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Dancing in the dark: darkness as a signal in plants

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Abstract

Daily cycles of light and dark provide an organizing principle and temporal constraints under which life on Earth evolved. While light is often the focus of plant studies, it is only half the story. Plants continuously adjust to their surroundings, taking both dawn and dusk as cues to organize their growth, development and metabolism to appropriate times of day. In this review, we examine the effects of darkness on plant physiology and growth. We describe the similarities and differences between seedlings grown in the dark versus those grown in light–dark cycles, and the evolution of etiolated growth. We discuss the integration of the circadian clock into other processes, looking carefully at the points of contact between clock genes and growth-promoting gene-regulatory networks in temporal gating of growth. We also examine daily starch accumulation and degradation, and the possible contribution of dark-specific metabolic controls in regulating energy and growth. Examining these studies together reveals a complex and continuous balancing act, with many signals, dark included, contributing information and guiding the plant through its life cycle. The extraordinary interconnection between light and dark is manifest during cycles of day and night and during seedling emergence above versus below the soil surface.

Keywords

Arabidopsis; circadian; dark; growth; light; photobody; phytochrome; PIF; sugar

THE DARK SIDE OF PLANT PHYSIOLOGY

The day–night oscillations on Earth require photosynthetic organisms to maximize photon collection during the light period. The nature of photosynthetic carbon fixation and the importance of this process for agriculture promote an attention bias toward physiological functions of and in light. Indeed, many model organism studies default to growing plants under constant light to accelerate the life cycle, eliminating the dark phase out of experimental simplicity (discussed at length in Velez-Ramirez *et al.* 2011). However, time-resolved studies are beginning to illuminate the intricate predictive and reactive physiological adjustments that plants make based on time of day. Additionally, etiolated

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CONFLICT OF INTEREST STATEMENT

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growth provides a pattern of dark growth that is conserved across many land plants as the newly germinated seedling grows up through the substrate. In this review, we examine the effects of darkness on plant physiology. By comparing growth mechanisms in dark-grown plants and those grown in day–night cycles, we describe several ways in which darkness exerts influence. The term ‘growth’ is primarily used here to refer to hypocotyl elongation growth, but other types of growth are specifically noted. We examine common pathways used under different conditions, the relationship of the circadian clock system to growth and metabolism, and the diurnal and circadian regulation of carbon catabolic pathways. A renewed focus on the physiology of darkness is applicable to studies of early plant growth through soil and in day–night cycles. These ideas may also be important for understanding growth and patterning differences between parts of the plant that are differentially exposed to light, as are the roots and shoots, although we do not discuss it in-depth here. These processes are strongly dependent on darkness of varying duration, and we suggest that attention bias toward processes occurring exclusively in light severely limits our understanding of metabolism, signaling and growth.

LIFE UNDERGROUND

A plant that germinates in a dark environment, such as in soil, must overcome specific challenges. The embryonic plant transitions from heterotrophic growth, using maternally deposited energy reserves, to autotrophic growth relying on photosynthesis. Depending on environmental light conditions, a plant must decide between two developmental programmes. Light promotes photomorphogenesis, or de-etiolated growth. This is characterized by deceleration of hypocotyl elongation, opening of the apical hook, unfolding and expansion of the cotyledons, increased synthesis of protective anthocyanin pigments and maturation of chloroplasts, including assembly of the photosynthetic machinery and elaboration of internal membranes. If light is limited, the seedling will use skotomorphogenesis, or etiolated growth. This program is characterized by an elongated hypocotyl (embryonic stem) with apical hook, tightly closed embryonic leaves (cotyledons) protecting the shoot apical meristem as it pushes through the soil, and undifferentiated chloroplast precursors (etioplasts) (Arsovski *et al.* 2012; Chory *et al.* 1996; Chory *et al.* 1989). These processes are accomplished largely by promoting the activity of the basic helix-loop-helix (bHLH) phytochrome-interacting factors (PIFs) transcription factors and by degrading transcription factors via the ubiquitin-proteasome system (COP/DET/FUS – see succeeding texts). These factors are general regulators of growth and have been found to act downstream of almost every environmental stimulus that changes growth rate, including shade, temperature and high light (de Wit *et al.* 2016; Fraser *et al.* 2016; Martin *et al.* 2016; Nozue *et al.* 2015; Quint *et al.* 2016). For the purpose of this review, we will focus on their roles in skotomorphogenesis and during the day–night cycle. Brassinosteroids, gibberellins, auxin and ethylene phytohormones have important roles in the control of etiolated growth. The functions of these hormones have been reviewed in depth and will not be covered here (Arsovski *et al.* 2012; de Wit *et al.* 2016; Rodrigues *et al.* 2014; Symons & Reid 2003; Vanstraelen & Benkova 2012; Wang *et al.* 2012).

