Downstream of the plant circadian clock: output pathways for the control of physiology and development. Sally Adams and [1]Isabelle A. Carré

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Biographical notes:

Dr Sally Adams carried out her PhD studies at the University of Bath, where she investigated the role of DNA methylation in genomic imprinting in Arabidopsis. As part of her first post-doctoral position at the Friedrich Miesher Institute in Basel and the University of Geneva, she investigated the epigenetic basis of hybrid vigour. She then moved to the University of Leicester to examine chloroplast division in the unicellular green alga, Chlamydomonas. This was followed by further projects looking at the modulation of flowering time by light quality in Arabidopsis, and the integration of cold and light responses. She now works at the University of Warwick, where she uses a systems biology approach to investigate the global regulation of rhythmic transcription by the circadian clock.

Dr Isabelle Carré carried out her PhD studies at the University of Stony Brook where studied the mechanism by which the circadian clock controls the timing of cell division in *Euglena*. She then moved on to the University of Virginia to investigate the regulation of circadian gene expression in *Arabidopsis*. She is now an Associate Professor at the University of Warwick, where she uses a combination of molecular, genetic and biochemical approaches to study the mechanisms underlying circadian rhythms and photoperiodic responses in plants. She collaborates with mathematicians, statisticians and bioinformaticians to (i) investigate and model the function of gene networks underlying circadian oscillators and photoperiodic flowering, and (ii) decipher the regulatory logic within gene promoters that underlies specific temporal patterns of transcription.

Abstract.

The plant circadian clock controls many aspects of growth and development, allowing an individual to adapt its physiology and metabolism in anticipation of diurnal and seasonal environmental changes. Circadian regulation of hormone levels and hormonal signaling modulates many features of development, including daily growth patterns and the breaking of seed dormancy. The clock also plays a role in seasonal day-length perception allowing plants to optimally time key development transitions, such as reproduction. Moreover, the clock restricts (gates) the sensitivity of a plants response to environmental cues, such as light and stress, to specific times of the day, ensuring the plant can distinguish between normal fluctuations and longer term changes. The central oscillator controls many of these output pathways via rhythmic gene expression, with several of the core clock components encoding transcription factors. Post-transcriptional processes are also likely to make an important contribution to the circadian regulation of output pathways. The plant circadian clock plays a role in regulating fitness, hybrid vigour and numerous stress responses. Thus elucidating the complexities of the circadian output mechanisms and their regulation may provide new avenues for crop enhancement.

Introduction.

Plants are sessile organisms, which as such are incapable of evading adverse environmental conditions. To compensate, plants exhibit a very high level of phenotypic plasticity enabling their adaptation to variable growth conditions. In addition, they rely on their circadian clock in order to modify their physiology and metabolism in anticipation of predictable changes in environmental light and temperature conditions. Approximately 12% of Arabidopsis genes are controlled by the clock and exhibit circadian rhythmicity in constant light [1]. The percentage of rhythmic genes increases to 89% under varied diurnal environmental cycles. The plant circadian clock plays an important role in photosynthesis by ensuring that expression of many of the genes involved in the light harvesting reactions takes place at the optimal time of the day [2]. Furthermore, it controls the degradation of starch during the night to ensure that reserves last until dawn [3]. The clock also mediates the coordination of metabolic pathways for nitrogen assimilation and utilization [4].

Possessing a clock whose period matches the environmental cycle was shown to confer adaptive fitness in terms of growth rates and seedling survival [5]. Under natural conditions, the clock is normally entrained to diurnal light-dark cycles, the timing of rhythms relative to the day-night cycle (commonly referred to as “phase”) is determined in part by the free-running period in constant conditions [6]. In hybrid and allopolyploid plants, increased chlorophyll, sugar and starch contents was found to correlate with altered epigenetic regulation of the core oscillator genes *LATE ELONGATED HYPOCOTYL* (*LHY)* and *CIRCADIAN CLOCK ASSOCIATED-1* (*CCA1),* which resulted in altered amplitude of oscillations of their transcripts. This suggested that the increased growth vigour of these plants may be explained by modified circadian regulation of physiological and metabolic pathways [7]. Thus, developing a detailed understanding of circadian output pathway mechanisms may suggest new strategies for crop improvement by altering the timing of specific rhythms relative to cyclic environmental changes.

The oscillator mechanism.

