Dawn and photoperiod sensing by phytochrome A

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In plants, light receptors play a pivotal role in photoperiod sensing, enabling them to track seasonal progression. Photoperiod sensing arises from an interaction between the plant's endogenous circadian oscillator and external light cues. Here, we characterise the role of phytochrome A (phyA) in photoperiod sensing. Our meta-analysis of functional genomic datasets identified phyA as a principal regulator of morning-activated genes, specifically in short photoperiods. We demonstrate that *PHYA* expression is under the direct control of the PHYTOCHROME INTERACTING FACTOR transcription factors, PIF4 and PIF5. As a result, phyA protein accumulates during the night, especially in short photoperiods. At dawn phyA activation by light results in a burst of gene expression, with consequences for physiological processes such as anthocyanin accumulation. The combination of complex regulation of *PHYA* transcript and the unique molecular properties of phyA protein make this pathway a sensitive detector of both dawn and photoperiod.

phytochrome | photoperiodism | systems biology

Introduction

As photosynthetic organisms, plants are highly tuned to the external light environment. This exogenous control is exerted by photoreceptors, such as five member phytochrome family (phyA-E), that, in turn, regulate the activity of key transcription factors. An important feature of phytochrome signalling is that it can be strongly influenced by the plants internal circadian clock, which operates as a master regulator of rhythmic gene expression (1). The interplay between phytochrome signalling and the clock aligns daily gene expression profiles to shifts in day-length. These adjustments and associated post-transcriptional events form the basis of photoperiodic sensing, coordinating molecular, metabolic and developmental responses to the changing seasons.

Earlier work has shown that light and the clock interact through so called "external coincidence" mechanisms to deliver photoperiodic control of responses such as flowering time and seedling hypocotyl growth (2, 3). Previously we used a modelling approach to assess the functional characteristics of these two external coincidence mechanisms (4). An important component of our study was the analysis of published genomics data that allowed us to identify new network properties and to test the applicability of our model to the broader transcriptome. This work highlighted the huge potential of data mining approaches to uncover new molecular mechanisms of external coincidence signalling.

A well characterised external coincidence mechanism involves the PHYTOCHROME INTERACTING FACTOR transcription factors PIF4 and PIF5, that regulate rhythmic seedling hypocotyl growth in short photoperiods. Sequential action of the clock Evening Complex (EC) and phyB defines the photoperiodic window during which PIF4/5 can accumulate. Light activated phyB negatively regulates PIF4/5 by triggering their proteolysis and by sequestering PIFs from their target promoters (5, 6). The EC, comprising EARLY FLOWERING 3 (ELF3), EARLY FLOWERING 4 (ELF4), and LUX ARRHYTHMO (LUX), is a transcriptional repressor that has a post-dusk peak of activity. Nights longer than 10-12h exceed the period of EC action, allowing *PIF4/5* to accumulate and regulate gene expression specifically in long nights. The period of PIF activity is abruptly terminated at dawn, following activation of phyB by light. This external coincidence module therefore delivers a diurnal control of growth that is only active in short-day photocycles and becomes more robust as the night lengthens.

The diurnal PIF growth module is a clear example of how phyB contributes to photoperiod sensing. The phytochrome family share a set of core characteristics that enable tracking of changes in light quality and quantity, such as those that occur at dawn. The phytochrome chromoproteins exist in two isomeric forms, inactive Pr and active Pfr, that absorb in the red (peak 660nm) and far-red light (peak 730nm), respectively. Red light (R) drives photoconversion from Pr to Pfr, while far-red (FR) light reverses this process. This so called R/FR reversibility allows phytochromes to operate as biological light switches that respond to light spectra and intensity. Once formed, the active Pfr translocates from the cytosol to the nucleus to perform its signalling functions.

The photochemistry of phytochrome signalling is conserved across the phytochrome family. However, phyA exhibits unique signalling features, including nuclear translocation kinetics and protein stability. As a result, the responses of phyA to light are distinctive. For example, phyB-E responses are classically R/FR reversible, while phyA responses are not. Instead, phyA is tuned to detect continuous FR-rich light, indicative of close vegetation, in the so-called far-red high irradiance responses (FR-HIR) (7). phyA also initiates very low fluence responses that are important for activating germination and de-etiolation in low light scenarios (e.g. when shielded by vegetation). Another distinguishing feature is that unlike phyB-E, that are light stable, phyA is unstable in the presence of light. These characteristics mean that in photoperiodic conditions phyA protein levels are

Significance

The changing seasons subject plants to a variety of challenging environments. In order to deal with this, many plants have mechanisms for inferring the season by measuring the duration of daylight in a day. A number of well-known seasonal responses such as flowering are responsive to daylength or photoperiod. Here, we describe how the photoreceptor protein phytochrome A senses short photoperiods. This arises from its accumulation during long nights, as happens during winter, and subsequent activation by light at dawn. As a result of this response, the abundance of red anthocyanin pigments is increased in short photoperiods. Thus, we describe a mechanism underlying a novel seasonal phenotype in an important model plant species.

