Award Review

From a Repressilator-Based Circadian Clock Mechanism to an External Coincidence Model Responsible for Photoperiod and Temperature Control of Plant Architecture in Arabidopsis thaliana

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Circadian clocks enable organisms to define subjective time, that is, to anticipate diurnal day and night cycles. Endogenous circadian rhythms regulate many aspects of an organism’s physiological and morphological growth and development. These daily oscillations are synchronized to the environment by external cues such as light and temperature, resulting in enhanced fitness and growth vigor in plants. Recent findings concerning biochemical properties of central oscillators in Arabidopsis thaliana have advanced our understanding of circadian clock function. Central oscillators are composed of three classes of transcriptional repressors. The interactions among them include a repressilator structure. Output from the circadian clock is transduced through regulating transcription of downstream genes directly by the oscillator components. The essential role of the output pathway in the circadian system is to make different elementary steps responsible for daily cellular processes exert maximum effects at specific times of the day. Recently, significant progress was made in defining the mechanisms by which plant growth on a day-to-day basis is activated at specific times of the day in a manner dependent on photoperiod and temperature conditions. Plant growth is controlled by the clock through interactions with light and phytohormone signaling. This review focuses on the node that connects clock output to light and phytohormone signaling that coordinates plant growth with rhythmic changes in the environment.

Key words: Arabidopsis thaliana; circadian clock; external coincidence; light signaling; phytohormone signaling

I. Transcriptional Regulation-Dependent Circadian Clock Mechanism in A. thaliana

Circadian clocks are intrinsic, entrainable mechanisms that generate biological rhythms with approximately a 24-h period. Circadian rhythms are widespread in many biological processes of almost all eukaryotic organisms including plants. A clock provides plants with an adaptive advantage to anticipate and respond to daily changes in environmental conditions such as day and night, and seasonal changes in photoperiod. Endogenous circadian rhythms were first studied by observation of daily leaf movements in plants. An insightful experimental approach to monitor bioluminescence of transgenic plants harboring clock or clock-controlled gene promoters was developed by Millar and Kay, making it possible to characterize biological rhythms more precisely in a nondestructive manner. In a model higher plant A. thaliana, genetics, reverse genetics and biochemical study uncovered various types of clock-associated genes in concert with progress in the monitoring technique of clock functions. Based on current knowledge of the molecular functions of clock-associated genes, supported by modeling and simulation, central oscillator components to define the molecular mechanism of circadian clock are classified into three classes of transcriptional regulators: Myb-related proteins, CCA1 and LHY, pseudo-response regulators with the CCT motif (PRR family), PRR9, PRR7, PRR5 and TOC1 and a recently identified evening complex (EC) composed of LUX/PCL1 (GARP family protein), ELF3 and ELF4 (Fig. 1). Transcripts of these clock genes are regulated in a circadian fashion. CCA1 and LHY are expressed with sharp peaks in the morning. Circadian wave of sequential PRR9, PRR7 and PRR5 transcripts is observed during the day. The EC genes (LUX, ELF3 and ELF4) and TOC1 are expressed from evening to midnight. Importantly, mutational lesions in the genes encoding these clock oscillators affect the robustness of circadian rhythms severely. A cca1 lhy double mutant exhibits dampened circadian rhythm with a short period. A ppr9 ppr7 ppr5 triple mutant shows an arrhythmic circadian phenotype. Loss of function mutants of any one of the genes encoding EC also result in arrhythmia. These results suggest that each class of clock compo-
ments plays a phase-specific essential role in oscillator functions, determining temporal vulnerability of the clock network.Clarification of the biochemical activity of these clock components has advanced our understanding of clock mechanism. CCA1 and LHY directly repress the expression of TOC1, LUX and ELF4 by binding specifically to a cis-element within their promoters known as the evening element (EE: AAAAT-ATCT), a motif that is overrepresented in the promoters of clock-regulated evening expressed genes.15,22–25 It was recently unraveled that PRR9, PRR7, and PRR5 act as transcriptional repressors in the clock network. They associate with CCA1 and LHY promoters in vivo and repress these genes from early daytime until the middle of the night.26 The molecular properties of TOC1 have also been characterized, demonstrating it is a DNA-binding transcriptional repressor of CCA1 and LHY.10,27,28 In addition to CCA1 and LHY, TOC1 binds to the promoters and inhibits the expression of almost all the above-mentioned central oscillator components, PRR9, PRR7, PRR5, LUX, and ELF4.27,28 Combinatorial analysis of genome-wide expression study and chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) lead to identification of direct target genes for PRRs29 and TOC1.27,28 The DNA binding motifs enriched in these PRR family protein share weak sequence similarity, making it difficult to identify the consensus sequence.25–29 This suggests that the PRR family functions as transcriptional repressors in combination with other transcription factors to define the sequence specificity for promoter binding. In EC proteins, LUX is responsible for the DNA binding activity for target genes. Protein binding microarray (PBM) experiments identified the consensus LUX binding site (LBS: GAT[A/T]CG).30 LBS is present at the promoters of PRR9, PRR7, TOC1, LUX, and ELF4.10,30 Recent studies indicate that LUX functions as a transcriptional repressor of PRR9, TOC1, and its own promoter,10,30 and that ELF3 and ELF4 function as transcriptional repressors of PRR9 and PRR7.31 Taken together, it was proposed that the clock gene circuit in A. thaliana includes a repressilator structure (Fig. 1).10,32