The mechanism of the central oscillator has been reviewed in detail elsewhere [8]. Briefly, the plant clock is similar to that of animals, being composed of interlocked transcriptional feedback loops (Figure 1). However, the genetic components of these feedback loops are completely distinct. A core feedback loop comprises two MYB transcription factors, LHY and CCA1, with largely overlapping functions. These proteins are expressed in the morning and act to repress transcription of the *TIMING OF CAB-1* (*TOC1)* gene, which encodes a pseudo-response regulator. LHY and CCA1 levels decline in the evening, allowing *TOC1* expression to resume. This in turn results in activation of *LHY* and *CCA1* transcription in the morning [9]. A second feedback loop is mediated by *PSEUDO-RESPONSE REGULATOR-7* and *-9* (*PRR7* and *PRR9*). Transcription of these genes is promoted by LHY and CCA1, and accumulation of their protein products subsequently results in downregulation of *LHY* and *CCA1* transcription [10]. A mathematical model incorporating these two feedback loops, with a third mediated by TOC1 and an unknown component termed "Y", recapitulated most of the experimental data available[11]. The nuclear protein GI was suggested to function as a factor of "Y", as its temporal expression pattern matches that of the predicted component . The current model now requires revision as the GIGANTEA (GI) protein was recently shown to not act in a transcriptional feedback loop, but modulate turn-over of the TOC1 protein via its interaction with the F-box photoreceptor ZEITLUPE (ZTL). Binding of the rhythmically expressed GI protein to ZTL in the evening results in its stabilisation. ZTL and GI then form a complex with TOC1 and target it for degradation by the proteasome (Figure 1). Although not essential for clock function, this process was shown to be important to maximize the amplitude of TOC1 protein oscillations [12]. The pseudo-regulator protein PRR5 appears to carry out a dual function. It acts as part of loop 1 to enhance TOC1 activity by promoting its phosphorylation, nuclear accumulation and recruitment to nuclear foci [13], and as part of loop 2 to mediate repression of LHY and CCA1 transcription in a manner similar to PRR7 and PRR9 [14].

The circadian oscillator is likely to comprise additional levels of regulation as a number of further components have been identified whose function remains to be mapped to

this network. These include CASEIN KINASE 2 (CK2) [15], the GARP transcription factor LUX ARRHYTHMO (LUX), also described as PHYTOCLOCK1 (PCL1) [16, 17], the novel nuclear proteins EARLY FLOWERING 4 (ELF4), TIME FOR COFFEE (TIC), FIONA1 (FIO1) and XAP5 CIRCADIAN TIME KEEPER (XCT) [18-20], the small GTPase LIGHT-INSENSITIVE PERIOD-1 (LIP1) [21] and two LIGHT-REGULATED WD proteins (LWD1 and LWD2) [22]. Mutations of *ELF4* and *LUX* produce arrhythmic phenotypes, suggesting that these genes function close to or as part of the core mechanism of the oscillator. On the other hand, genes that only affect period length may be associated with processes that modulate clock function but are not essential for circadian rhythmicity. For example, nitrogen metabolism can feedback on the function of the core oscillator [4]. The phytohormone abscisic acid (ABA), whose expression and effect is modulated by the clock, can also affect its period. In constant light, application of ABA to plants causes a lengthening of the period of gene expression from the *CCA1*, *AtGRP7* and *CAB2/LHCB1*1* promoters. Conversely, in the dark, the period of rhythmic expression from the *AtGRP7* promoter is shortened in ABA-mutants [23]. Recent work showed that expression of the core clock gene TOC1 is acutely induced by ABA, providing a clue to the mode of action of this hormone. Furthermore, TOC1 binds the promoter of the ABA-related gene (ABAR) and the resulting circadian expression of ABAR contributes to the gating of ABA effects on TOC1 [24]. Thus ABAR and TOC1 function together in a regulatory feedback loop that is not essential for the function of the circadian oscillator but that may modulate it in response to to developmental or environmental signals. Cytokinins also have been found to delay phase, whilst the addition of brassinosteriods can change circadian periodicity [25]. Thus, multiple metabolic and hormone pathways can feedback into the circadian system to regulate the function of the clock. Indeed, a proportion of the mutations affecting circadian period may reflect alterations of these more peripheral processes.

Recent evidence indicates that the mechanism of the clock may vary between tissues and organs. The pseudo-regulator protein PRR3 was shown to function specifically in vascular tissue to regulate stability of the TOC1 protein [26]. Furthermore, the circadian clock in Arabidopsis shoots was shown to consist of a simplified version of the clock in shoots [27]. In roots, two of the clock feedback loops are disengaged because the

transcription factors CCA1 and LHY are unable to inhibit expression of *TOC1* and *GI.* Under diurnal light-dark cycles the shoot and root clock are synchronized through the action of an unknown metabolic signal.