The PIFs are functionally redundant in promoting growth but have specific roles in responses to different environmental signals. During etiolated growth PIF1, 3, 4 and 5

appear to play a dominant role, regulating more than 2000 genes, and promoting rapid elongation growth (Pfeiffer *et al.* 2014; Zhang *et al.* 2013). Arabidopsis mutants lacking these four PIFs (pifq) resemble light-grown plants when grown in the dark (Leivar *et al.* 2008). In the transition from dark to light, active phytochromes interact with PIFs (PIF1 and PIF3 can interact with PHYA or PHYB while other PIFs interact mainly with PHYB) and induce phosphorylation and degradation of the PIFs by the proteasome (reviewed in Leivar & Quail 2011) in a photobodydependent mechanism (see photobody section). PIF3 can simultaneously bind DNA and PHYB (Martinez-Garcia *et al.* 2000). ChIP-seq of PHYA and PHYB proteins showed that both are also present on chromatin, possibly through an interaction with PIFs or other transcription factors (Chen *et al.* 2014; Jung *et al.* 2016). The blue/UV-A photoreceptor cryptochrome 2 (CRY2) forms a complex with PIF4 and PIF5 on DNA when blue light intensity is low (Pedmale *et al.* 2016). Together, these findings suggest that PIFs act downstream of multiple photoreceptors, and by multiple mechanisms.

A second group of genes that are crucial for the repression of photomorphogenesis were identified in genetic screens for mutants that de-etiolate in the dark (COP: COnstitutively Photomorphogenic; DET: De-ETiolated; FUS: FUSCA) (Chory 1992; Chory *et al.* 1989; Deng & Quail 1992; Deng *et al.* 1991, Pepper *et al.* 1994). These genes form three distinct protein complexes. Two E3 ubiquitin ligase complexes, COP1–suppressor of PHYA-105 (SPA) and COP10–DET1–DDB1 (CDD), mediate the degradation of the photomorphogenesis promoting factors. A third complex, the COP9 signalosome (CSN), is a general regulator for all CULLIN-RING E3 ligases (CRLs) via de-neddylation (reviewed in these complexes are all important in the ubiquitin proteasome system and functionally connected through CULLIN4 (CUL4), a scaffold protein of CRLs (Huang *et al.* 2014; Nezames & Deng 2012). Interestingly, human DET1 and COP1 form a complex with CUL4–DDB1. Arabidopsis DET1 and COP1 have not been shown to physically associate; however, DET1 is required for COP1 nuclear localization by an as yet unknown mechanism (von Arnim *et al.* 1997; Wertz *et al.* 2004). When grown in the dark, plants carrying mutations in *cop1*, *det1* or other proteins in these complexes exhibit the morphology of light growing seedlings and partially developed chloroplasts. Indeed, gene expression profiles of *det1* and *cop1* mutants are highly similar to those of light-grown wild type, suggesting that these factors are responsible for coordinating much of the photomorphogenesis program (Ma *et al.* 2003).