![Fig. 1. A Model for Simplified Transcriptional Repression-Based Interactions among Central Oscillators in A. thaliana.](image)

and LHY indirectly through the direct inhibition of PRRs, which are direct repressors of CCA1 and LHY. TOC1 affects every class of oscillator components directly as a transcriptional repressor (Fig. 1). In good agreement with this model, a toc1 mutant exhibits a short period and overexpression of TOC1 results in an arrhythmic phenotype.33,34 The real biological clock system of A. thaliana is more complicated than the simplified structure focused around the repressilator. In fact, it is suggested that direct and/or indirect transcriptional activation function of the above-mentioned central oscillators (CCA1,35 LUX,15 ELF3,17 and ELF4,18,36) and other oscillators (NOX,37 LWD1,38 and RVE8,39,40) are incorporated into this clock network. Autoregulation of CCA1/LHY,11,12 the PRR family,29 and the EC genes30,36 is also suggested to be involved in the clock mechanism. Furthermore, the whole structure the plant clock includes post-translational modification of the oscillator components such as CK2 mediated phosphorylation of CCA1 and LHY,25 phosphorelation and ZTL mediated degradation of PRRs,44–46 regulation of the EC by COP1 and GI,47 which is probably responsible for cellular localization, activity, and/or stability of the proteins. Despite the complexity of the circadian clock mechanism, the direct transcriptional repression-based interactions among the central oscillators (CCA1/LHY, PRRs, and EC) to establish the repressilator structure shown in Fig. 1 might be important for the Arabidopsis clock to generate robust (self-sustaining) and entrainable biological rhythms in response to external time cues such as light and temperature.

### II. External Coincidence Mechanism to Regulate Plant Architecture under Day and Night Cycle Conditions in A. thaliana