Control of rhythmic gene expression

As transcriptional regulation forms the basis of the oscillator mechanism, the root of many circadian output pathways is expected to reside at the level of rhythmic gene expression. Several of the clock genes encode transcription factors and regulate the oscillatory expression of many downstream genes. For example, CCA1 and LHY are believed to repress expression of evening-specific genes by binding to the Evening Element (EE: AAATATCT; a sequence identified as being overrepresented in sets of evening-specific promoters) [2]. LHY and CCA1 may also promote transcription of morning and mid-day specific genes through interactions with the CCA1-binding site (CBS: AAAAATCT; originally identified in the promoter of mid day-specific *light-harvesting chlorophyll a/b binding (LHCB)* genes) [28, 29]. Both CBS and EE elements are sufficient for cyclic transcription as synthetic promoters comprising multimerised copies of these sequences can confer rhythmic expression to luciferase reporter constructs [30, 31]. Genome-wide, ChIP-Seq identification of in vivo binding sites for LHY indicated that over 1000 promoters were recognized by this transcription factor, which represents approximately 3% of the genome (Sally Adams, Siren Veflingstad, Sascha Ott and Isabelle Carre, unpublished results). Thus, regulation by LHY alone may account for a significant fraction of rhythmic gene expression observed in Arabidopsis. However, not all of LHY target genes exhibited cyclic expression and the phase of peak expression of rhythmic target genes ranged from late morning (ZT4) till late night (ZT20). Modulation of LHY activity by other transcription factors may account for this broad range of expression patterns. A number of other transcription factors and transcription factor binding sites have been shown to contribute to rhythmic gene expression (Table 1). In addition, changes in chromatin structure may act to modulate the timing and amplitude of circadian gene expression. For example, rhythmic transcription of the *TOC1* locus is associated with rhythmic acetylation of histones at the promoter [32]. Moreover, treatment of plants with Trichostatin A, an inhibitor of histone

deacteylases, modified the phase and amplitude of TOC1 oscillations, resulting in an altered period length. The genome-wide contribution of epigenetic modifications to circadian-regulated gene expression remains to be determined.

Post-transcriptional processes also play a role in the regulation of gene expression. Expression of the glycine-rich RNA binding protein AtGRP7 (also known as CCR2) is regulated both by the clock and various biotic stresses [33] and modulates tolerance to cold, drought and high salinity [34]. AtGRP7 exhibits negative autoregulation by binding to its own pre-mRNA and promoting alternative splicing; the alternatively spliced transcript is then degraded. This negative feedback loop acts downstream of the clock as part of a slave oscillator, signaling temporal information via the regulation of target transcripts [35]. For example, during cold stress AtGRP7 is involved in the transport of mRNA from the nucleus to the cytoplasm and may act as a RNA chaperone, modulating transcript folding to facilitate nuclear export [34].

Gating and anticipation of environmental responses

Plants are highly responsive to changes in environmental conditions. However, it is important to make the distinction between “normal”, daily fluctuations and longer-term environmental changes. Thus, one of the roles of the circadian clock is to minimize inappropriate responses by restricting sensitivity to specific intervals of the day (a phenomenon described as “gating”). For example, the clock modulates light responses, to ensure that maximum sensitivity coincides with the middle of the day and that light signals perceived during the night have little or no effect. Expression of the *LHCB* genes was induced in response to light signals given during the subjective day, but minimal responses were observed during the subjective night [36]. The circadian clock itself is modulated by light, and this ensures its entrainment to light-dark cycles. This effect of light on the clock is also limited to specific times of the day. ELF3, a clock associated protein, plays an important role in the gating of light responses, by attenuating light responses during the night [37]. ELF3 modulates light input to the clock by regulating the proteasomal degradation of the GI protein [38]. In the light, GI forms a complex with the F-box blue-light photoreceptor ZTL and promotes its accumulation [12, 39]. ZTL in turn interacts with one of the core components of the oscillators, TOC1, and targets it

for degradation [40].It is proposed that at night ELF3 acts as a substrate adaptor allowing the ring finger ubiquitin ligase (CONSTITUTIVE PHOTOMORPHOGENIC 1) COP1 to interact with GI and promote its degradation. The resulting destabilization of the ZTL protein would limit light input to the clock during the night [38].

The circadian clock also gates a plants response to various stresses. In Arabidopsis, for example, cold treatments applied during the day (ZT4) induced cold responses more effectively than those during the night (ZT16) [41]. Many clock-regulated genes (68%) were also identified as stress-responsive genes, suggesting that another important function of the clock is to enable anticipation of daily stress conditions, such as falling temperatures in the evening. In support of this hypothesis, disruption of circadian clock function in the arrhythmic *prr9/prr7/prr5* mutant resulted in upregulation of a significant number of cold-responsive genes. These plants also showed increased tolerance to various stresses including cold, drought and high salinity [42]. Furthermore,TOC1- and ABAR-RNAi and overexpressing plants exhibited defective responses to drought, suggesting that the ABAR/TOC1 feedback loop described earlier is important to ensure survival under dry environments [24].