Reserved for Publication Footnotes

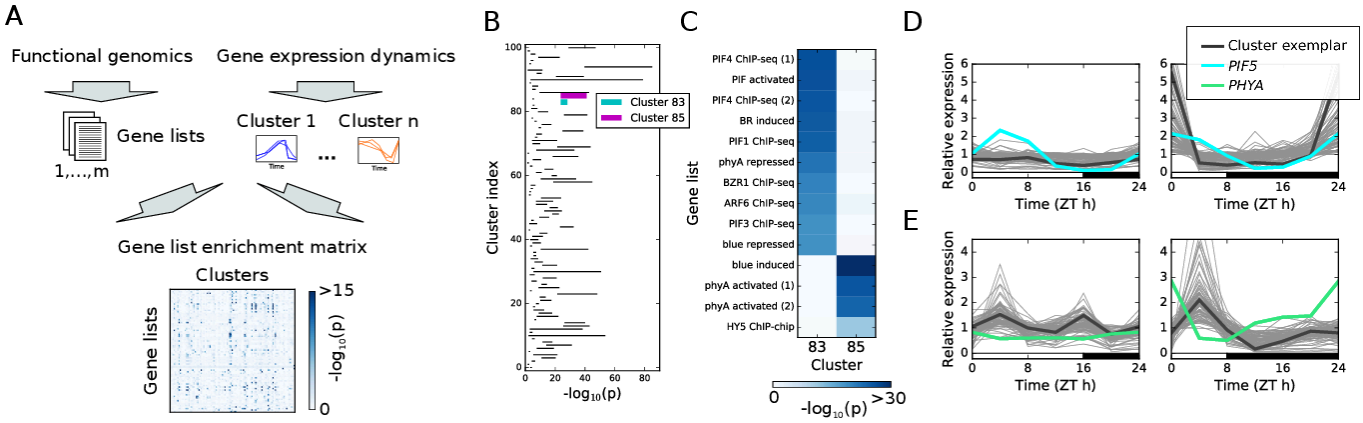

Fig. 1. Mining functional genomic data for active gene regulatory networks. (A) Flowchart of data integration. Genes were clustered together according to their dynamics in a range of conditions. Functional genomic datasets (e.g. ChIP-seq, RNA-seq) were curated from literature in the form of gene lists. Each cluster was then tested for over-enrichment of each gene list (hypergeometric test). (B) Top gene list enrichment scores across all clusters. Vertical lines indicate the range spanned by the three top-scoring enrichments. (C) Highlighted enrichment tests for clusters 83 and 85, which are enriched for distinct subsets of phytochrome-related gene lists. (D) Short day, night-specific expression of cluster 83, and its relationship with *PIF5* expression. (E) Short day, morning-specific expression of cluster 85, and its relationship with *PHYA* expression.

robustly diurnal (8), though it is not clear what drives phyA re-accumulation during the night.

Considerable progress has been made in understanding the molecular mechanisms of phyA signalling (7). Upon exposure to R or FR light, phyA is activated and moves from the cytosol to the nucleus. Nuclear import requires the NLS-containing helper proteins FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-like (FHL) (9). In the nucleus, phyA Pfr negatively regulates several proteins through direct interaction, including the PHYTOCHROME INTERACTING FACTOR (PIF) transcription regulators, the E3 ligase component CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), and SUPPRESSOR OF PHYA-105 1-4 (SPA1-4) (10, 11). The COP1/SPA complex targets several transcription regulators, including LONG HYPOCOTYL 5 (HY5), LONG HYPOCOTYL IN FAR-RED 1 (HFR1), and LONG AFTER FAR-RED LIGHT 1 (LAF1), for degradation (12-14). Through the regulation of this suite of transcription factors, phyA can modulate the expression of thousands of genes (15-17).

The activity of the phyA signalling pathway is regulated at multiple levels. The timing of *PHYA* expression is controlled by the circadian clock (18), and by light, though the underlying molecular mechanisms are unknown. phyA protein is both activated and destabilised by light (19). Thus, understanding phyA signalling requires understanding the interplay between these layers of regulation. This can be achieved by analysing dynamics of phyA regulation and action through different photoperiods where the competing regulatory signals converge at different times. Previously we have constructed mathematical models to understand photoperiodic control of flowering and PIF-mediated growth (4). This approach has been particularly useful for identifying non-intuitive pathway behaviours that arise from complex regulatory dynamics.

In this paper, we combine analysis of genome-scale datasets, mathematical modelling, and experimentation to unravel the molecular mechanisms of phyA regulation in light/dark cycles. We show that *PHYA* is directly targeted by the transcription factors PIF4 and PIF5. These transcription factors are under the dual control of light (via phytochromes (5)) and the circadian clock (via the evening complex (20)). This regulation results in dynamic regulation of *PHYA* transcript abundance, leading to high accumulation at night in short photoperiods. At dawn, phyA then induces the expression of hundreds of genes, including genes involved in anthocyanin biosynthesis. This firmly establishes a role for phyA as a sensor of dawn and short photoperiods.

Results

Data mining identifies phyA as a potential short-photoperiod sensor. Our previous work applied data mining methods to derive new molecular understanding of light signalling (4). In this study we used data mining to identify gene regulatory mechanisms that respond to changing photoperiod. This approach was made possible by the high quality transcriptomic and ChIP data available for diurnal and light-controlled gene expression (Table S1; Datafile 1). To do this we developed a computational workflow combining co-expression clustering and gene set enrichment (Fig 1A). First, genes were clustered on the basis of expression in a variety of conditions, focussing on different light conditions, and mutants of circadian and light signalling pathways (see Table S1 for a description of datasets). Importantly, this included gene expression in long days (16h light: 8h dark (8L:16D); LDs) and short days (16L:8D; SDs). This procedure identified 101 co-expression clusters (Datafile 2).

To identify regulatory mechanisms, we assessed a broad range of potential regulatory pathways. To do this, we consolidated 527 gene lists from available datasets. This consisted of 140 gene lists from 47 papers, covering a broad range of regulatory pathways (e.g. hormone signalling, transcription factors; see Datafile 1 for descriptions), combined with a further 387 transcription factor binding datasets generated in high throughput by DNA affinity purification sequencing (DAP-seq) (21). For each cluster of co-expressed genes, if there is a significant overlap between a particular gene list and the genes in a particular cluster, it can suggest regulatory mechanisms. Here, enrichment was quantified by the p-value of overlap between gene sets and clusters (hypergeometric test; see Datafile 3 for all calculated values). Similar approaches have previously been used to identify gene regulatory networks in a variety of contexts (e.g. (22, 23)). Analogous approaches include the identification of promoter motifs by enrichment in given gene sets (e.g. (24)). We developed a simple software tool, AtEnrich ("Arabidopsis thaliana gene list Enrichment analysis"), for performing enrichment analysis of these gene lists (https://github.com/danielseaton/atenrich).

