LETTERS

Rhythmic growth explained by coincidence between internal and external cues

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Most organisms use circadian oscillators to coordinate physiological and developmental processes such as growth with predictable daily environmental changes like sunrise and sunset. The importance of such coordination is highlighted by studies showing that circadian dysfunction causes reduced fitness in bacteria[1] and plants[2], as well as sleep and psychological disorders in humans[3]. Plant cell growth requires energy and water—factors that oscillate owing to diurnal environmental changes. Indeed, two important factors controlling stem growth are the internal circadian oscillator[4–6] and external light levels[7]. However, most circadian studies have been performed in constant conditions, precluding mechanistic study of interactions between the clock and diurnal variation in the environment. Studies of stem elongation in diurnal conditions have revealed complex growth patterns, but no mechanism has been described[8–10]. Here we show that the growth phase of *Arabidopsis* seedlings in diurnal light conditions is shifted 8–12 h relative to plants in continuous light, and we describe a mechanism underlying this environmental response. We find that the clock regulates transcript levels of two basic helix–loop–helix genes, phytochrome-interacting factor 4 (*PIF4*) and *PIF5*, whereas light regulates their protein abundance. These genes function as positive growth regulators; the coincidence of high transcript levels (by the clock) and protein accumulation (in the dark) allows them to promote plant growth at the end of the night. Thus, these two genes integrate clock and light signalling, and their coordinated regulation explains the observed diurnal growth rhythms. This interaction may serve as a paradigm for understanding how endogenous and environmental signals cooperate to control other processes.

Most core circadian clocks consist of a conserved oscillatory mechanism using a transcriptional negative feedback loop. In plants, two Myb-like transcription factors (CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY)) and a pseudo-response regulator (TIMING OF CAB EXPRESSION 1 (TOC1)) are thought to be components of the classical central oscillator[11]. Overexpression of *CCA1* or *LHY* causes arrhythmia in circadian-controlled gene expression and growth[6,12,13]. In addition to the core components, *EARLY FLOWERING 3* (*ELF3*) is required to restrict light input to the clock and other signalling pathways[14,15]. Under constant dim light, hypocotyl elongation is rhythmically controlled in a process that requires *CCA1* and ELF3 (refs 6, 16).

Light, perceived by phytochrome and cryptochrome photoreceptors, strongly reduces seedling growth rate[7]. Phytochromes signal in part by inducing degradation of a family of basic helix–loop–helix transcription factors known as PIFs or PIF3-likes (PILs) that, when present, act primarily to inhibit light responses[17]. The phytochrome and cryptochrome signalling pathways converge at other transcription factors, including the basic leucine zipper factor HY5 (ref. 18).

To examine how internal rhythms and photoperception interact to control growth, we asked whether rhythmic hypocotyl growth is light-dependent. As previously reported[6], under continuous light wild-type hypocotyls exhibited rhythmic growth, peaking at subjective dusk, whereas the arrhythmic mutant *elf3* grew continuously (Fig. 1a and Supplementary Fig. 1). Although the clock is known to function in continuous darkness[3], growth in continuous darkness was rapid and arrhythmic (Fig. 1b). This indicated that observable circadian growth control is light-dependent, and suggested that the clock and photoreceptor signalling pathways might interact to control normal growth. To investigate the interactions further, plants growing in short-day conditions were examined. The growth pattern of wild-type hypocotyls under short-day conditions was strikingly different from that seen in continuous light: peak growth occurred at dawn instead of at subjective dusk (Fig. 1c). This interaction between the clock and light signalling has been missed in previous studies because they were performed in continuous light conditions.

To investigate further the requirement for light, we asked whether photoreceptor signalling is required for rhythmic growth. Growth rhythms were weak or absent in *hy5* mutants (Fig. 1c), which are deficient in both phytochrome and cryptochrome signalling[18]. Additionally, rhythmic growth was very weak or absent in *hy2* mutants, which lack the phytochrome chromophore[19], suggesting that phytochrome signalling is necessary for rhythmic growth. The observed growth patterns could reflect the kinetics of signal transduction through the photomorphogenic pathways directly, or could result from interactions between the clock and light signalling. To distinguish these possibilities, we examined growth patterns in arrhythmic clock mutants. Unlike the wild type, arrhythmic *CCA1*-overexpressing (*CCA1*-OX) and *elf3* plants seemed to respond directly to changes in light: growth ceased at dawn and resumed at dusk (Fig. 1c). Quantification of these results using a dark responsiveness index (see Methods) confirmed that arrhythmic *elf3* and *CCA1*-OX plants were considerably more responsive to darkness than the wild type or light-signalling mutants (Supplementary Fig. 2). These results show: first, that arrhythmic and light-signalling mutants have distinct growth patterns, suggesting that they do not impair the same pathway; second, that the transition from light to darkness can quickly increase elongation rates, but these effects are normally buffered by the clock; and, third, that under diurnal cycles the clock functions to maintain the growth-repressing function of light during the first half of the night. (See Supplementary Discussion and Supplementary Figs 3, 4, 7 and 8 for detailed discussion of growth patterns in other clock mutants and diurnal versus continuous photoperiods.)