The characterization of COP/DET/FUS targets is an active area of research. Recently, it was shown that DET1 and COP10 interact *in vivo* with long hypocotyl in far-red (HFR), and the authors suggest that the CDD–CUL4 E3 ligase can act as substrate receptors to directly target HFR1 for degradation (Shi *et al.* 2015). New targets of COP1-mediated degradation are identified on a regular basis. This list includes members of the B-box, bHLH, ERF/AP2, bZIP, MYB and GATA transcription factor families (Datta *et al.* 2008; Holm *et al.* 2002; Jang *et al.* 2010; Lau & Deng 2012; Luo *et al.* 2010; Osterlund *et al.* 2000; Seo *et al.* 2004; Seo *et al.* 2003; Xu *et al.* 2016a; Xu *et al.* 2016b; Yang *et al.* 2005). COP1 is required for the degradation of activated phytochromes in the dark, while phytochromes inhibit the COP1–SPA complex in the light (Jang *et al.* 2010; Seo *et al.* 2004; Sheerin *et al.* 2015). COP1 is also involved in the regulation of circadian clock proteins, interacting with early flowering 3 (ELF3) and destabilizing *gigantea* (GI) (Park *et al.* 2004; Yu *et al.* 2008).

The molecular function of DET1 is less well understood, in part because the CDD-CUL4 complex lacks a DWD protein with a WDXR motif, which is the typical substrate recognition motif for CUL4–DDB1 ligases. This makes it more challenging to understand the mechanism of this E3 ligase complex. In addition to its role in the CDD complex, DET1 has been implicated in chromatin regulation and shown to act as a transcriptional co-repressor (Benvenuto *et al.* 2002; Dong *et al.* 2014). It has repression activity *in vivo* and can interact with the core clock genes circadian clock associated 1 (CCA1) and late elongated hypocotyl 1 (LHY) to mediate the repression of timing of cab expression 1 (TOC1) (Fig. 1) (Dong *et al.* 2014; Lau *et al.* 2011). In fact, *det1* mutants have an extraordinarily short circadian period, running at ~18.5 h in constant darkness, and ~20 h in constant light (Millar *et al.* 1995). Recent work has shown that, in the dark, PIF1 interacts with COP1 and enhances E3 ligase activity for elongated hypocotyl 5 (HY5) degradation while, in the light, the phosphorylated form of PIF1 is recruited to the COP1 complex for rapid ubiquitylation and subsequent degradation (Zhu *et al.* 2015). Dong *et al.* (2014) showed that DET1 stabilizes PIF proteins and that 56% of genes that are regulated by light and DET1 are also regulated by PIFs. All together, it seems that COP/DET/FUS and the PIFs work in concert to repress photomorphogenesis.

Of the photomorphogenesis-promoting transcription factors, HY5 is among the best studied and it has some level of interaction with each of the photomorphogenesis-repressing factors mentioned in the preceding texts. *hy5* mutants are characterized by large cotyledons, long hypocotyls under all lighting conditions and reduced chlorophyll and anthocyanin levels. It was the first identified target of COP1, and its accumulation depends on DET1, although a direct HY5-DET1 interaction has not been found (Ang *et al.* 1998; Osterlund *et al.* 2000). In the light, the nuclear abundance of COP1 and DET1 is reduced, allowing HY5 to accumulate (Pacin *et al.* 2014; Pepper *et al.* 1994; von Arnim & Deng 1994; von Arnim *et al.* 1997). As a result, HY5 interacts with other transcription factors, and with DNA, to change the expression of more than 2000 genes (Kurihara *et al.* 2014; Lee *et al.* 2007; Zhang *et al.* 2011). HY5 interacts with PIF1 and PIF3 *in vivo*, co-regulating and competing for binding at G-box motifs in the promoters of photosynthetic genes (Chen *et al.* 2013). In a different study, HY5 expression was found to be dependent on both COP1 and PIFs, and the authors suggested a model in which PIFs work as cofactors of COP1 (Xu *et al.* 2014). There are also indications that HY5 can regulate the circadian clock. During the day, ELF4 expression is directly activated by HY5. The clock components CCA1 and LHY bind the ELF4 promoter in the morning, inhibiting this activation. Interestingly, HY5 and CCA1 can bind, promoting interaction with the DNA and potentially influencing clock progression (Andronis *et al.* 2008; Li *et al.* 2011). The observation that B-BOX22 (BBX22), which interacts with HY5 to repress growth, can change the growth rate at the end of the night suggests some involvements of HY5 (Chang *et al.* 2011; Datta *et al.* 2008). The *bbx22* mutant did not change the timing, but increased the magnitude of the peak growth rate. Given the demonstrated interconnections between HY5, PIFs, the COP1/DET1 complexes and the clock, it will be of interest to examine the potential effects of HY5 on temporally restricted growth patterns.