Enrichment analysis identified many significant associations, with 37 of 101 clusters enriched with at least one gene set at $p < 10^{-20}$ (Fig 1B). As expected, this highlighted roles for circadian and light signalling factors in controlling the diurnal

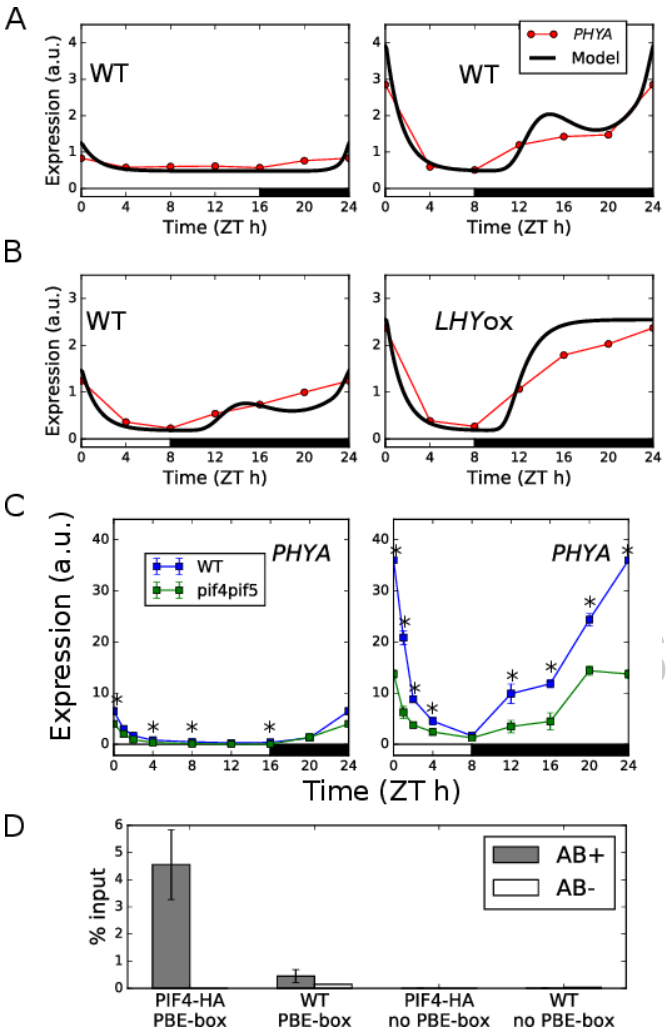

Fig. 2. *PHYA* expression is directly regulated by PIF4 and PIF5 (A, B) Comparison of model simulations and microarray data for *PHYA* in short compared to long photoperiods (A) and WT (Ler) compared to *LHYox* in 8L:16D light/dark cycles (B) (data from (24)). (C) PHYA expression in short and long photoperiods, in the WT (Col-0) and the *pif4 pif5* mutant. Plants were grown for 2 weeks in the given photoperiod. Expression was measured relative to *ACT7*. (n=3, error bars represent SEM, ZT0 timepoint re-plotted at ZT24). (D) ChIP-qPCR of PIF4 binding to the *PHYA* promoter. Plants were grown for two weeks in short days (8L:16D white light, 100 µmol m^{-2} s^{-1}) at 22°C, and samples were collected at the end of the two weeks at ZT0 (n=3, error bars represent SEM).

dynamics of gene expression. For example, Cluster 83 is regulated by the *PIF4/PIF5* pathway, that controls changes in hypocotyl elongation with photoperiod (4, 25) (Fig1C,D). Targets of the PIF family of transcription factors have been identified by ChIP-seq (26-28), as have targets of PIF-interacting proteins AUXIN RESPONSE FACTOR 6 (ARF6) and BRASSINAZOLE-RESISTANT 1 (BZR1) (29). Cluster 83 is strongly enriched for all of these gene lists ($p<10^{18}$; hypergeometric test; Fig 1C). The expression profile of cluster 83 genes in long days (16L:8D) and short days (8L:16D) is consistent with regulation by the PIF4 and PIF5 transcription factors. This is illustrated in Fig 1D, with higher night-time levels of *PIF5* transcript in short photoperiods, and higher night-time expression of genes in this cluster. As expected, this cluster includes well-known markers of PIF activity including *ATHB2*, *IAA29*, *HFR1*, and *CKX5* (30).

Phytochrome signalling, and in particular phyA, is also implicated in the regulation of cluster 85. This cluster is enriched for genes responding rapidly to red light in a phyA-dependent manner (16), and genes responding to far red light in a phyA-dependent manner (15) (Fig 1C). Furthermore, it is enriched for genes bound by the transcription factor HY5 (31), which is stabilised by phyA via its interaction with COP1 (32). This cluster of genes also displays a pattern of gene expression consistent with sensitivity to light, with a peak in expression following dawn (Fig 1E). The size of this peak changes with photoperiod, and is especially pronounced in short photoperiods (Fig 1E). Interestingly, the expression of these genes in the morning is correlated with expression of *PHYA* during the preceding night, which is higher during the night in short photoperiods (Fig 1E). Therefore, we proceeded to investigate the photoperiodic regulation of *PHYA* expression, and the implications of this for the seasonal control of gene expression of this set of genes.

A model of PIF activity predicts *PHYA* expression dynamics. Previous reports have indicated that phyA protein accumulates in etiolated seedlings and during the night in a diurnal cycle through an unknown process (7,33). As highlighted by earlier studies and our clustering analysis, the PIF family of transcription factors display a similar pattern of activity (3, 4, 25). Furthermore, our previous analysis of gene expression dynamics identified *PHYA* as a putative target of PIF4 and PIF5 (4).