To confirm this hypothesis, we examined growth under an 8 h T-cycle (repeating 4 h light:4 h dark treatments, 4L:4D; Fig. 1d). Wild-type plants exhibited frequency demultiplication[3]: growth cues from

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two 4L:4D cycles among three were ignored, producing a 24 h rather than an 8 h rhythm. A simple interpretation is that the circadian clock gates growth in the dark. However, because rhythmic growth is not seen in entrained plants shifted to constant darkness (Fig. 1b), previous light exposure is required to see this effect. Thus, the clock gates the rate at which light-signalling is inactivated on transfer to darkness. Under these 8 h T-cycles, *CCA1*-OX and *elf3* grew during every dark period (Fig. 1d), confirming that plants without a functional clock respond directly to light/dark transitions and that the clock is responsible for the frequency demultiplication seen in the wild type.

Clock-regulated transcription is thought to be an important mechanism for circadian control of physiological processes[3]. We therefore hypothesized that the clock might regulate hypocotyl elongation at the transcriptional level. To find genes that might link the clock and light-signalling pathways, we compared expression profiles in plants that either did or did not respond to darkness with growth. Specifically, we sampled both wild-type Columbia (Col) and *CCA1*-OX plants at two time points in the dark, one when Col is growing and one when it is not (see Supplementary Fig. 5 and Supplementary Discussion). When we used whole-genome expression analysis to contrast both elongating versus non-elongating Col and elongating *CCA1*-OX versus non-elongating Col, we identified 38 genes that are upregulated and 23 genes that are downregulated in growing plants (Supplementary Tables 1, 2 and Supplementary Discussion). Among these, we identified two closely related basic helix–loop–helix genes, *PIF4* (ref. 20) and *PIF5* (also known as *PIL6*; refs 21, 22), as the two strongest candidates on the basis of their high ranking by rank-product analysis (Fig. 2a and Supplementary Table 1 and previously published data). These two genes code for phytochrome-B-interacting proteins that negatively regulate light signalling and positively regulate growth[20,22,23]. Expression of *PIF4* and *PIF5* messenger RNA is clock-regulated in continuous light[21] (Supplementary Fig. 6), and both proteins interact with a central clock component, TOC1 (ref. 17), suggesting *PIF4* and *PIF5* as possible links between the clock and light signalling. Because knockout or overexpression of either gene does not alter central clock properties (Supplementary Fig. 6), there seems to be a regulatory cascade: clock to PIF4 and PIF5, and from PIF5 to light signalling, as previously suggested for PIF5 (ref. 23).

To complement our microarray data (Fig. 2a), we examined expression of *PIF4* and *PIF5* in plants grown in short-day conditions by quantitative PCR with reverse transcription (qRT–PCR) (Fig. 2b, c). In wild-type plants, expression of both genes decreased soon after lights were turned off and began to rise in the mid- to late-night, correlating well with the observed phase of hypocotyl growth in short-day conditions. In *CCA1*-OX plants, these genes cycle with low amplitude, if at all, such that levels are much higher during the early and middle night in *CCA1*-OX compared with the wild type, which is consistent with growth patterns in this genotype.

We hypothesized that lack of plant growth in the early night might be due to clock-mediated repression of *PIF4* and *PIF5* expression. To test this, we examined growth in plants overexpressing *PIF4* or *PIF5*. Similar to the arrhythmic lines, these plants grew immediately in response to darkness both in short-day conditions and in 8 h T-cycles (Fig. 2d, e, and Supplementary Fig. 2). These plants do not show decreased growth in the light during the first few days of the short-day experiment, probably owing to the very high levels of *PIF4* or *PIF5* expression—approximately ten times higher than in *CCA1*-OX (Supplementary Fig. 6). Together with the microarray and qRT–PCR expression data, the growth patterns in the OX lines show that the clock gates the dark growth response by regulating *PIF4* and *PIF5* expression.

Hypocotyl growth is inhibited by light in wild-type and *PIF4*- and *PIF5*-OX plants, as well as in *CCA1*-OX plants, indicating that *PIF4* and *PIF5* mRNA levels are not the only factors determining growth. Light triggers degradation of PIF3 and PIF1 (also known as PIL5) proteins[24–26]—close paralogues of PIF4 and PIF5. Indeed, we found that PIF4 and PIF5 protein levels rapidly decrease in the light and increase in response to darkness (Fig. 2f and g), correlating well with observed growth responses in overexpressors (Fig. 2d and e). This correlation strongly supports the idea that PIF4 and PIF5 proteins are key regulators of diurnal growth rhythms. This notion is further supported by *pif4* or *pif5* single-mutant or *pif4 pif5* double-mutant plants, which show a partial or complete loss, respectively, of the dawn growth peak (Fig. 2e). Thus, PIF4 and PIF5 are partially redundant in function, but both are required to fully promote growth in diurnal light conditions.