It is generally admitted that growth is controlled through interactions between light and phytohormone signaling.45,46 Photoreceptor mutants and mutants of phytohormone biosynthesis or signaling exhibit abnormalities in photomorphogenesis processes during desetiolation including elongation response of the embryonic stem, the hypocotyl.47 Elongation of hypocotyls is also observed in shade avoidance responses under low red/far-red (R/FR) ratios and/or low blue light intensity conditions.47 Involvement of phytohormones as well as photoreceptor mediated light signaling is evident also in the control of growth under a canopy.48 On the other hand, it was reported from a long time ago that circadian dysfunction causes aberrant hypocotyl elongation patterns.49 In fact, almost all the central oscillator mutants of Arabidopsis show longer or shorter hypocotyls than wild-type.50 Circadian clocks enable organisms to define subjective time, that is, to anticipate diurnal day and night cycles. The essential role of the output pathway in the circadian system is to make different elementary steps (events) responsible for daily cellular processes exert maximum effect at the specific times of the day. It was a long mystery how the above three independent mechanisms (light signaling, phytohormone signaling, and circadian clock) to serve plant growth interact among themselves. The finding that the growth of hypocotyls in seedlings reaches maximum
rates at dawn under short-day conditions, using infrared light imaging technique done by Nozue and colleagues, advanced our understanding about this issue. Compared with de-etiolation and shade avoidance responses, growth is regulated on a daily basis. It is well known that Arabidopsis growth under long-day conditions results in short hypocotyls, and short-day; long hypocotyls. The critical photoperiod to cause hypocotyl elongation of Arabidopsis green seedlings is duration of around 12 h dark. It is demonstrated that phytochrome-interacting bHLH transcription factor, PIF4 and PIF5 (Fig. 2) is predominantly involved in the photoperiodic control of hypocotyl growth. A pif4 pif5 double mutant almost or completely abolishes the photoperiodic response of hypocotyl elongation. The primary sequences of the PIF family have characteristic domain structures. They contain the considerably conserved bHLH domains that are responsible for dimerization and DNA binding to target genes (Fig. 2). N-Terminal regions containing about 100 amino acids designated the active phytochrome-binding (APB) domain (also called the PIL motif), which is necessary and sufficient for phyB interaction is conserved among eight members of the PIF family. Sequence alignments showed that they share in common a significantly conserved APB core motif (Fig. 2). It is demonstrated that the interaction with photo-activated phyB induces rapid phosphorylation, which leads to the 26S proteasome-mediated degradation of PIF1, PIF3, PIF4, PIF5, and PIF7. In the context of relationship between circadian clock and light signaling, PIF4, PIF5, and PIF7 are distinguishing, because each of their transcripts shows a robust circadian rhythm with a peak in the middle of the day, indicating they are under the control of the circadian clock. It was reported recently that EC directly represses the transcription of PIF4 and PIF5. While PIF4 is expressed both in daytime and nighttime on short-days, it is expressed exclusively in the daytime under long-day conditions.
day conditions to induce elongation of hypocotyls, PIF4 is activated at the end of night only under short-clock-dependent mechanisms, it was speculated that regulations described above. Through these phyB- and clock-dependent mechanisms, it was speculated that PIF4 is activated at the end of night only under short-day conditions to induce elongation of hypocotyls, because it is only when transcription of PIF4 coincides with PIF4 protein stabilization that the transcriptional activity is activated.\(^{51,53,55}\) Inspection of genome-wide transcriptome database combined with ChIP analysis led to identification of ATHB2 as one of the direct targets of PIF4 and PIF5.\(^{69}\) Considering the expression profiles of the ATHB2 gene under various photoperiod conditions, it is suggested that PIF4 is actually activated at the end of night specifically under short-day conditions (Fig. 4A).\(^{69}\) The coordinate interplay between photoreceptor phyB and circadian clock makes it possible for seedlings to grow in such a way that elongation of hypocotyls is accelerated at the end of night specifically in short-days due to coincidence between circadian clock-dependent internal and photoperiod-dependent external time cues (Fig. 3). PIF3 is a foundation member of the bHLH family and is involved in many aspects of phyB signaling including seed germination and chlorophyll biosynthesis as well as hypocotyl elongation.\(^{70–72}\) It was reported recently that, although PIF3 is not regulated in a circadian fashion, basal expression of PIF3 is also involved in the photoperiodic control of plant growth together with PIF4 and PIF5.\(^{73}\) The functional broadness of PIF3 might be important in the PIF family to understand phytochrome-mediated signaling. In the context of relationship between circadian clock and phytohormones signaling, gibberellic acid (GA) and brassinosteroid (BR) are particularly important for the photoperiodic control of plant growth. It is shown that GA signaling is gated by the circadian clock through transcriptional regulation of the GA receptors, resulting in higher stability of the DELLA proteins (DELLAs) during daytime and higher sensitivity at night,\(^{74}\) because GA perceived by the receptors leads to degradation of DELLAs.\(^{75}\) In addition, blue light causes a considerable reduction in internal active GA in seedlings through induction of GA2ox transcripts (GA2ox1 and GA2ox2) and rapid reduction of GA20ox transcripts (GA20ox1).\(^{65,76}\) These two mechanisms of the regulation act on the cellular contents of the DELLAs synergistically under day and night cycle conditions, consistently with the earlier observation that the accumulation of DELLAs is more stimulated in long-days than in short-days.\(^{77}\) Importantly, it is demonstrated that DELLAs interact with PIF3 and PIF4 to inhibit the transcriptional activity of them.\(^{78,79}\) Reductions in endogenous GA levels, through the application of a GA biosynthesis inhibitor or a mutant of GA biosynthesis (ga1), results in complete inhibition of hypocotyl elongation.\(^{77}\) Thus GA is prerequisite for photoperiodic control of plant growth. Considering these facts, the gated GA signaling may also be involved in the mechanism to activate PIF4 transcriptional activity at the end of night specifically under short-day conditions independently of phyB (Figs. 3 and 4A).\(^{77}\) phyB and DELLAs might therefore play the essentially same role in the photoperiodic control of plant growth. In the case of BR signaling, BR biosynthetic genes are diurnally regulated in such a way that BR accumulates in the middle of the light phase.\(^{80}\) BR perceived by the receptor leads to activation of BZR1 family transcription factors.\(^{81}\) It was found recently that activated BZR1 and PIF4 interact with each other to regulate common target genes.\(^{82,83}\) It was also reported that relationship between GA and BR signaling is important for positive regulation of seedling growth.\(^{84}\) Under low blue light conditions such as shade, GA and BR levels might increase even in the daytime. The involvement of PIF4 and PIF5 in shade avoidance responses under low blue light conditions is evident because the responses are significantly impaired in a pif4 pif5 double mutant.\(^{85}\) Taken together, it might be possible that BZR1-PIF4/5 complex is activated in the daytime under low blue light conditions (Fig. 3). In relevant to the shade avoidance responses, physiological function of PIF7 was characterized recently. It was found that PIF7 is predominantly required in shade avoidance responses under low R/FR conditions,\(^{66}\) though PIF4 and PIF5 are also involved in it (Fig. 3).\(^{83}\) Another interesting aspect with respect to relationship between the circadian clock and phytohormone signaling is that expressions of a set of phytohormone biosynthesis or