In order to assess the plausibility of the hypothesised regulation of *PHYA* expression by PIF4/5, we tested whether our model of PIF4/5 activity could explain *PHYA* dynamics in different photoperiods and circadian clock mutants, as measured by microarray experiments in a previous study (24). In short days (8L:16D), both model and data exhibit rhythmic *PHYA* expression with an end of night peak (Fig 2A). In long days (16L:8D), however, expression is low throughout the day and night (Fig 2A). The model also matches the measured response of *PHYA* expression at end of night and end of day across multiple photoperiods (Fig S1). Finally, the model matches the exaggerated nocturnal rise in *PHYA* observed in two circadian clock mutants - the *lux* mutant and *LHY* overexpressor (Fig 2B, Fig S3A). These mutants are notable for exhibiting weak evening complex activity, with a resultant increase in *PIF4* and *PIF5* expression during the night. In summary, a model of PIF4/5 regulation of *PHYA* is able to explain differences in *PHYA* expression across environmental conditions and genotypes. Interestingly, the *PHYA* cofactor *FHL* (also identified as a likely PIF4/5 target in (4)) shows similar patterns of expression across the microarray datasets inspected here, and its expression can also be explained by the model of PIF4/5 activity (Figs S2, S3). This suggests that PIF4/5 regulate both *PHYA* and *FHL*, and therefore may exert significant influence on the activity of the phyA signalling pathway.

PIF4 and PIF5 directly regulate *PHYA* expression. To further establish a role for PIF4 and PIF5 in regulating *PHYA* and *FHL* expression, we measured mRNA levels by qPCR in Col-0 (wild type) and *pif4 pif5* plants, in short (8L:16D) and long (16L:8D) photoperiods. This revealed the expected *PHYA* expression profile, with transcript levels rising to much higher levels during the night in a short day compared to in a long day, and markedly reduced in the *pif4 pif5* mutant specifically in short photoperiods (Fig 2C). This was reduced further in the *pifQ* mutant, that lacks PIF1 and PIF3 in addition to PIF4 and PIF5 (Fig S4). Furthermore, a similar pattern was observed for *FHL* (Fig S4). As for transcript, phyA protein accumulates to higher levels in short days compared to long days (Fig S5A), and its levels at ZT0 in short days are reduced in the *pif4 pif5* and *pifQ* mutants (Fig S5B). These data suggest that PIFs may act collectively to regulate phyA abundance.

The strong coordination between *PHYA* expression and PIF activity across many conditions suggested that this regulation might be direct. Numerous ChIP-seq analyses of the PIF family have been performed across a range of conditions (26-28, 33).

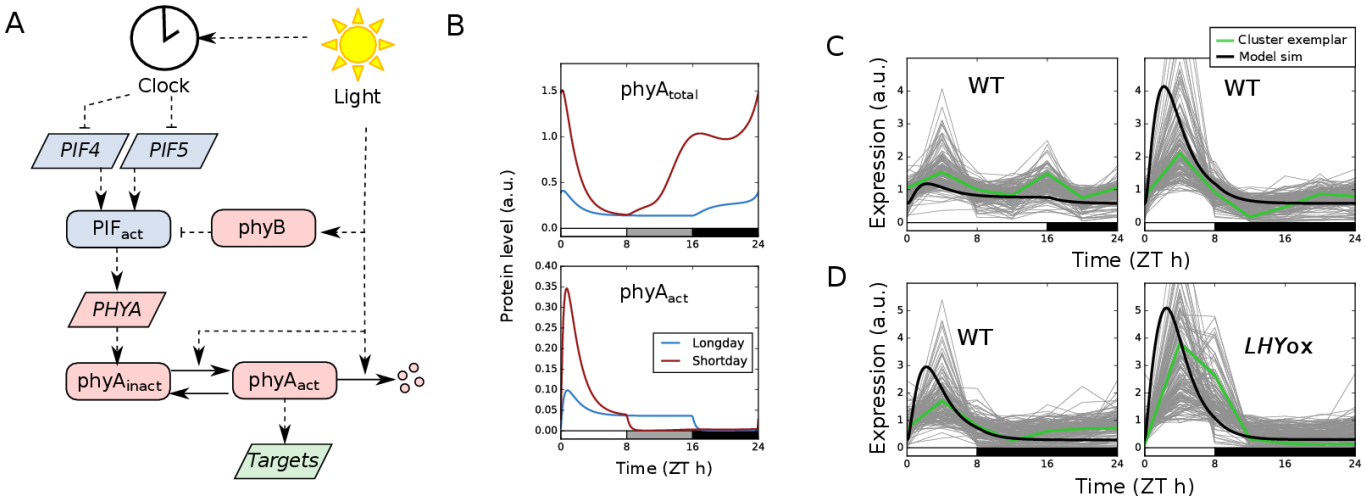

Fig. 3. A model of phyA signalling predicts gene expression dynamics. (A) Model schematic. Solid lines represent mass transfer, dashed lines represent regulatory effects. Transcripts are represented by trapezoids, proteins by rectangles. (B) Simulation of a simple model of phyA signalling in short and long photoperiods. (C, D) Gene expression of the putative phyA-regulated cluster of co-expressed genes, compared to model simulations, in photoperiods (C), and LHYox (D) (data from (24)).