On the basis of our observations we propose a model for circadian and light regulation of growth (Fig. 2h): during the day, light inhibits

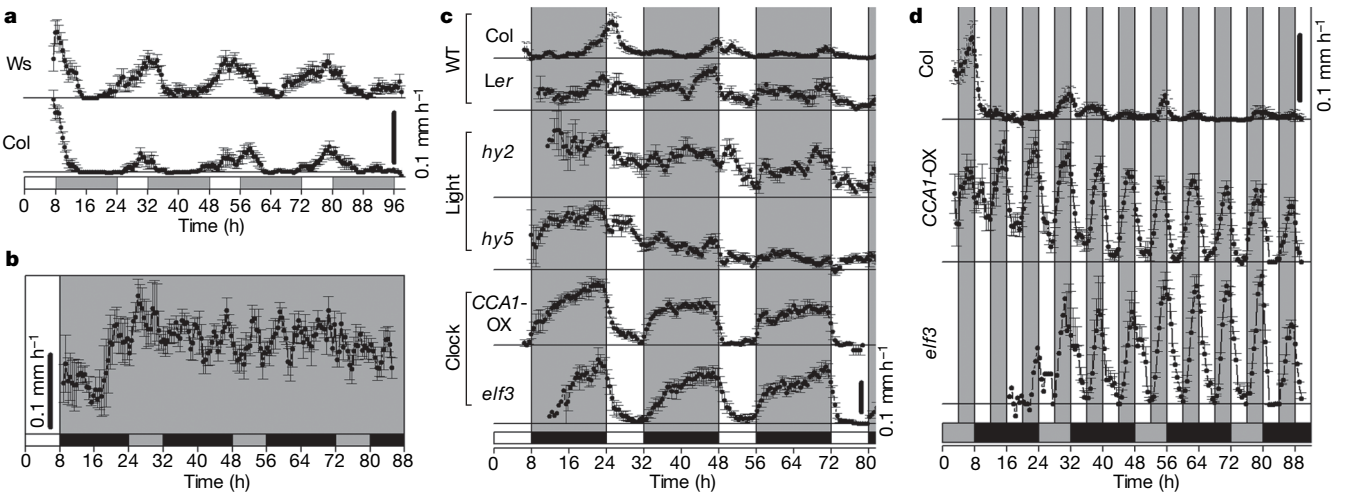

Figure 1 | Diurnal rhythms of hypocotyl elongation require light and the circadian clock. Plants were entrained for three days under short-day conditions and then switched to continuous light (**a**) or 4L:4D (**d**). Alternatively, plants were entrained for four days under short-day conditions and then switched to continuous darkness (**b**) or kept in short-day conditions (**c**). We used infrared imaging to monitor seedling growth (see Methods). Growth rate is plotted as a function of time; zero indicates dawn of the fourth day. The vertical scale bar indicates 0.1 mm h^{-1}. Measurements were started when hypocotyls were easily discernible, typically $t = 8$. The mean ± s.e.m. of at least two independent experiments is shown; $n \geq 6$ seedlings. In all plot areas, times of true light and darkness are indicated by clear and grey rectangles, respectively; see below for meaning of *x* axis rectangles. **a**, Rhythmic elongation of wild-type Col and Wassilewskija (Ws) hypocotyls in continuous light. White and grey bars on the *x* axis indicate subjective day and night, respectively. **b**, Continuous hypocotyl elongation of wild-type (WT) Col in continuous darkness. Grey and black bars on the *x* axis indicate subjective day and night, respectively. **c**, Hypocotyl elongation in short-day conditions. Col is the wild-type background for *CCA1*-OX, *elf3* and *hy2*; Landsberg *erecta* (L*er*) is the wild-type background for *hy5*. **d**, Growth in 4L:4D conditions is altered in clock mutants. Grey and black rectangles on the *x* axis indicate subjective day and night, respectively.

growth in part by inactivating the growth-promoting transcription factors PIF4 and PIF5, probably by degradation (S.L., P.D.D. and C.F., unpublished). During the first half of the night, the clock prevents growth by repressing transcription of *PIF4* and *PIF5*, maintaining the light-signalling pathway in an activated state. Closer to dawn, clock-mediated transcriptional repression of PIF4 and PIF5 fades, allowing their expression and the subsequent promotion of growth. Light is necessary for growth rhythms because it is required to inactivate or degrade PIF4 and PIF5; similarly, normal growth rhythms depend on the downregulation of the *PIF4* and *PIF5* message by the clock. Thus, normal diurnal growth patterns depend on interactions between internal (circadian) and external (light) cues. This is an example of an external coincidence model, originally proposed by Bünning[27] to explain photoperiodic regulation of a seasonal response—the transition from vegetative to reproductive growth.