One particular challenge for future study will be dissecting these interactions in a tissue-specific and cell type-specific manner. This may elucidate the different growth responses of

the hypocotyl (inhibition of elongation) and the cotyledons (promotion of growth and expansion) during de-etiolation. Some progress has been made in this direction, with two studies suggesting that at least part of the light signaling converges on the PIFs in both organs. In the first study, Sentandreu *et al.* (2011) showed that four different direct targets of PIF3 regulate specific signatures of etiolation in the apical hook, cotyledon or hypocotyl. In the second study, Sun *et al.* (2016) found that a group of small auxin up RNA (SAUR) genes were light-induced in cotyledons and/or repressed in hypocotyls and that PIF3 and PIF4 directly bind and regulate expression of these genes. Interestingly, recent work has shown that chromatin organization in the cotyledons and hypocotyl are differentially regulated during de-etiolation, and these differences are dependent on COP1 and DET1 (Bourbousse *et al.* 2015). More detailed analysis of specific tissue layers can yield important insights, as demonstrated by several papers describing the importance of the epidermis in regulating hypocotyl elongation in shade conditions (Kim *et al.* 2016; Procko *et al.* 2016; Savaldi-Goldstein *et al.* 2007). These approaches are likely to be quite valuable for future work on growth in the dark. Further development of tissue-specific tools for manipulation of gene expression will be critical to form a more detailed understanding of these growth processes and gene regulatory networks (GRNs) at the organ and tissue level.

Another group of mutants specifically modifies hypocotyl growth in the dark and are related to maintenance of the cell wall. Two cellulose synthase mutants, *prc1* (CesA6) and *rsw1* (CesA1), cause structure defects in the expanding cell wall, and their hypocotyls are dramatically shorter in the dark (Desnos *et al.* 1996; Fagard *et al.* 2000). These phenotypes can be suppressed by light in a fluence rate dependent manner and by mutations in *det1* or *cop1*. These interactions suggest that the rapid elongation seen in the dark depends on the integrity of the cell wall. Mutations affecting the cell wall may not have serious consequences in the background of these constitutively short mutants.

EVOLUTION OF THE ETIOLATED BODY PLAN: IN THE BEGINNING, WAS THERE LIGHT?

The precursors to land plants are thought to have been aquatic, with few interruptions in the light/dark cycle expected in between the surface of the seas and the sky. Light provides both energy and risk for photosynthetic organisms in this environment. The ‘escape from light’ hypothesis proposed that photo-damage from the ultraviolet (UV) part of the spectrum may have provided pressure to protect the genome from light during the day, while taking advantage of the dark to carry out DNA replication and cell division (Pittendrigh 1993). While this is a difficult hypothesis to test directly, clocks have been shown to increase fitness in oscillating environments in multiple species, including cyanobacteria and *Arabidopsis* (Dodd *et al.* 2005; Woelfle *et al.* 2004). One key aspect of clock-driven physiology is that it runs in a pattern matching the environment and relies on accurate predictions of day and night length. However, an early phase of life for many land plants occurs with severely limited environmental light input and thus damped external rhythmic stimulus.

There has been some debate as to the relationship between the growth programmes used during long-term darkness versus oscillating light–dark cycles. Xing-Wang Deng’s group

has suggested that photomorphogenesis is the default developmental pathway, whereas skotomorphogenesis is a specialized and evolutionarily derived developmental pattern (Jiao *et al.* 2005; McNellis & Deng 1995; Wei *et al.* 1994). They supported this assertion using two arguments. Firstly, while many recessive mutations have been found that display a light-grown phenotype in the dark, no single mutants had been identified with growth patterns closely resembling dark-grown plants in the light. Secondly, basal plants such as gymnosperms and algae usually form chloroplasts in the dark and are not able to etiolate (tend to follow similar developmental patterns in light and darkness). Both of these arguments have since been challenged, and it is likely that the true origin of skotomorphogenesis is more complex.