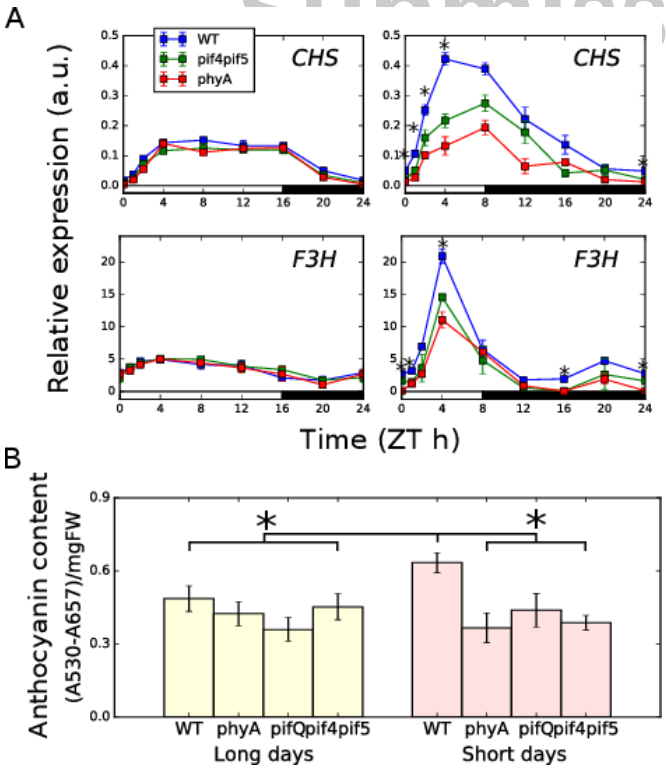

Fig. 4. Anthocyanin accumulation is regulated by phyA in a photoperiod-specific manner. (A) qPCR timecourse data for *F3H* and *CHS* in long and short days (LD, SD, respectively), in WT (Col-0), *pif4 pif5*, and *phyA*. Expression is relative to *ACT7*. Plants were grown for 2 weeks at 22°C under 100 µmol m-2 s-1 white light in the specified photoperiod (* indicates significant difference at p<0.05 between WT and both *pif4 pif5* and *phyA*, two-tailed t-test, n = 3, error bars represent SEM) (B) Anthocyanin accumulation in the same conditions as (A), also including the *pifQ* mutant. (* indicates difference from WT in short days at p < 0.01, one-tailed t-test, n = 3, error bars represent SD).

Among these, only Oh *et al.* (33) has found direct binding of a PIF (PIF4) to the *PHYA* promoter, in deetiolated seedlings. In order to test direct regulation of *PHYA* by PIFs in our conditions, we performed ChIP for PIF4-HA and PIF5-HA on the *PHYA* promoter in plants grown in short days, focussing on a region with a PIF-binding E-box (PBE) element (CACATG; (28)). The results of this are shown in Fig 2D (PIF4) and Fig S6 (PIF5), with enrichment of PIF4-HA and PIF5-HA at the *PHYA* promoter. Thus, PIF4 and PIF5 appear to regulate *PHYA* expression by direct binding to its promoter in short days.

PIFs regulate phyA action specifically in SDs. Additional support for PIF4 and PIF5 as SD regulators of *PHYA* comes from a hypocotyl elongation experiment. When supplied continuously, far-red light activates phyA in an HIR mode (19). We used this unique photochemical property to provide a readout for phyA activity through the night of SD- and LD-grown seedlings. Our data show that 4h of FR light (delivered at the end of the night (EON)) suppresses hypocotyl elongation in a phyA and PIF-dependent manner in SDs but not LDs (Fig S7). To rule out any potential influence of phyB and other light stable phytochromes on phyA action we also provided brief end-of-day (EOD) far-red treatments that switch these phytochromes to their inactive Pr conformer. As expected, EOD deactivation of phyB enhanced hypocotyl elongation in WT and *phyA* seedlings, and this was more marked in SDs. Delivery of prolonged (EON) far-red to EOD-far-red treated seedlings led to phyA-suppression of hypocotyl elongation, a response that was markedly reduced in *pif4 pif5* and *pifQ* mutants. These photo-physiological experiments provide robust support for our central hypothesis that the photoperiodic phyA regulation is largely conferred by SD PIF action.

phyA mediates a photoperiod-dependent acute light response. Differences in phyA accumulation during the night are expected to result in differences in phyA activity during the following day. In order to assess this, we developed a model of phyA signalling mechanisms, combining our model of PIF regulation with a simplified version of the model of Rausenberger et al. (34) (see SI Appendix for details; Fig 3A). In this model, phyA signalling activity is high when light is present and phyA protein is abundant. The rapid decrease in the level of phyA protein after dawn means that phyA activity peaks in the early morning. This pulse in the expression of downstream genes is termed an 'acute light response' (35). This is illustrated in Fig 3B, showing simulations of the combined clock-PIF-phyA model in short and long photoperiods.

The model predicts that the changing activity of PIFs across different photoperiods and genotypes changes the amplitude of the acute light response (Fig 3B). In particular, it predicts that

the amplitude of the acute light response at dawn is increased in short photoperiods, as well as in the *LHYox* line and the *lux* mutant (i.e. conditions with high *PHYA* expression during the night). The genes in the putative phyA-regulated cluster (cluster 85) display these dynamics (Fig 3 C,D). The model is also able to make predictions for gene expression dynamics during seedling deetiolation, in which dark-grown seedlings are exposed to red light (Fig S8A). Here, the model predicts a diminished amplitude of response in the *pifQ* mutant during deetiolation in red light (Fig S8B). Again, the model correctly predicts the expression of genes in cluster 85 across these conditions in microarray data from plants grown in darkness and treated with red light for 1h, or grown in continuous red light (36) (Fig S8C). Together, these results demonstrate that our molecular understanding of this pathway is consistent with phyA regulation of cluster 85, as expected based on its enrichment for phyA-associated terms in our meta-analysis of functional genomic datasets (Fig 1C).