It has been suggested that strong responses to light/dark transitions obscure circadian regulation of growth[6]. However, our results show that the opposite is true. The clock is critical in specifying wild-type growth patterns under light/dark cycles and can block acute responses to light/dark transitions. Circadian gating of acute responses is widely

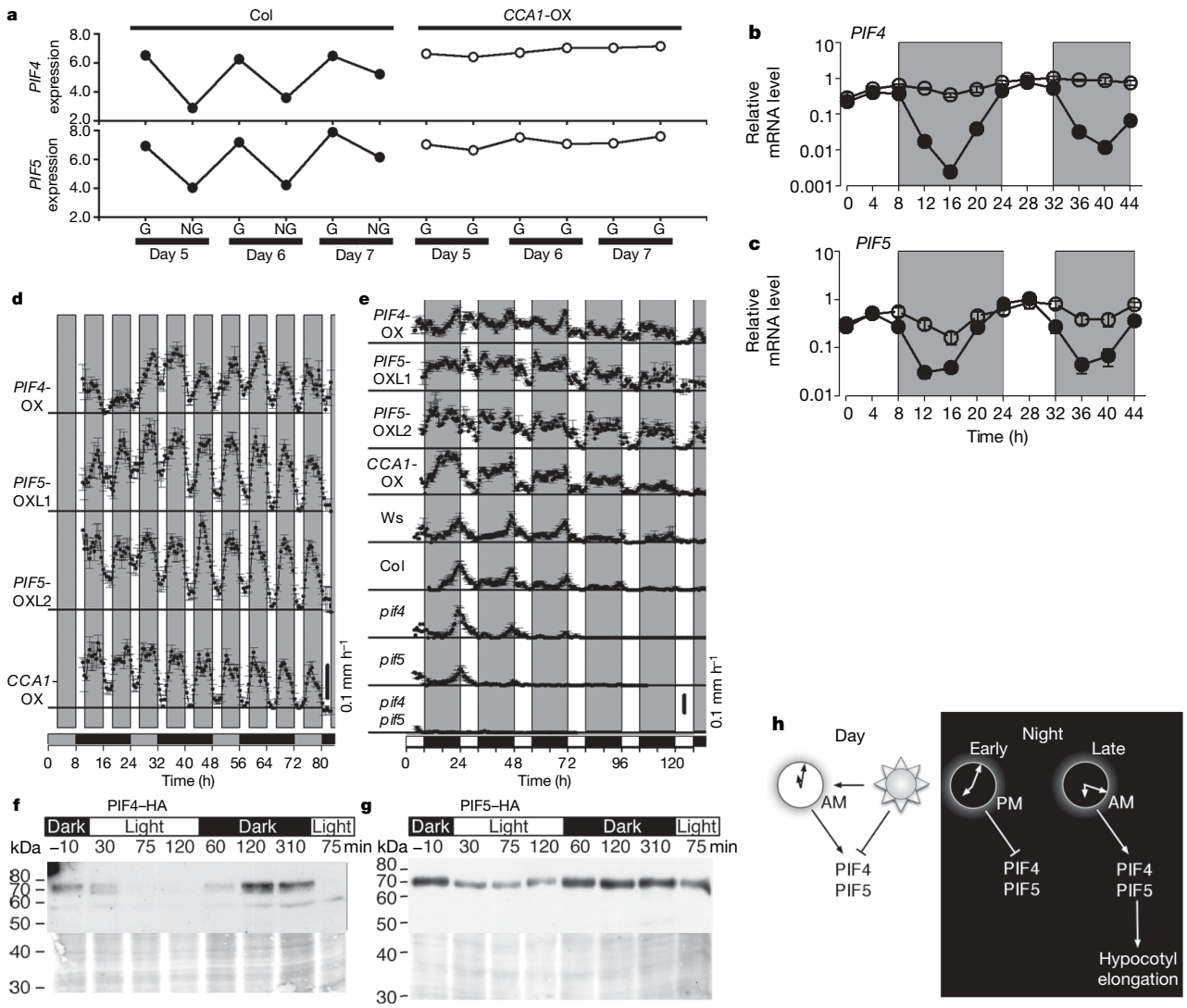

Figure 2 | Transcript and protein level regulation of light-signalling components in hypocotyl growth control. **a**, *PIF4* and *PIF5* expression patterns as assayed by microarray during 160 min light:320 min dark cycles (the light and dark period of a short day divided by 3, SD/3) are correlated with timing of hypocotyl elongation. Letters on the *x* axis indicate observed growth (G) or non-growth (NG) phenotypes at the time of collection. **b**, **c**, Diurnal expression patterns of *PIF4* (**b**) and *PIF5* (**c**) mRNA in wild-type and *CCA1*-OX plants as determined by qRT–PCR. Filled and open circles indicate expression level in Col and *CCA1*-OX, respectively. Zero indicates dawn of the fifth day. Shaded rectangles indicate darkness. The mean ± s.e.m. of two independent experiments, each with two replicates, are shown. **d**, *PIF4* and *PIF5* overexpression or knockout impairs clock regulation of growth. The experiment was performed as in Fig. 1d except SD/3 cycles were used instead of 4L:4D cycles and entrainment occurred over four days instead of three days. OXL1 and OXL2 refer to two independent lines overexpressing *PIF5* (see Methods). **e**, *PIF4* and *PIF5* overexpression or knockout alter rhythmic diurnal growth patterns. The experiment was performed as in Fig. 1c. **f**, **g**, PIF4 and PIF5 protein levels decrease in the light and increase in the dark. Lines constitutively expressing HA-tagged PIF4 (**f**) and PIF5 (**g**) were grown under SD/3. Times (in minutes) after transitions from dark to light and from light to dark are indicated. HA-tagged protein levels during the first cycle of day seven are shown in upper panels. Protein loading is shown in lower panels by Coomassie staining of blotted membranes. Two independent experiments were performed for two independent transgenic lines, and representative figures are shown here (PIF4–HA;OX5 in **f** and PIF5–HA;OX3 in **g**). kDa, kilodalton. **d**, **e**, *PIF4*-OX is driven by the PIF4 promoter, but expression is 25-fold higher than in the wild type[22]; **f**, *PIF4–HA* is driven from the cauliflower mosaic virus 35S promoter. **g**, Overexpression of PIF5 and PIF5–HA was achieved using the cauliflower mosaic virus 35S promoter. **h**, External coincidence model for rhythmic growth generation; see text for details.