The genetic argument is countered by the discovery of the recessive mutant *hemera* (*hmr*), which exhibits skotomorphogenesis-like phenotypes – a long hypocotyl and chloroplast defects that lead to an albino phenotype – when grown in light (Chen *et al.* 2010). As such, the *hmr* mutant may occupy the gene class that was presumed not to exist, being similar, in some important ways, to etiolated seedlings. In addition, based on their phylogenetic argument, the authors postulated that skotomorphogenesis evolved later on the evolutionary time scale and land plants adapted to terrestrial conditions such as soil and dense vegetation canopies by temporarily repressing photomorphogenesis. A recent study in pine seedlings demonstrated a difference in light dependent regulation between gymnosperms and flowering plants in the regulation of photosynthesis associated nuclear genes (PhANGs). In gymnosperms, the PhANGs are regulated by positive plastid biogenic signals largely independent of light, while in flowering plants the control of chloroplast development evolved through recruitment of COP1 to suppress the ability of PhANGs to respond to the positive signal coming from the plastid in the dark (Hills *et al.* 2015). This effect applies to the difference in chloroplast maturation between these lineages. However, the pine still exhibits strong elongation growth in the dark, emphasizing the point that the chloroplast maturation and elongation growth phenotypes commonly associated with etiolated growth are fully separable in evolutionary time. In agreement with this, Mathews (2006) argues that gymnosperm etiolation is very pronounced and notes that even quite early in the evolution of vascular plants etiolation-like growth patterns have been observed. Free-sporing vascular plants, such as ferns and lycophytes, and even algae showed some characteristics of etiolation, and mosses elongate and are pale when grown in the dark (Cove *et al.* 1978; Mathews 2006; Riese *et al.* 2008; Virgin 1978). It is therefore possible that common aspects of etiolation were either present in ancestral plants or were adopted independently in many lineages.

Differential production of reactive oxygen species may provide selective pressure on mechanisms of chloroplast maturation during skotomorphogenesis in various lineages. Angiosperms cannot green in the dark because of the light-dependent enzyme protochlorophyllide oxidoreductase (LPOR). In etiolated angiosperms, chlorophyll biosynthesis stops prior to formation of protochlorophyllide (Pchl_{id}). In the dark, Pchl_{id} is bound to LPOR. This complex can absorb light, catalyzing the reduction of Pchl_{id} to chlorophyllide in a light-dependent reaction and leading to accumulation of chlorophyll. This reaction generates large amounts of singlet oxygen when illuminated, which can cause severe damage and cell death. In gymnosperms and other lineages, a light-independent

enzyme designated dark-operative Pchlide oxidoreductase (DPOR) is present, allowing chlorophyll synthesis in dark and light (Reinbothe *et al.* 2010). Thus, the inclusion of chloroplast maturation in the etiolation versus de-etiolation programmes is flexible, and selective pressure may be partially determined by the nature of the POR enzyme.

Early growth stages are highly sensitive to environmental conditions, especially in annuals, and the threats and opportunities provided by environmental light provide strong selective pressures. Different local environmental conditions and lineage-specific constraints, such as the potential threat of lethal pulses of reactive oxygen species, may provide a molecular basis for some of the variation observed among plant taxa. However, the specific mechanisms underlying the evolution of skotomorphogenesis are likely more intricate than the mechanisms proposed and will require deeper evolutionary analysis of chloroplast maturation, photoreception and growth programmes. The discovery of hmr (and the implication that there are more mutants like it yet to be found) and the presence of etiolation-like growth patterns in many ancient plant lineages suggests that skotomorphogenesis is not simply tacked onto default photomorphogenic growth program for all terrestrial vascular plants. The capacity for alternate light and dark growth modes is deeply embedded in the GRNs that integrate lighting information to control growth.