Rhythmic expression of the cold-inducible gene *DREB1C* (an early component of cold and dehydration signaling pathways also described as *CBF2*) is mediated by the bHLH transcription factor PIF7. PIF7 represses expression of *DREB1C* via binding to a G-box element within the promoter. Its effect is potentiated by physical interaction with the clock protein TOC1, resulting in rhythmic inactivation of *DREB1C* transcription [43]. LHY and CCA1 may also contribute to cold responses, as bioinformatic studies showed that the EE sequence was enriched in the promoters of cold induced genes [44]. Mutational analysis of EEs and of shorter, EE-like (EEL) motifs in the promoters of the circadian-regulated and cold-inducible genes *COR27* and *COL1* showed that these sequences were required for cold induction and that their effect was amplified by interactions with ABA response element-like sequences (ABREL, sequence ACGTG) [45]. Statistical analyses indicated that combinations of EE, EE-like or CBS sequences with the ABREL were significantly enriched within sets of cold-regulated genes. Thus, cold induction of gene expression may be mediated via the interaction of LHY and CCA1, or related MYB

transcription factors, binding EE/EEL sequences with bZIP transcription factors binding the ABREL. Combinations of EE, EE-like or CBS sequences with a G-box motif (CACGTG) were also enriched within sets of cold-regulated genes, suggesting that LHY and CCA1 may also interact with bHLH transcription factors binding the sequence CANNTG to mediate cold-responses [45].
These findings suggest intimate links between the clock mechanism and stress response pathways. Circadian regulation of stress-response genes may serve to minimize their deleterious effects on plant growth, and modulation of the timing of expression of these genes may offer more promising avenues for crop improvement than constitutive overexpression.

Hormonal control of plant growth and development

In addition to its role in tuning plant physiology to the environment, the circadian clock regulates growth and development through its effects on phytohormones. Under diurnal conditions, bioactive levels of auxin, gibberellins, brassinosteriods, abscisic acid (ABA) and ethylene accumulate specifically in the morning [46]. The considerable overlap between sets of hormone and clock-regulated genes suggests that oscillations of hormone levels may underlie rhythmic expression of many cyclic genes [46, 47].

Many genes implicated in ABA synthesis are under clock control, with the majority showing peak expression during the morning [47]. This is likely to mediate both the rhythmic production of ABA, and indirectly the circadian regulation of ABA responsive genes, since expression of over 40% of ABA induced genes coincides with that of ABA biosynthesis genes. Similarly, the production of ethylene is rhythmic, with levels peaking in the middle of the subjective day [48]. The exact mechanism by which the clock controls cyclic ethylene production is unclear, however several ethylene synthesis genes exhibit circadian regulation and their peak of expression coincides with that of ethylene emission [47]. Key components of the ethylene signalling pathway (*EIN3* and *EIL1*) are also under clock control. As neither of these genes is regulated by ethylene, their circadian regulation must be independent of rhythmic ethylene emission. Thus the clock regulates hormone signaling at multiple levels. Genes involved in almost all aspects of auxin signalling display circadian regulation. Furthermore, transcriptional and

growth responses to application of exogenous auxin are gated by the clock, with maximum responsiveness in the subjective night [49]. Temporal co-ordination of phytohormone transcript abundance may be mediated, at least in part, via the Hormone Up at Dawn (HUD) element (Table 1), a short DNA sequence motif (CACATG) which is overrepresented in phytohormone gene promoters and when multimerised can confer time of day-specific expression to a reporter gene [46]. In addition, the circadian regulated transcription factor RVE1 is essential for diurnal auxin rhythms, and promotes production of free auxin during the day through transcriptional activation of the auxin biosynthetic gene *YUCCA8* [50]. Thus, RVE1 provides a mechanistic link between the clock- and auxin-signalling pathways.

The circadian regulation of hormone signalling components may allow the temporal integration of endogenous pathways with external environmental cues to fine-tune development. For example, Arabidopsis seedlings exhibit rhythmic growth of their hypocotyls, with elongation peaking at subjective dusk in continuous light [51]. However, under diurnal cycles the peak of growth shifts towards dawn, due to interactions between circadian and light cues [52]. Circadian and light-controlled patterns of phytohormone gene expression were shown to correlate well with these temporal patterns of hypocotyl growth. Peaks of phytohormone gene expression coincided with dawn under short day conditions and with dusk in continuous light, resulting in the contrasting patterns of growth [46].

Seed dormancy allows plants to time germination to correspond with suitable environmental conditions. The breaking of seed dormancy is dependent upon environmental factors such as light and temperature. In addition, a process called dry after-ripening promotes the loss of dormancy, and is thought to enable rapid germination following an extended drought period. Environmental cues have been shown to regulate seed dormancy and germination by modulating levels of two phytohormones with conflicting action. ABA acts to both establish and maintain seed dormancy and inhibit germination, whilst gibberellins promote the breaking of dormancy and subsequent germination [53]. As several enzymes involved in gibberellin and ABA metabolism are controlled by the circadian clock, it was perhaps unsurprising to find that

mutations that alter circadian rhythms also alter germination frequencies [54]. A number of Arabidopsis mutants with abnormal circadian clocks (including *lhy, cca1, gi, ztl* and *lux*) showed environmental sensing defects in seed, resulting in altered levels of dormancy. A mechanistic link between the circadian oscillator and dormancy control was suggested by the observation that TOC1 can bind to a central regulator of dormancy, ABI3, in yeast two-hybrid assays [55]. However the process by which the clock affects seed dormancy is unclear. The clock appears to be arrested in an evening-like state in dry seed, restarting when the seeds are imbibed. In response to imbibition, *CCA1* expression was sharply upregulated in non-dormant seed, but not in dormant seed. Distinct patterns of circadian-regulated gene expression followed [54].This suggests that signals modulating dormancy status act on an unknown factor that in turn regulates *CCA1* expression at the time of imbibition to specify subsequent patterns of circadian oscillations and modulate hormone levels.