In order to further test the model prediction of phyA activity, we investigated the regulation of the dawn-induced circadian clock gene *PSEUDO RESPONSE REGULATOR 9* (*PRR9*), a known target of phyA signalling (34). Measurement of *PRR9* expression in *pif4 pif5* and *phyA* demonstrates that *PRR9* is indeed regulated by phyA, with reduced expression in both mutants, specifically in short photoperiods (Fig S9A). Given the effect of phyA on *PRR9* expression, we hypothesised that this regulation would affect the expression of other circadian clock genes. However, the expression of core clock genes *PRR7*, *TOC1*, *GI*, *LUX*, and *ELF4* displayed limited changes in *phyA* and *pif4 pif5* mutants in short and long days (Fig S9B).

In summary, this cluster of putative phyA targets displays expression dynamics consistent with our mechanistic understanding of phyA signalling, as captured by our mathematical model. This further implicates phyA as a key regulator of these genes.

phyA confers photoperiodic control of anthocyanin accumulation. Our results demonstrate that phyA-mediated acute light responses are amplified in short photoperiods. Therefore, we expect short photoperiods to exaggerate *phyA* mutant phenotypes. In order to identify potential phenotypes of interest, we assessed enrichment of gene ontology (GO) terms within the cluster of putative phyA targets. This identified highly significant enrichment for anthocynanin and flavonoid biosynthesis (GO:0046283, GO:0009812; Table S2). This is consistent with the observation that phyA is involved in anthocyanin accumulation in far-red light (37), and regulates expression of *CHALCONE SYNTHASE* (*CHS*), an enzyme involved in the synthesis of flavonoid and anthocyanin precursors.

To test the phyA photoperiodic link, we measured expression of *FLAVANONE 3-HYDROXYLASE* (*F3H*) and *CHS* in short and long days, in WT (Col-0), *pif4 pif5*, and *phyA*. Although *CHS* was not identified in the phyA-regulated cluster (cluster 85), it is a well-known target of phyA signalling, and displays several of the expected features of induction by phyA in available microarray data, including a photoperiod-modulated dawn peak. Our timeseries qPCR data show that in short days *CHS* and *F3H* transcript levels rise rapidly post-dawn in WT, but this response is markedly reduced in *phyA* and *pif4 pif5* (Fig 4A). Contrasting with this, expression of *CHS* and *F3H* is similar in *phyA* and *pif4 pif5* through a long day (Fig 4A). This comparison was similar in experiments where the lights-on at dawn was simulated based on natural conditions (Fig S10; see SI Appendix for details), with a fast dawn (reaching 100 μmol m^{-2} s^{-1} after 50min), and a slow dawn (reaching 100 μmol m^{-2} s^{-1} after 90min). While the amplitude varied slightly, the expression profiles of *PHYA*, *F3H* and *CHS* in WT, *phyA*, *pif4 pif5* and *phyA pif4 pif5* were qualitatively similar in abrupt, fast and slow dawns. This response consistency most likely results from inherent photosensory properties that enable phyA to detect and react to very low fluence rate dawn light. These data are consistent with phyA being most active during the day in short photoperiods.

In order to test whether these differences in gene expression result in differences in metabolic phenotype, we measured anthocyanin accumulation in plants grown in short and long days. As expected, anthocyanin levels are highest in the WT in short days, and are reduced in the *phyA*, *pif4 pif5* and *pifQ* mutants, specifically in short days (Fig 4B). These results highlight a role for the PIF-phyA module in mediating seasonal changes in anthocyanin levels.

Discussion

Perception of light allows plants to prepare for the predictable daily and seasonal rhythms of the natural environment. We have delineated a role for the light photoreceptor phyA in both daily and seasonal responses. On a daily timescale, phyA acts as a precise sensor of dawn, peaking in activity following first light. On a seasonal timescale, the amplitude of this dawn peak in activity changes, and is especially pronounced in short photoperiods.

The ability of phyA to respond sensitively to dawn relies on two key properties: its ability to sense very low levels of light (38), and its accumulation in darkness (7,33) (8, 39). It is well established that the active Pfr form of phyA is light labile, and degrades fairly rapidly following light exposure. However, inactive phyAPr accumulates in seedlings that are kept in prolonged periods of darkness (8). A night-time rise in phyA protein levels has also been reported for seedlings grown in short days (39). Here, we have identified the PIF transcription factors as regulators of this nocturnal elevation in phyA, and linked this accumulation to the induction of hundreds of transcripts at dawn.

This cycle of accumulation and repression of photosensitivity across a dark-to-light transition is reminiscent of responses in the mammalian eye. A combination of physiological and molecular mechanisms heighten photosensitivity during prolonged darkness, but this sensitivity gradually diminishes during prolonged exposure to light (40). Such systems have been shown to enable sensitive responses to fold-changes in stimuli (41). This may be especially important in the case of phyA, as it allows a high-amplitude response at dawn, when there is a transition from darkness to low-intensity light. Furthermore, phyA is not the only light-labile photoreceptor: Cryptochrome 2 shows similar patterns of accumulation in darkness (39, 42). Thus, our analysis of phyA signalling may have implications for other light signalling pathways. In particular, it highlights the importance of studying such pathways in conditions that approximate the natural environment i.e. in photoperiods.

Our analysis suggests that nocturnal accumulation of phyA results in photoperiodic responses. In short photoperiods, higher levels of phyA are present during the night, leading to an enhanced sensitivity to light at dawn. Inspection of transcriptomic and functional genomic datasets revealed that this expectation is met in hundreds of phyA-induced genes. Furthermore, these changes in gene expression have consequences for plant metabolism and growth. For example, induction of genes involved in flavonoid and anthocyanin biosynthesis in short photoperiods is reflected in changes in anthocyanin accumulation in these conditions. A role for phyA in regulating anthocyanin metabolism has previously been demonstrated under far-red light (37). Here, we extend this role to plants grown under white light in short photoperiods. The potential relevance of increased anthocyanin accumulation to growth in short photoperiods remains to be understood, but may involve protection from photoperiod-specific stresses. For example, anthocyanins protect from oxidative stress (43), which is higher in short photoperiods (44).