INVASION OF THE (PHOTO)BODY SNATCHERS

Plants use many photoreceptors to sense their environments, providing critical information about the changes in light quality, intensity, direction and duration. In *Arabidopsis*, five classes of photoreceptors have been identified: five red/farred-absorbing phytochromes (PHYA-E), blue/UV-A-absorbing cryptochromes (CRY1, 2), phototropins (PHOT1,2), the LOV-F-Box-Kelch domain (ZTL, FKF and LKP2) families and the UV-B-absorbing receptor UVR8. Red light induces the major phytochrome, PHYB, to change its conformation from the red-absorbing inactive form (Pr) to the farred-absorbing active form (Pfr) (Burgie *et al.* 2014; Butler *et al.* 1959). Far-red light induces the return to the inactive Pr conformation, allowing the pool of PHYB to measure the red/far-red ratio and signal accordingly. The Pfr state can also spontaneously revert to Pr in the dark, the rate of which may be modulated by phosphorylation (Medzihradsky *et al.* 2013). The structural biochemistry of the complete phytochrome photocycle has been reviewed in detail (Burgie *et al.* 2014). The Pfr form can enter the nucleus where they form subnuclear foci, called phytochrome nuclear bodies or photobodies. Photobodies appear to be heterogeneous, changing their composition over the course of the day. They are dependent on either hemera (HMR) or tandem zinc knuckle/plus3 (TZP) and integrate light signals into coordinated transcriptional responses (Chen & Chory 2011; Chen *et al.* 2010; Huang *et al.* 2016b; Kaiserli *et al.* 2015).

Hemera-dependent photobodies have been proposed to be sites of protein turnover in which phytochromes induce rapid phosphorylation of PIFs (most likely PIF1 and PIF3), tagging them for ubiquitination and degradation by the proteasome, and perhaps making them more transcriptionally active, although this is not the sole mechanism of modulating PIF activity (Kircher *et al.* 2002; Van Buskirk *et al.* 2012; Yamaguchi *et al.* 1999). Activated phytochromes, primarily PHYB, form photobodies during the day/night cycle, forming rapidly at lights-on and dissipating after several hours in the dark (Huang *et al.* 2016b;

Kircher *et al.* 2002). The spontaneous reversion rate in the dark (from Pfr back to the Pr ground state) is on the order of 4 h, although activated PHYB may be further stabilized in photobodies during the first hours of the night. These pools of active PHYB may be able to prevent the accumulation of PIF until late in the night (Fig. 2) (Klose *et al.* 2015; Medzihradzsky *et al.* 2013; Van Buskirk *et al.* 2014). PHYA, which is activated by far-red and destabilized by red light, can also form short-lived photobodies with PIF3, but the function, composition, and dynamics are not well understood (Bauer *et al.* 2004).

Tandem zinc knuckle/plus3-containing photobodies not only contain activated PHYB but also include the circadian Evening Complex (EC, Fig. 1) components ELF3, ELF4 and LUX, in addition to TOC1, COP1, SPA1–3 and PIF7 (Huang *et al.* 2016a). These interactions were shown by isolating the EC, and therefore may not be a complete list of TZP binding partners. However, the specific inclusion of PIF7 and the absence of HMR suggest that at least two types of photobody play important roles in controlling growth, possibly via controlling discrete subsets of PIF transcription factors. However, HMR may not be a direct member of these complexes. Direct isolation of TZP complexes will be particularly important for understanding the full range of interacting proteins, as opposed to the indirect detection via interaction with the EC. This will allow characterization of the order-of-assembly and formation and dissociation kinetics, which may encode temporal information. These functions of PHYB in photobodies provide compelling mechanisms for limiting PIF accumulation and activity to the late night and suggest a possible mechanism for generating specificity among PIFs (Fig. 2). It will be of interest to determine if indeed there are distinct types of photobodies that are identifiable based on the presence of HMR, TZP or subsets of the PIF family. Additionally, it will be important to see if there are genetic interactions between HMR and TZP, such as testing if HMR is required for the TZP-containing photobodies to form, or if there are two or more types of photobodies. Such avenues are likely to provide insight into how light signals through a limited set of photoreceptors, particularly phytochromes, and lead to diverse and specific downstream events.

HELLO DARKNESS, MY OLD FRIEND

The high degree of influence of environmental oscillations on life is reflected in the multiple evolutionary origins of circadian clock systems. These systems, usually incorporating transcriptional/translational feedback loops, are biochemical oscillators that are set by regular environmental changes including the major signifiers of day (light, warmer temperature) and night (dark, cooler temperature). These oscillators have strong influences on other physiological processes by regulating gene expression according to the time of day. The circadian clock in plants maintains a strong influence on growth, reproductive development, and metabolism (reviewed in depth in Hsu & Harmer 2014; McClung 2011; Sanchez & Kay 2016). The clock allows the plant to anticipate daily changes in the environment, such as dawn and dusk, and regulates gene expression programmes based on time of day. This includes key light signaling and photosynthetic components. This, combined with the observation of phase-specific gene expression, requires that conversations about the influence of darkness on growth and physiology take the clock into account (Covington *et al.* 2008; Michael *et al.* 2008).