known[3], but the mechanisms are mostly unidentified. Our finding that a dark-induced growth response is transcriptionally regulated by the clock provides a model that can be used to examine other gated responses. In addition, our finding that growth-promoting transcription factors are controlled by light and the clock by means of different regulatory mechanisms could provide a model for how other types of signalling pathways converge on common regulatory factors.

It is worth considering an ecological reason for rapid growth during late night. One possibility is that this allows plants to time growth to coincide with maximum water availability, because growth is among the first responses to be limited by water[28,29]. Another possibility is that this system allows plants to buffer their responses to acute changes in light, thereby growing only in response to extended periods of darkness.

Many modern studies of biological rhythms have been carried out in constant environmental conditions—a reductionist approach that has yielded important insights into the nature of the circadian clock. However, an understanding of how the circadian system functions in the real world will require more complex experimental conditions that better approximate the natural world. Using such conditions, we have uncovered a novel interaction among well-known regulatory networks that together regulate plant growth. Further investigation of how molecular networks respond to diurnal conditions will improve our understanding of how organisms live in and respond to their natural environment.

METHODS SUMMARY

Plant materials and growth condition. Plant materials are described in detail in the Methods. Sterilized seeds were plated in square plates. Seeds were stratified for four days and incubated under short-day cycles (8 h white fluorescent light:16 h dark (short-day conditions)) for three days. In most cases, monitoring of plant growth started from day four.

Time-lapse photography and image analysis. To acquire images in the dark, five seconds of background infrared illumination was given by infrared light-emitting diode (infrared-LED), and plant images were captured by an infrared-sensitive charge-coupled device camera. The infrared-LED was controlled via a USB port. Images were captured at 30-min intervals. Hypocotyl length was measured by Image J software. Data analysis was performed in the statistical environment R (http://www.R-project.org/).

RNA extraction, whole-genome expression analysis and qRT–PCR analysis. RNA was extracted by RNeasy Plant Mini kit (Qiagen). Affymetrix ATH1 genome arrays were used for the whole-genome expression assay. Robust multi-array averaging and rank product analysis were performed using Bioconductor in the R environment (Bioconductor /R) Detailed information is found in the Methods.

