found in Table S2. Protein extracts were prepared from seedlings grown under short-day conditions as described (29). ChIP assays were performed as previously described (8) using short-day grown seedlings during the third day of growth at the indicated times. Primers used in the detection of each gene by qRT-PCR can be found in Table S2. Co-immunoprecipitation (CoIP) assays were performed using short-day grown seedlings at ZT16 during the third day of growth as described (30), with modifications specified in the SI. Bimolecular Fluorescence Complementation (BiFC): The coding regions of *PIF3* and *TOC1* were PCR-amplified and cloned into pGWnY and pGWcY vectors (31). Details of all reagents and procedures are provided in the SI Materials and Methods.

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