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**Fig. 4.** Schematic Representation of External Coincidence Models. External coincidence models to explain photoperiod (A) and temperature (B) controls of plant growth, and photoperiodic control of flowering (C). Diurnal elongation growth responses under natural day- and night-cycle conditions in plants are accomplished by the interactions between circadian clock and light signaling, and between circadian clock and phytohormone signaling (D).
signaling-associated genes correlate with time of hypocotyl growth, that is, they are diurnally induced at the end of night in short-days.\textsuperscript{90} It is possible that PIF4 and PIF5 participate in this time of day- and photoperiod-specific gene expression. In fact, a genome-wide approach by ChIP-Seq analysis identified about 1,000 PIF5 binding sites on the chromosome under shade conditions, and auxin biosynthesis or signaling-associated genes are significantly detected as target genes of PIF5.\textsuperscript{91} These are consistent with the earlier finding that transcriptional responses to auxin are gated by the circadian clock with the maximum sensitivity just before dawn.\textsuperscript{88} Although it is poorly understood how auxin is linked to the clock based plant growth, it is evident that auxin is involved in the elongation responses to serve plant growth.\textsuperscript{89-91} It was confirmed recently that not only auxin-associated genes (\textit{GH3.5}, \textit{IAA19}, \textit{IAA29}), but also genes associated with other growth-regulating hormones such as BR (\textit{BR6ox2}), GA (\textit{GA1}), ethylene (\textit{AC38}), and cytokinin (\textit{CKX5}) are induced in a time-of-day- and photoperiod-specific manner in accordance with the PIF4 and PIF5 dependent external coincidence model, suggesting that the circadian clock orchestrates a variety of hormone-signaling pathways to regulate the photoperiod-dependent morphogenesis.\textsuperscript{92} As described above, the time-of-day- and photoperiod-specific plant growth is best explained by stimulation of the PIF4 transcriptional activity at the end of night specifically in short-days, due to the coincidence between the internal (circadian rhythm) and external (photoperiod) time cues, but flexibility of the external coincidence model was challenged by the recent finding that elongation of hypocotyls was markedly promoted at a high growth temperature \textit{(i.e., 28 °C)} in a PIF4-dependent manner.\textsuperscript{93,94} It was found that the PIF4 and PIF5-mediated external coincidence model is applicable to temperature control of diurnal plant growth, based on the observation that transcription of \textit{PIF4} occurs precociously at the end of night under high temperature conditions even in long-days (Fig. 4B).\textsuperscript{95} In accord with this, target genes of PIF4 were also induced at the end of night under these conditions, verifying the model (Fig. 4B).\textsuperscript{95} PIF4 regulates auxin biosynthesis at high temperature.\textsuperscript{96,97} In addition to auxin, other phytohormones network is induced at high temperature as in short-days.\textsuperscript{95} The extended external coincidence mechanism explains how two environmental cues (\textit{i.e., photoperiod and temperature}), both of which vary on daily and season-to-season bases, are integrated into the same clock and PIF4-mediated output pathway that regulate a hormone-signaling network to fit plant architectures properly to domestic habitats. The circadian clock can control multiple output pathways, depending on when the clock opens the gate to the output pathways. The external coincidence mechanism of photoperiodic control of plant growth is similar to that of the photoperiodic control of flowering time, in which the CO transcription factor serves as the critical integrator for both clock and light signals.\textsuperscript{98,99} In the former case, the clock opens the gate to PIF4/5 activity during \textit{extended-night (end of night)} in short-days. In the latter case, the clock opens the gate to CO activity during \textit{extended-daytime (end of day)} in long-days.\textsuperscript{100-102} These mechanisms are mirror images of each other (Fig. 4C).