Western blot analysis. Details of generation of *Arabidopsis* plants overexpressing haemagglutinin (HA)-tagged PIF4 or PIF5 protein and methods for detecting PIF4–HA or PIF5–HA proteins levels are described in the Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K. & Johnson, C. H. The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. *Curr. Biol.* **14,** 1481–1486 (2004).
2. Dodd, A. N. *et al.* Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309,** 630–633 (2005).
3. Dunlap, J. C., Loros, J. J. & DeCoursey, P. J. (eds) *Chronobiology* (Sinauer Associates, Sunderland, Massachusetts, 2005).
4. Lecharny, A. & Wagner, E. Stem extension rate in light-grown plants. Evidence for an endogenous circadian rhythm in *Chenopodium. Physiol. Plant.* **60,** 437–443 (1984).
5. Ibrahim, C. A., Lecharny, A. & Millet, B. Circadian endogenous growth rhythm in tomato. *Plant Physiol.* **67,** 113 (1981).
6. Dowson-Day, M. J. & Millar, A. J. Circadian dysfunction causes aberrant hypocotyl elongation patterns in *Arabidopsis. Plant J.* **17,** 63–71 (1999).
7. Chen, M., Chory, J. & Fankhauser, C. Light signal transduction in higher plants. *Annu. Rev. Genet.* **38,** 87–117 (2004).
8. Bertram, L. & Karlsen, P. Patterns in stem elongation rate in chrysanthemum and tomato plants in relation to irradiance and day/night temperature. *Sci. Hortic.* **58,** 139–150 (1994).
9. Tutty, J. R., Hicklenton, P. R., Kristie, D. N. & McRae, K. B. The influence of photoperiod and temperature on the kinetics of stem elongation in *Dendranthema grandiflorum. J. Am. Soc. Hortic. Sci.* **119,** 138–143 (1994).
10. Bertram, L. & Lercari, B. Kinetics of stem elongation in light-grown tomato plants. Responses to different photosynthetically active radiation levels by wild-type and *aurea* mutant plants. *Photochem. Photobiol.* **66,** 396–403 (1997).
11. Gardner, M. J., Hubbard, K. E., Hotta, C. T., Dodd, A. N. & Webb, A. A. How plants tell the time. *Biochem. J.* **397,** 15–24 (2006).
12. Schaffer, R. *et al.* The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* **93,** 1219–1229 (1998).
13. Wang, Z. Y. & Tobin, E. M. Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* **93,** 1207–1217 (1998).
14. McWatters, H. G., Bastow, R. M., Hall, A. & Millar, A. J. The ELF3 *zeitnehmer* regulates light signalling to the circadian clock. *Nature* **408,** 716–720 (2000).
15. Covington, M. F. *et al. ELF3* modulates resetting of the circadian clock in *Arabidopsis. Plant Cell* **13,** 1305–1315 (2001).
16. Thain, S. C. *et al.* Circadian rhythms of ethylene emission in *Arabidopsis*. *Plant Physiol.* **136,** 3751–3761 (2004).
17. Duek, P. D. & Fankhauser, C. bHLH class transcription factors take centre stage in phytochrome signalling. *Trends Plant Sci.* **10,** 51–54 (2005).
18. Osterlund, M. T., Hardtke, C. S., Wei, N. & Deng, X. W. Targeted destabilization of HY5 during light-regulated development of *Arabidopsis. Nature* **405,** 462–466 (2000).
19. Kohchi, T. *et al.* The *Arabidopsis HY2* gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. *Plant Cell* **13,** 425–436 (2001).
20. Huq, E. & Quail, P. H. PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in *Arabidopsis*. *EMBO J.* **21,** 2441–2450 (2002).
21. Yamashino, T. *et al.* A link between circadian-controlled bHLH factors and the *APRR1/TOC1* quintet in *Arabidopsis thaliana*. *Plant Cell Physiol.* **44,** 619–629 (2003).
22. Khanna, R. *et al.* A novel molecular recognition motif necessary for targeting photoactivated phytochrome signaling to specific basic helix–loop–helix transcription factors. *Plant Cell* **16,** 3033–3044 (2004).
23. Fujimori, T., Yamashino, T., Kato, T. & Mizuno, T. Circadian-controlled basic/helix–loop–helix factor, *PIL6*, implicated in light-signal transduction in *Arabidopsis thaliana. Plant Cell Physiol.* **45,** 1078–1086 (2004).
24. Park, E. *et al.* Degradation of phytochrome interacting factor 3 in phytochrome-mediated light signaling. *Plant Cell Physiol.* **45,** 968–975 (2004).
25. Shen, H., Moon, J. & Huq, E. PIF1 is regulated by light-mediated degradation through the ubiquitin–26S proteasome pathway to optimize photomorphogenesis of seedlings in *Arabidopsis*. *Plant J.* **44,** 1023–1035 (2005).
26. Al-Sady, B., Ni, W., Kircher, S., Schafer, E. & Quail, P. H. Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. *Mol. Cell* **23,** 439–446 (2006).
27. Bünning, E. Die endogene Tagesrhythmik als Grundlage der photoperiodischen reaktion. *Ber. Dtsch. Bot. Ges.* **54,** 590–607 (1936).
28. Tsuda, M. & Tyree, M. T. Plant hydraulic conductance measured by the high pressure flow meter in crop plants. *J. Exp. Bot.* **51,** 823–828 (2000).
29. Hsiao, T. C. Plant responses to water stress. *Annu. Rev. Plant Physiol.* **24,** 519–570 (1973).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions K.N. performed all experiments. Statistical analysis of growth and microarray data was done by J.N.M. K.N. and J.N.M. wrote the paper. P.D.D., S.L. and C.F. contributed HA-tagged protein overexpressing plants, western blot protocols, and *pif4 pif5* double-mutant seed. M.F.C. contributed microarray experimental design. K.N., J.N.M. and S.L.H. contributed to project design. All authors discussed the results and commented on the manuscript.

Author Information The microarray data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE6906. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J.N.M. (jnmaloof@ucdavis.edu).

METHODS

Plant materials and growth conditions. Seeds of *CCA1*-OX (also known as *CCA1-34*) (ref. 13), *toc1-2* (ref. 30), *elf4-1* (ref. 31) and *hy2-103* (ref. 19) were provided by E. Tobin (University of California), S. Kay (The Scripps Research Institute), A. Millar (University of Edinburgh) and J. C. Lagarias (University of California, Davis), respectively. 35S-driven *PIF5*-OX lines (PIF5-OXL1 and 2, used in Fig. 2d and e and in Supplementary Figs 2 and 6) and *pif5*-knockout seeds[23] were provided by T. Mizuno (University of Nagoya). *PIF4*-OX and *pif4*-knockout (also called *slr2*) seeds[20] were provided by P. Quail (University of California, Berkeley). In this PIF4 overexpression line, designated *PIF4-OX* in this paper, PIF4 is driven by its native promoter but is expressed approximately 25-fold higher than in the wild type, presumably owing to the insertion site of the transgene[22]. This line was used for Fig. 2d and e and Supplementary Figs 2 and 6. Wild-type seeds (Col, C24, L*er* and Ws), *elf3-1*, *gi-2* and *hy5-1* were supplied by the *Arabidopsis* Biological Resources Center. Isolation of *pif4-101*, a T-DNA insertion allele, *pif4-101 pif5* double-mutants and plants overexpressing PIF4–HA or PIF5–HA constructs will be described elsewhere (S.L., P.D.D. and C.F., unpublished). The *PIF4–HA* and *PIF5–HA* genes are driven from the 35S promoter and were used for experiments shown in Fig. 2f and g. These constructs rescue their respective knockouts, showing that they encode functional proteins (data not shown). *PIF5–HA* shows growth patterns similar to the untagged *PIF5*-OX, whereas the *PIF4–HA* growth pattern is more similar to the wild type, owing to low expression relative to *PIF4*-OX.