Responses to day-length

The plant circadian clock also mediates perception of seasonal changes in day-length in order to modulate flowering time and alter plant architecture.

The ability of plants to regulate the transition from vegetative to reproductive growth in response to day-length allows them to initiate reproduction at the most favorable time of the year. The photoperiodic regulation of flowering has been covered in detail in recent reviews. Thus, in brief, Arabidopsis, the transcription factor CONSTANS (CO) plays a central role in the perception of day-length and flowering. Expression of *CO* is finely tuned by the circadian clock, with RNA levels peaking 16 hours after dawn [56]. Under short day conditions, expression of the *CO* mRNA occurs after dark. The CO protein product is rapidly degraded in these conditions and flowering is inhibited. The dark-dependent degradation of CO is regulated by COP1, which targets the protein to the proteasome [57]. In contrast, under long days, the peak of expression occurs during day-light hours, when the activity of COP1 is inhibited by the action of the cryptochromes and by its exclusion from the nucleus [58]. As a result, CO protein accumulates and promotes flowering via induction of the floral integrators, *FT* and *TSF* [59, 60]. Thus, the photoperiodic regulation of flowering is mediated by the coincidence

of an endogenous rhythm (CO) with an external signal (light). Another level of external coincidence is mediated by the F-box protein, blue light photoreceptor FKF1, which acts together with GI to promote the degradation of the transcriptional repressor CDF1 and enable CO transcription at the end of a long day [39]. The CO-FT pathway is conserved in a wide variety of plant species and a functional CO homologue has been identified in *Chlamydomonas*, a unicellular photosynthetic green alga, suggesting that this pathway developed early in the chlorophyte lineage to regulate development in response to photoperiod [61].

Plant growth is also responsive to day-length. The bHLH transcription factors *PIF4* and *PIF5* allow adjustment of growth patterns to seasonal changes in photoperiod. Regulation takes place through an external coincidence mechanism similar to that described for the photoperiodic control of flowering. Transcription of the *PIF4* and *PIF5* genes is under circadian control, and stability of the protein products is modulated by light [52]. Under short day conditions, expression of *PIF4* and *PIF5* is restricted to the dark portion of the cycle. Both proteins accumulate to a high level, promoting hypocotyl elongation. Under long-day conditions, however, expression of the *PIF4* and *PIF5* genes coincides with light and their protein products fail to accumulate, resulting in a short-hypocotyl phenotype [62].

Conclusion and perspectives

Evidence so far suggests that the mechanism of circadian output pathways is largely transcriptional. Transcription factor binding sites that are associated with rhythmic gene expression are gradually being uncovered. A major challenge, however, will be to understand how such a great diversity of gene expression patterns can be mediated by an oscillator composed of a small set of genes, and an even smaller set of transcription factors. Rhythmic hormone accumulation is likely to regulate an important fraction of the circadian transcriptome. Regulated protein turn-over may represent a key regulatory mechanism, as this contributes both to the function of the oscillator and to the photoperiodic regulation of flowering. The F-box proteins ZTL and FKF1 are likely to target proteins other than TOC1 and CDF1 for ubiquitination and proteasomal degradation. Similarly, the ELF3 protein may direct other proteins than GI to the

ubiquitin ligase COP1. Protein phoshorylation may also play a role. For example, the maize transcription factor Opaque 2 is expressed constitutively but activated periodically through rhythmic phosphorylation [63]. A small number of microRNAs display circadian regulation in Drosophila, suggesting a role in regulating transcript stability and translational efficiency [64]. No such data are available for plants, although diurnal accumulation of four microRNAs has been reported in Arabidopsis [65]. It also remains to be determined whether circadian oscillations of cytoplasmic calcium concentrations contribute to rhythmic changes in plant physiology.

The photoperiodic regulation of flowering time and that of hypocotyl elongation are mediated by similar external coincidence mechanisms, involving rhythmic expression of one of more transcription factors and light-regulated protein turnover. This suggests that other photoperiod-sensitive aspects of development such as germination, the formation of vegetative organs such as bulbs and tubers, and leaf abscission may be regulated by similar mechanisms.

