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.

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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 had 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. Night's 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 isoforms, 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 responses are 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 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.
robustly diurnal (8), though it is not clear what drives phyA reaccumulation 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 HYOCOTYL 1 (FYH1) and FYH1-like (FHL) (9). In the nucleus, phyA Pfr negatively regulates several proteins through direct interaction, including the PHOTOCYCLE 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 HYOCOTYL 5 (HY5), LONG HYOCOTYL 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 transcription (20). Here, 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; Datatfile 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 an array of conditions, including dark (16h light: 8h dark (16L:16D); LDs) and short days (16L:8D; SDs). This procedure identified 101 coregulated clusters (Datafile 2).

To identify regulatory mechanisms, we assessed a broad range of potential regulatory pathways, consolidating 527 gene lists from the literature. This consisted of 140 gene lists from 47 papers, covering a broad range of regulatory pathways (see Datafile 1 for description), combined with a further 387 transcription factor binding datasets generated in high throughput by DNA affinity purification sequencing (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, AnEnrich, for performing enrichment analysis of these gene lists (26).

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 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) (Fig 1C, 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^{-15}; hypergeometric test; Fig 1C; Datasets 3). 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 transcription 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 ATTHB2, IAA429, HFR1, and CXX3 (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 for genes responding to far red light in a phyA-independent 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 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 (8, 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 PIF4/5 regulation of PHYA expression, 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 exhibited rhythmic PHYA expression with an end of night peak (Fig 2A). In long days (16L:8D), however, expression was low throughout the day and night (Fig 2A). The model also matched the measured response of PHYA expression at end of night and end of day across multiple photoperiods (Fig S1). Finally, the model matched the exaggerated nocturnal rise in PHYA observed in two circadian clock mutants - lux and LHYox (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. 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 was also explained by the model (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 and pif5 mutants, 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 accumulated to higher levels in short days compared to long days (Fig S5A), and its levels at ZT0 in short days were 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. Several ChIP-seq analyses of the PIF family have been performed across a range of conditions (26-28, 34). Among these, only Oh et al. (34) 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...
PHYA at the PHYA revealed enrichment of PIF4-HA (Fig 2D) and PIF5-HA (Fig S6) at p < 0.05 between WT and both pif4 pif5 s-1 white light in the specified photoperiod (* indicates significant difference relative to pif4 pif5 days (LD, SD, respectively), in WT (Col-0), specific manner. (A) qPCR timecourse data for Anthocyanin accumulation is regulated by phyA in a photoperiod-

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 the phyA signalling model 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); model simulations re-scaled to match arbitrary scaling of normalized microarray data).

PIFs regulate phyA action specifically in short days. Additional support for PIF4 and PIF5’s role as short day 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 short- and long-day-grown seedlings. Our data showed that 4h of FR light (delivered at the end of the night (EON)) suppressed hypocotyl elongation in a phyA and PIF-dependent manner specifically in short days (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, this enhanced hypocotyl elongation in WT and phyA seedlings, and this was more marked in short days. 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 pif4pif5 and pifQ mutants. These photo-

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).

with a PIF-binding E-box (PBE) element (CACATG; (28)). This revealed enrichment of PIF4-HA (Fig 2D) and PIF5-HA (Fig S6) at the PHYA promoter. Thus, PIF4 and PIF5 appear to regulate PHYA expression by direct binding to its promoter in short days.

phyA mediates a photoperiod-dependent acute light response. Differences in phyA accumulation during the night are expected to affect phyA activity during the following day. In order to assess this, we developed a mathematical model of phyA signalling mechanisms, combining our model of PIF regulation with a simplified version of the model of Rausenberger et al. (35) (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’ (36). This is illustrated in Fig 3B, showing simulations of the combined clock-PHYA-phyA model in short and long photoperiods.

The model predicted that the changing activity of PIFs across different photoperiods and genotypes changes the amplitude of the acute light response (Fig 3B). In particular, it predicted that the amplitude of the acute light response at dawn is increased in short photoperiods, as well as in LHYox and las mutants (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 also matched...
gene expression dynamics during seedling deetiolation, in which
dark-grown seedlings are exposed to red light (Fig S8A). Here,
the model predicted a diminished amplitude of response in the
phyA of the deetiolation in red light (Fig S8B). Again, the
model correctly predicted 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 (37) (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 terms.

In order to further test the model predictions 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 (35). Measurement of PRR9
expression in pif4 pif5 and phyA demonstrated 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. How-
ever, the expression of core clock genes PRR7, TOC1, GLU, LUX,
and ELF4 displayed limited changes in pif4 and pif4 pif5 mutants
in short and long days (Fig S9B).

In summary, this cluster of putative phyA targets displays ex-
pression 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 accumu-
lation.** Our results demonstrate that phyA-mediated acute light
responses are amplified in short photoperiods. Therefore, we ex-
pect 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 enrich-
ment for anthocyanin 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 (38), 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-HYDOXYLASE (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
an expression in metabolic datasets (Fig 1C). Although

As expected, anthocyanin levels were highest in the WT in short
days, and were reduced in the phyA, pif4 pif5 and pifQ mutants,
specifically in short days (Fig 4B). These results demonstrate how
the PIF-phyA module mediates 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 (39),
and its accumulation in darkness (8, 33). It is well established
that the active Pfr form of phyA is light labile, and degrades
fairly rapidly following light exposure. However, inactive phyA
Pr 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 (33). 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 dark-
ness, 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 (33, 42). Thus, our analysis
of phyA signalling may have implications for other light signaling
pathways. In particular, it highlights the importance of studying
such pathways in conditions that approximate the natural envi-
ronnement 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 transcrip-
tomic and functional genomic datasets revealed that this expec-
tation is met in hundreds of phyA-induced genes. Furthermore,
these changes in gene expression have consequences for plant
development, with an increased sensitivity to light and a
reduction in growth in short days (45). The precise regulatory mechanisms involved
in flavonoid and anthocyanin biosynthesis in short photoperiods
are 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 (38). 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 un-
derstood, but may involve protection from photoperiod-specific
stressors. 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 demon-
strated a role for phyA, alongside other phytochromes, in biomass
production (46), while others have shown that phyA regulates
flowering (33). 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
Materials and methods

25. Kanimoto A, Yamashita T, Nakamichi N, Iwao Y, Nakashiki H, Mizuno T. Phytochrome Columbia-0 (Col-0) wild type, and mutants in this background, were used for experiments. See SI Appendix, SI Materials and Methods for detailed descriptions of the plant materials and growth conditions. Experimental methods (qPCR, ChIP, Western blotting, anthocyanin measurement), data analysis methods (coexpression clustering, enrichment analysis), and the mathematical modeling methods are also provided in the SI Appendix, SI Materials and Methods.

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Supporting Information

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