For hypocotyl growth rate measurements, seeds were surface-sterilized with 70% ethanol and 0.1% Triton X-100 for 5 min followed by 95% ethanol for 1 min. Sterilized seeds were resuspended in sterile water and plated in two rows on 40 ml of 1 × MSMO (Murashige and Skoog minimal organics medium without sucrose; M6899, Sigma), 3% sucrose and 1% Phytagar (Invitrogen) in a 10 cm square plate (4021, Nalge Nunc). Seeds were stratified at 4 °C for four days and incubated under short-day cycles for three days. In most cases, monitoring of plant growth started from day four. Light was provided by cool white fluorescent lamps (OSRAM Sylvania) with a fluence rate of 62 μmol m^{-2} s^{-1}. Three layers of screens (Silver Gray Fibreglass, Phifer Wire Products) were used to reduce light intensity to 12 μmol m^{-2} s^{-1} for continuous light and long-day experiments; one layer of screen and adjustment of distance from the light source was used to reduce light intensity to 47 μmol m^{-2} s^{-1} for 12L:12D experiments. The Petri dishes were tilted about 30° from the vertical during entrainment and assays.

For RNA extractions for microarray analysis, ~100 seedlings were grown on filter paper laid on 1 × MSMO, 3% sucrose and 0.9% agar (Cat A1296, Sigma) in 6 cm round Petri dishes. Seeds were stratified as above and then incubated for four days at 20 °C under short-day conditions. The Petri dishes were tilted about 30° from vertical in a growth chamber equipped with red and blue LEDs (70.7 μE red light and 15.3 μE blue light). Starting from subjective dawn at day five, cycles of 160 min light and 320 min dark (SD/3) were given for three days (Supplementary Fig. 5). Samples were collected two hours after lights were turned off, transferred to 1.5 ml tubes, and immediately frozen by liquid nitrogen and stored at −80 °C. Samples were collected using infrared goggles (Night Vision model NCB4, Night Owl Optics) to avoid exposing plants to visible light.

For qRT–PCR, 10–20 seedlings were grown on 1 × MSMO, 3% sucrose and 0.9% agar plates without filter paper. Seedlings were collected at four-hour intervals from day four to day six (for those under short-day conditions) or from day five to day six under continuous light after four days of short-day entrainment (for those in continuous light).

For western blot analysis, 10–20 seedlings were grown on 1 × MSMO, 3% sucrose and 0.9% agar plates without filter paper. Plants were entrained in short-day conditions for four days and then switched to SD/3 conditions. During the first SD/3 cycle of the ninth day, seedlings were collected 10 min before lights were turned on, then after 30, 75 or 120 min in the light, then after 60, 120 or 310 min in the subsequent dark period, and finally after 75 min in the following light period.

Time-lapse photography and image analysis. To acquire images in the dark, five seconds of background infrared illumination was given by infrared-LED (276-143, Radioshack) with a diffusion filter (R111, Rosco), and plant images were captured by an infrared-sensitive charge-coupled device camera (MEGA-DCS, Videre Design) equipped with a close-up lens (HF25HA-1B, Fujinon). The infrared-LED was controlled by a USB controller (RUSB-PO8/8(R), Rabbit House) using a C^{++} program. Images (1,280 × 960 pixels) were captured at 30-min intervals by a custom-written C^{++} program through a FireWire connection. Hypocotyl length was measured by Image J software (http://rsb.info.nih.gov./ij/). Data analysis was performed in the statistical environment R (ref. 32; http://www.R-project.org/). To compare dark responsiveness of different genotypes (Supplementary Fig. 2), the average growth rate during the last three hours of the light period was subtracted from the average growth rate during the first three hours in the dark and then averaged across seedlings. Larger values of this 'dark responsiveness index' indicate larger growth rate increases in response to darkness. R scripts are available on request.