Summary

- The plant circadian clock is similar to animal clocks in that it is composed of interlocked transcriptional feedback loops. However its genetic components are distinct.
- Immediate output pathways are mediated through transcriptional regulation by the master clock genes *LHY/CCA1* and *TOC1*. Rhythmic protein degradation regulated by GI, ELF3 and the blue light photoreceptor, F-box protein ZTL may also mediate circadian output pathways.
- Downstream events include the rhythmic regulation of phytohormone synthesis and signaling.
- Circadian gating of light-responses is mediated, at least in part, by the ELF3-mediated targeting of the GI protein for ubiquitination and proteasomal degradation.
- Day-length regulation of growth and flowering are mediated by similar external coincidence mechanisms involving the rhythmic accumulation of a light-labile

protein that only accumulates to active levels under short-day conditions, or of a dark-labile protein that only accumulates to active levels under long-day conditions.

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Table 1. Promoter motifs regulating circadian gene expression.

Motif name	***Consensus sequence***	***Phase of Expression***	***Notes and References***
Morning Element	CCACAC	Dawn/ Morning	[1, 31, 47]
G-box	CACGTG	Dawn/ Morning	Bound by bZIP and bHLH transcription factors [66, 67]
HUD element	CACATG	Morning (short days)	Overrepresented in phytohormone gene promoters [46].
Hex element	TGACGTGG	Day to evening	Bound by bZIP proteins. Over-represented in PPR gene promoters including morning expressed PRR5/7/9 and evening expressed TOC1(PRR1) [68, 69].
TBS motif	GGNCCCAC	Dawn	CHE, a TCP transcription factor, binds to the TBS motif in the CCA1 promoter and represses transcription [70].
5A motifs	5(W)-CC-5(W)KW	Dawn	Required for the correct expression of LHY. Shows similarity to CArG boxes which are bound by MADS box transcription factors [71].
CCA1 binding site (CBS)	AAAAATCT	Morning	Bound by LHY and CCA1 [29, 30]

GATA element	GGATAAG/GATAA	Late day / evening	Bound by GATA factors, type IV zinc finger transcription factors with a role in light, circadian and nitrogen-dependent control of transcription [1, 47].
Evening element (EE)	AAAATATCT	Evening	Bound by the MYB transcription factors LHY and CCA1 [9].
Protein box	ATGGGCC	Midnight	[1]
Starch box	AAGCCC	Midnight	[1]
Telo box	AAACCCT	Midnight	[1]

Figure legends

Figure 1: The circadian oscillator of Arabidopsis and its immediate output pathways.

The Arabidopsis circadian oscillator is composed of at least three interlocked regulatory feedback loops. A core feedback loop (1) is composed of the transcription factors LHY and CCA1 and the pseudoresponse regulator TOC1. LHY and CCA1 are expressed at dawn and repress transcription of the *TOC1* gene during the day. Their expression resumes in the evening once LHY and CCA1 levels have subsided. The TOC1 protein acts to promote transcription of the *LHY* and *CCA1* genes late in the night, thus completing the cycle. TOC1 activity is promoted by its interaction with PRR5, which enhances its phosphorylation, nuclear accumulation and recruitment to nuclear foci. A second feedback loop (2) is mediated by LHY and CCA1, promoting transcription of the *PRR9, 7* and *5* genes. The PRR9, 7 and 5 protein products accumulate sequentially during the day and act to maintain repression of *LHY* and *CCA1* transcription. A third regulatory loop (3) is mediated through LHY/CCA1-mediated, rhythmic expression of *GI*. The GI protein accumulates late in the day and forms a complex with the ZTL photoreceptor in the light. The GI-ZTL complex is then stabilized and acts to target the TOC1 protein for ubiquitination and subsequent proteasomal degradation. This process is inhibited during the subjective night by the accumulation of PRR3.

Entrainment of the oscillator to diurnal light-dark cycles is mediated by multiple light input pathways. In the morning, light signals mediated by phytochromes and cryptochromes induce *LHY* and *CCA1* transcription. In the evening, light signals mediated by the ZTL photoreceptor regulate the turn-over of the TOC1 protein. The latter effect of light is gated through the action of the ELF3 protein (dark grey box). ELF3 acts during the night to target the GI protein to the ubiquitin ligase COP1. This limits the abundance of the GI-ZTL complex and therefore reinforces the effect of PRR3 to enable TOC1 accumulation.