Previously, substantial focus has been placed on the role of phyA in seedling establishment (19, 45). We recently demonstrated a role for phyA, alongside other phytochromes, in biomass

production (46), while others have shown that phyA regulates flowering (47). The precise regulatory mechanisms involved in each process are likely to be context-dependent. For example, in seedlings grown in constant far-red light, loss of PIF4 and PIF5 does not affect phyA protein abundance (45). These conditions differ substantially from the conditions used in this study, where a change in photoperiod is required to promote transcription of *PIF4*, *PIF5*, and their target *PHYA*. This illustrates the potential for the same regulatory network to be deployed in different ways depending on the developmental and environmental context.

In summary, our study firmly positions phyA as a photoperiodic dawn sensor that is tuned to detect the very low light levels that signify dawn onset in the natural environment. This property ensures that phyA is a very reliable sensor of dawn transition in nature, where weather, local and seasonal changes can profoundly affect the intensity of morning light.

Supporting Information

SI Appendix. Supplementary Figures S1-S11, Supplementary Tables S1-S4, Models and methods.

Acknowledgements

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1. Greenham K, McClung CR. Integrating circadian dynamics with physiological processes in plants. Nat Rev Genet. 2015;16(10):598-610.
2. Yanovsky MJ, Kay SA. Molecular basis of seasonal time measurement in Arabidopsis. Nature. 2002;419:308-12.
3. Nozue K, Covington MF, Duek PD, Lorrain S, Fankhauser C, Harmer SL, et al. Rhythmic growth explained by coincidence between internal and external cues. Nature. 2007;448:358-61.
4. Seaton DD, Smith RW, Song YH, MacGregor DR, Stewart K, Steel G, et al. Linked circadian outputs control elongation growth and flowering in response to photoperiod and temperature. Molecular Systems Biology. 2015;11.
5. Leivar P, Quail PH. PIFs: pivotal components in a cellular signaling hub. Trends in Plant Science. 2011;16:19-28.
6. Park E, Park J, Kim J, Nagatani A, Lagarias JC, Choi G. Phytochrome B inhibits binding of phytochrome-interacting factors to their target promoters. The Plant Journal. 2012;72:537-46.
7. Possart A, Fleck C, Hiltbrunner A. Shedding (far-red) light on phytochrome mechanisms and responses in land plants. Plant Science. 2014;217–218:36-46.
8. Sharrock RA, Clack T. Patterns of expression and normalized levels of the five Arabidopsis phytochromes. Plant Physiol. 2002;130(1):442-56.
9. Hiltbrunner A, Tscheuschler A, Viczián A, Kunkel T, Kircher S, Schäfer E. FHY1 and FHL Act Together to Mediate Nuclear Accumulation of the Phytochrome A Photoreceptor. Plant and Cell Physiology. 2006;47:1023-34.
10. Krzymuski M, Cerdán PD, Zhu L, Vinh A, Chory J, Huq E, et al. Phytochrome A antagonizes PHYTOCHROME INTERACTING FACTOR 1 to prevent over-activation of photomorphogenesis. Molecular Plant. 2014;7:1415-28.
11. Yoo J, Cho M-H, Lee S-W, Bhoo SH. Phytochrome-interacting ankyrin repeat protein 2 modulates phytochrome A-mediated PIF3 phosphorylation in light signal transduction. The Journal of Biochemistry. 2016;160:243-9.
12. Osterlund MT, Hardtke CS, Wei N, Deng XW. Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature. 2000;405(6785):462-6.
13. Seo HS, Yang JY, Ishikawa M, Bolle C, Ballesteros ML, Chua NH. LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. Nature. 2003;423(6943):995-9.
14. Jang IC, Yang JY, Seo HS, Chua NH. HFR1 is targeted by COP1 E3 ligase for post-translational proteolysis during phytochrome A signaling. Genes Dev. 2005;19(5):593-602.
15. Chen F, Li B, Li G, Charron J-B, Dai M, Shi X, et al. Arabidopsis Phytochrome A Directly Targets Numerous Promoters for Individualized Modulation of Genes in a Wide Range of Pathways. The Plant Cell. 2014;26:1949-66.
16. Tepperman JM, Hwang Y-S, Quail PH. phyA dominates in transduction of red-light signals to rapidly responding genes at the initiation of Arabidopsis seedling de-etiolation. The Plant Journal: For Cell and Molecular Biology. 2006;48:728-42.
17. Tepperman JM, Zhu T, Chang HS, Wang X, Quail PH. Multiple transcription-factor genes are early targets of phytochrome A signaling. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:9437-42.
18. Hall A, Kozma-Bognár L, Tóth R, Nagy F, Millar AJ. Conditional Circadian Regulation of PHYTOCHROME A Gene Expression. Plant Physiology. 2001;127:1808-18.
19. Casal JJ, Candia AN, Sellaro R. Light perception and signalling by phytochrome A. Journal of Experimental Botany. 2014;65:2835-45.
20. Nusinow DA, Helfer A, Hamilton EE, King JJ, Imaizumi T, Schultz TF, et al. The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature. 2011;475:398-402.
21. O'Malley RC, Huang S-sC, Song L, Lewsey MG, Bartlett A, Nery JR, et al. Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. Cell. 2016;165:1280-92.
22. Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Research. 2016;44:W90-7.