RNA extraction and microarray analysis. Our experimental design is based on the observed growth patterns of wild type and *CCA1*-OX under 4L:4D cycles. We reasoned that expression of genes of interest would correlate with growth patterns in the dark. Plants were grown in SD/3 conditions (see above) and whole seedlings were collected 280, 1,240, 1,720, 2,680, 3,160 and 4,120 min after dawn on the fifth day (Supplementary Fig. 5). These time points occur 2 h after lights were turned off in the first and third dark period of each day, over three days. Col grows during the first but not the third dark period, so time points 280, 1,720 and 3,160 were coded as growing (G) for Col and were treated as biological replicates. Time points 1,240, 2,680 and 4,120 were coded as non-growing (NG) for Col and were treated as replicates. All time points were coded as growing for *CCA1*-OX and were treated as replicates. 100 mg frozen tissue samples were ground by pestle and electric motor. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen). From 5 μg of total RNA, complementary RNA was made and labelled with biotin (Affymetrix) according to the protocol used in ref. 33. 15 μg of the resulting fragmented cRNA was hybridized to Affimetrix ATH1 genome array and images were taken by the Affymetrix GeneChip 3000 Scanner. Hybridization and scanning were done at the UC Davis School of Medicine Microarray Core Facility (http://www.ucdmc.ucdavis.edu/medmicro/microarray.html). Robust multi-array averaging[34] and rank product analysis[35] were performed using the affy and RankProd packages, respectively, in Bioconductor /R[36]. Two contrasts were made by Rank Product: first, Col G versus Col NG (growing versus non-growing conditions) and, second, CCA1 G versus Col NG (also growing versus non-growing). Genes were designated as being significantly up- or down-regulated if they had a false-discovery rate value less than 0.1 for both of these contrasts. Gene annotations are based on the latest version of the *Arabidopsis* genome (TAIR6) at TAIR (http://www.arabidopsis.org/) and published literature.

RNA extraction and qRT–PCR analysis. Total RNA was extracted from about 10–20 seedlings using RNeasy Plant Mini kit (Qiagen). The RNA was treated with DNaseI on columns (Qiagen), and 500 ng of eluted RNA was used for complementary DNA synthesis using iScript (Biorad) for samples grown in short-day conditions or SuperScript III reverse transcriptase (Invitrogen) with custom-made oligo-dT (18 nucleotides T). 4 μl of 40-fold diluted cDNA was added to 25 μl PCR buffer containing SYBR Green I and TAQ polymerase (IQ SYBR Green Supermix, BioRad), and fluorescence was detected using an iCycler (Biorad). Primers and PCR conditions for *PP2A* subunit (At1g13320), *PIF4* and *PIF5* were as described in refs 37, 38, except that 50 cycles of two-step PCR were used. Primers for *CCA1*, *TOC* and *GIGANTEA* (*GI*) are described in ref. 39. Data were obtained from two replicate samples in each of two duplicate experiments. The expression of each gene within each sample was normalized against *PROTEIN PHOSPHATASE 2A* (*PP2A*) subunit, and expressed relative to a calibrator sample with the use of the formula $2^{-\Delta\Delta CT}$ as described in ref. 40. ΔΔCT is defined as: [CT gene of interest (unknown sample)–CT *PP2A* (unknown sample)]–[CT gene of interest (calibrator sample)–CT *PP2A* (calibrator sample)], where CT denotes the threshold cycle for detection of PCR product. The expression of *PP2A* does not vary significantly across public microarray data[38]. The calibrator sample was designated as the most highly expressed time point for each gene of interest, and therefore has an expression of 1.0.

Western blot analysis. Generation of *Arabidopsis* plants overexpressing HA-tagged PIF4 or PIF5 protein will be described elsewhere (S.L., P.D.D. and C.F., unpublished). Methods for detecting PIF4–HA or PIF5–HA protein levels are described in ref. 41, except that anti-HA-peroxidase (Roche) was used at 1:1,000 dilution. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) was used for the peroxidase substrate to detect signals.

30. Strayer, C. *et al.* Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* **289**, 768–771 (2000).
31. Doyle, M. R. *et al.* The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. *Nature* **419**, 74–77 (2002).
32. R Development Core Team. *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Vienna, Austria, 2005).
33. Schmid, M. *et al.* A gene expression map of *Arabidopsis thaliana* development. *Nature Genet.* **37**, 501–506 (2005).
34. Irizarry, R. A. *et al.* Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264 (2003).
35. Breitling, R., Armengaud, P., Amtmann, A. & Herzyk, P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett.* **573**, 83–92 (2004).
36. Gentleman, R. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).

37. Czechowski, T., Bari, R. P., Stitt, M., Scheible, W.-R. & Udvardi, M. K. Real-time RT–PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant J.* **38,** 366–379 (2004).
38. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K. & Scheible, W.-R. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* **139,** 5–17 (2005).
39. Mockler, T. C. *et al.* Regulation of flowering time in *Arabidopsis* by K homology domain proteins. *Proc. Natl Acad. Sci. USA* **101,** 12759–12764 (2004).
40. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25,** 402–408 (2001).
41. Duek, P. D., Elmer, M. V., van Oosten, V. R. & Fankhauser, C. The degradation of HFR1, a putative bHLH class transcription factor involved in light signaling, is regulated by phosphorylation and requires COP1. *Curr. Biol.* **14,** 2296–2301 (2004).