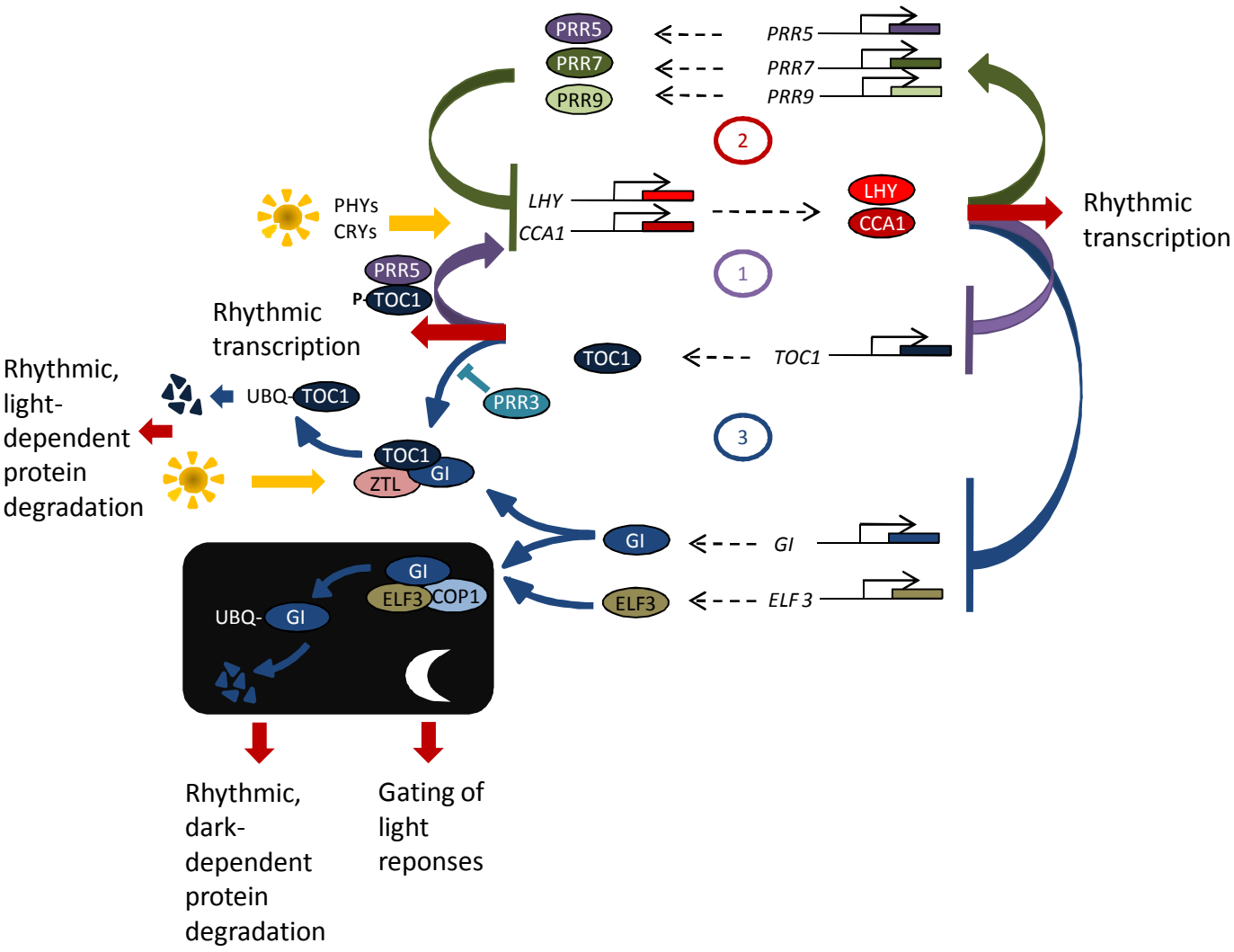
Immediate output pathways are indicated by red arrows. Transcriptional regulation by LHY/CCA1 and TOC1 is likely to play a major role. Rhythmic protein degradation may also play an important role in circadian output pathway, as the ZTL and ELF3 proteins may target other proteins than TOC1 and GI for degradation.