23. Kulkarni SR, Vaneechoutte D, Van de Velde J, Vandepoele K. TF2Network: predicting transcription factor regulators and gene regulatory networks in Arabidopsis using publicly available binding site information. Nucleic Acids Research.
24. Michael TP, Breton G, Hazen SP, Priest H, Mockler TC, Kay SA, et al. A morning-specific phytohormone gene expression program underlying rhythmic plant growth. PLoS biology. 2008;6:e225.
25. Kunihiro A, Yamashino T, Nakamichi N, Niwa Y, Nakanishi H, Mizuno T. Phytochrome-interacting factor 4 and 5 (PIF4 and PIF5) activate the homeobox ATHB2 and auxin-inducible IAA29 genes in the coincidence mechanism underlying photoperiodic control of plant growth of Arabidopsis thaliana. Plant & Cell Physiology. 2011;52:1315-29.
26. Hornitschek P, Kohnen MV, Lorrain S, Rougemont J, Ljung K, López-Vidriero I, et al. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. The Plant Journal: For Cell and Molecular Biology. 2012;71:699-711.
27. Pfeiffer A, Shi H, Tepperman JM, Zhang Y, Quail PH. Combinatorial complexity in a transcriptionally centered signaling hub in Arabidopsis. Molecular Plant. 2014;7:1598-618.
28. Zhang Y, Mayba O, Pfeiffer A, Shi H, Tepperman JM, Speed TP, et al. A quartet of PIF bHLH factors provides a transcriptionally centered signaling hub that regulates seedling morphogenesis through differential expression-patterning of shared target genes in Arabidopsis. PLoS genetics. 2013;9:e1003244.
29. Oh E, Zhu J-Y, Bai M-Y, Arenhart RA, Sun Y, Wang Z-Y. Cell elongation is regulated through a central circuit of interacting transcription factors in the Arabidopsis hypocotyl. eLife. 2014;3.
30. Nomoto Y, Nomoto Y, Kubozono S, Yamashino T, Nakamichi N, Mizuno T. Circadian clock- and PIF4-controlled plant growth: a coincidence mechanism directly integrates a hormone signaling network into the photoperiodic control of plant architectures in Arabidopsis thaliana. Plant & Cell Physiology. 2012;53:1950-64.
31. Lee J, He K, Stolc V, Lee H, Figueroa P, Gao Y, et al. Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell. 2007;19(3):731-49.
32. Hardtke CS, Gohda K, Osterlund MT, Oyama T, Okada K, Deng XW. HY5 stability and activity in arabidopsis is regulated by phosphorylation in its COP1 binding domain. EMBO J. 2000;19(18):4997-5006.
33. Oh E, Zhu J-Y, Wang Z-Y. Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nature Cell Biology. 2012;14:802-9.
34. Rausenberger J, Tscheuschler A, Nordmeier W, Wüst F, Timmer J, Schäfer E, et al. Photoconversion and nuclear trafficking cycles determine phytochrome A's response profile to far-red light. Cell. 2011;146:813-25.
35. Anderson SL, Somers DE, Millar AJ, Hanson K, Chory J, Kay SA. Attenuation of phytochrome A and B signaling pathways by the Arabidopsis circadian clock. Plant Cell. 1997;9(10):1727-43.
36. Leivar P, Tepperman JM, Monte E, Calderon RH, Liu TL, Quail PH. Definition of Early Transcriptional Circuitry Involved in Light-Induced Reversal of PIF-Imposed Repression of Photomorphogenesis in Young Arabidopsis Seedlings. The Plant Cell. 2009;21:3535-53.
37. Neff MM, Chory J. Genetic Interactions between Phytochrome A, Phytochrome B, and Cryptochrome 1 during Arabidopsis Development. Plant Physiology. 1998;118:27-35.
38. Shinomura T, Nagatani A, Hanzawa H, Kubota M, Watanabe M, Furuya M. Action spectra for phytochrome A- and B-specific photoinduction of seed germination in Arabidopsis thaliana. Proceedings of the National Academy of Sciences. 1996;93:8129-33.
39. Mockler TC, Michael TP, Priest HD, Shen R, Sullivan CM, Givan SA, et al. The Diurnal Project: Diurnal and Circadian Expression Profiling, Model-based Pattern Matching, and Promoter Analysis. Cold Spring Harbor Symposia on Quantitative Biology. 2007;72:353-63.
40. Lamb TD, Pugh EN. Dark adaptation and the retinoid cycle of vision. Progress in Retinal and Eye Research. 2004;23:307-80.
41. Shoval O, Goentoro L, Hart Y, Mayo A, Sontag E, Alon U. Fold-change detection and scalar symmetry of sensory input fields. Proceedings of the National Academy of Sciences. 2010;107:15995-6000.
42. El-Din El-Assal S, Alonso-Blanco C, Peeters AJM, Raz V, Koornneef M. A QTL for flowering time in Arabidopsis reveals a novel allele of CRY2. Nature Genetics. 2001;29:435-40.
43. Akula R, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior. 2011;6:1720-31.
44. Petriacq P, de Bont L, Genestout L, Hao J, Laureau C, Florez-Sarasa I, et al. Photoperiod Affects the Phenotype of Mitochondrial Complex I Mutants. Plant Physiol. 2017;173(1):434-55.
45. Lorrain S, Trevisan M, Pradervand S, Fankhauser C. Phytochrome interacting factors 4 and 5 redundantly limit seedling de-etiolation in continuous far-red light. Plant J. 2009;60(3):449-61.
46. Yang D, Seaton DD, Krahmer J, Halliday KJ. Photoreceptor effects on plant biomass, resource allocation, and metabolic state. Proceedings of the National Academy of Sciences. 2016;113:7667-72.
47. Sarid-Krebs L, Panigrahi KCS, Fornara F, Takahashi Y, Hayama R, Jang S, et al. Phosphorylation of CONSTANS and its COP1-dependent degradation during photoperiodic flowering of Arabidopsis. The Plant Journal: For Cell and Molecular Biology. 2015;84:451-63.