### III. Concluding Remarks and Future Perspectives

Circadian clock, light signaling, and phytohormone signaling can be studied independently. Progress in each research consequently led to better understanding of plant growth on a day and night cycle basis. Some of the de-etiolation mechanisms under the control of light and phytohormone signaling might be evolutionally co-opted in the photoperiod and temperature controls of diurnal plant growth. Since plants are sessile, adaptation to daily changes in environmental conditions brings great advantages to them. Also, since light and temperature vary on time-to-time, season-to-season, and place-to-place bases, the plant circadian clock plays a pivotal role in it. The external coincidence mechanism responsible for photoperiod and temperature controls of plant growth is established by the interactions between circadian clock and light signaling, and between circadian clock and phytohormones signaling (Fig. 4D). In this model, two external cues (\textit{i.e., photoperiod and temperature}) modify the phase of the rhythmic expression of \textit{PIF4} independently, but are integrated into the same circadian clock, light and phytohormone signaling-regulated PIF4 (and/or PIF5) output pathway, and this in turn coordinately regulates a hormone-signaling network to fit plant architectures appropriately to natural domestic habitats. A long photoperiod inhibits elongation of hypocotyls and petiols, and a short photoperiod enhances it. On the other hand, high temperature enhances plant growth, and a low temperature inhibits it. Long photoperiods coincide with high temperatures, and short photoperiods with low temperatures in the natural environment. The plant circadian clock might buffer exaggerated responses throughout the whole plant life cycles by integrating both the photoperiod and temperature signals into the external coincidence mechanism to serve diurnal plant growth, because plant architecture is critically important in long distance transport for cell-to-cell communications (\textit{e.g., sink-source relationship and signal transduction}) to organize multi-cellular systems. However, it is poorly understood how photoperiod and/or high temperature affect the clock function \textit{per se} that is responsible for the phase control of rhythmic expression of \textit{PIF4}. Functional modification of the activity of EC might be involved in the sensitivity of \textit{PIF4} expression phase to photoperiods, because it represses the transcription of \textit{PIF4} and \textit{PIF5} directly.\textsuperscript{14} Marvelously fascinating evidence was presented recently with regard to the thermosensory mechanism in \textit{A. thaliana}.\textsuperscript{103} The nucleosome structures of some temperature sensitive genes (\textit{e.g., HSP70}) are modified by the incorporation of histone H2A.Z in place of H2A as ambient temperature decreases.\textsuperscript{104} H2A.Z occupancy defines the state of transcriptional activity of the genes. This finding might also help in understanding how circadian rhythms are modified in output pathways by temperatures. At the protein level, furthermore, the activities of PIF4 and PIF5 are thought to be regulated intricately. In addition to DELLLAs and BZR1, PIF family protein HFR1 and HLH protein PAR1 interact with PIF4 and prevent it from binding to the target DNA.\textsuperscript{105,106} Stability of PIF4 is down-regulated by low temperature.\textsuperscript{107} It is known that PIF family
protein SPT is involved in the control of plant growth under low temperature conditions. Regulation of protein SPT is involved in the control of plant growth and its output mechanism in plants. Though this suggestion is interesting, it is not well understood how the PIF4-downstream hormone signaling network coordinately regulates plant architecture in response to changes in environmental conditions. Clarification of these subjects on the external coincidence model will contribute to comprehend circadian clock and its output mechanism in plants.

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References