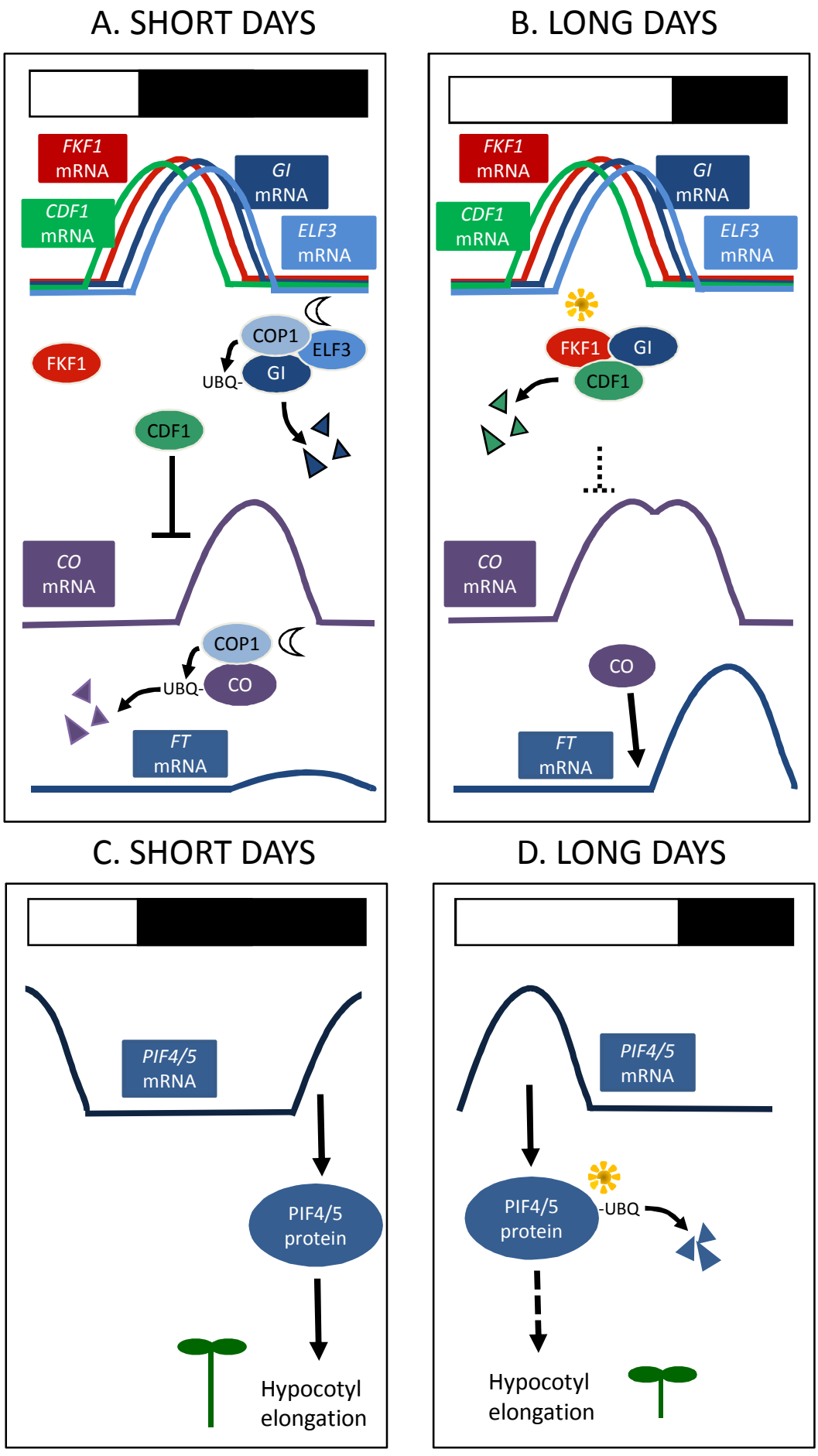
Figure 2: External coincidence models for photoperiodic responses in Arabidopsis.

A, B. Photoperiodic regulation of flowering. Expression of the *CO* mRNA is repressed during the day by the action of CDF1, and this repression may be lifted via the action of FKF1 and GI. Under short-day conditions, ELF3 acts to recruit GI to the ubiquitin ligase COP1, leading to its degradation. CDF1-mediated repression of *CO* expression is then maintained until night-time, when CDF1 expression declines. Expression of the CO protein during the night results in its immediate degradation through the dark-dependent action of the COP1 protein. Under long-day conditions, however, FKF1 is activated by light, leading to complex formation with GI and degradation of CDF1. This enables CO

transcription towards the end of the day. The COP1 protein is inactive in the light and therefore the CO protein can accumulate to active levels and promote transcription of *FT*, leading to flowering.

C,D. Photoperiodic regulation of hypocotyl elongation. Expression of the *PIF4/5* mRNAs is rhythmic and peaks in the morning. However, the protein products are light-labile. Under long-day conditions, the PIF4/5 proteins are synthesized in the light and fail to accumulate to active levels. Under short-day conditions, transcription of the *PIF4/5* genes anticipates dawn. Significant levels of the protein products accumulate before dawn and act to promote hypocotyl elongation.


PRR5
PRR7
PRR9
PRR5
PRR7
PRR9
2
LHY
CCA1
LHY
CCA1
PHYs
CRYs
Rhythmic transcription
1
PRR5
P-TOC1
Rhythmic transcription
TOC1
TOC1
Rhythmic, light-dependent protein degradation
UBQ-TOC1
PRR3
3
TOC1
GI
ZTL
GI
GI
ELF3
GI
ELF3
COP1
UBQ-
GI
Rhythmic, dark-dependent protein degradation
Gating of light reponses


A. SHORT DAYS
FKF1 mRNA
GI mRNA
CDF1 mRNA
ELF3 mRNA
FKF1
COP1
ELF3
UBQ-
GI
CDF1
CO mRNA
COP1
UBQ-
CO
FT mRNA
B. LONG DAYS
FKF1 mRNA
GI mRNA
CDF1 mRNA
ELF3 mRNA
FKF1
GI
CDF1
CO mRNA
CO
FT mRNA
C. SHORT DAYS
PIF4/5 mRNA
PIF4/5 protein
Hypocotyl elongation
D. LONG DAYS
PIF4/5 mRNA
PIF4/5 protein
-UBQ
Hypocotyl elongation