The rate of hypocotyl elongation in *Arabidopsis* seedlings is controlled by photoperiod and gated by the circadian clock. Dowson-Day & Millar (1999) showed that the hypocotyl growth rate oscillates with an ~25 h period in constant light conditions. This period becomes shorter in a *toc1* mutant and becomes arrhythmic in an *elf3* mutant, agreeing with known effects of these mutations on reporters of circadian-controlled gene expression (Hicks *et al.* 1996; Somers *et al.* 1998). While these data strong mutations on reporters of circadian-controlled gene expression (Hicks *et al.* 1996; Somers *et al.* 1998). While these data strongly support a role for the clock in controlling elongation, the authors did not examine this process under light–dark cycles. A subsequent study found a striking function for the clock in controlling the temporal growth pattern in oscillating conditions. Nozue *et al.* (2007) compared wild-type and clock mutant plants growing in short days (8 h light at 62 μ E, 16 h dark). While the wild-type (Columbia) elongation rate reliably peaked in the late night and early morning, two arrhythmic lines – CCA1-ox (which over-expresses a major circadian transcriptional repressor – Wang & Tobin 1998) and *elf3*– began growing rapidly upon lights-off, maintaining a high rate of growth until lights-on, at which point the growth rate slowed. In a particularly illuminating experiment, this group directly tested the interaction between the clock and photoperiod in growth rate control by examining growth in plants entrained to short days and shifted to 4 h light, 4 h dark (8 h T-cycle) conditions. Under this regime, wild-type plants maintained a 24 h rhythm in peak growth rate, while CCA1-ox and *elf3* mutants responded purely to the change in light, rapidly increasing growth rate in the dark and reducing growth in the light. These data reveal a layered system of growth control in the shoot; growth is acutely promoted in the dark, but is restricted to a particular time of day by the circadian clock. When the clock is removed, this temporal gating function is lifted and the underlying control system, ‘sense fewer photons; promote growth’, is revealed.

Hypocotyl elongation is photoperiod-sensitive, but this sensitivity is not linear in wild-type plants. Studying the nature of this non-linearity, Niwa *et al.* (2009) tested many circadian clock mutants and plotted ‘photoperiod response curves’ of hypocotyl length as a function of the duration of the dark period ranging from constant light to constant dark. Wild-type plants maintain a short (~1–2 mm) hypocotyl in constant light up to a 12 h dark period, with the hypocotyl length increasing with longer nights to a maximum (~8–10 mm) in constant darkness. Strikingly, the strongest clock perturbations used in this study [*CCA1-ox* and the *pseudo-response regulator (prr) 5 prr7 prr9* triple mutant] convert this pattern to a nearly linear function, with the mutant plants elongating in long photoperiods at a rate that arrives at a similar maximum length in constant darkness. This finding is consistent with the Nozue study, in that the elongation growth that occurs in wild-type occurs only after a prolonged period of darkness. Stopping the clock removes an inhibitory signal, allowing growth to occur in shorter photoperiods. In the clock mutants, some growth can occur in dark periods as short as 3 or 6 h and accumulate over several light–dark cycles, resulting in a longer than normal hypocotyl.

How does the circadian clock connect to and control growth-promoting pathways? Microarray analysis of transcripts from wild-type and CCA1-ox plants in growth and non-growth phases identified the growth-promoting PIF4 and PIF5 as constitutively highly expressed when the clock is stopped by CCA1-ox (Nozue *et al.* 2007). Over-expression lines for both of these PIFs in the 8 h T-cycle condition showed the same growth pattern as

CCA1-ox: immediate growth promotion at the onset of dark. The *pif4 pif5* double mutant did not rhythmically elongate in the dark under their conditions. Consistent with this, hypocotyl growth showed a linear response to increasing the dark period in PIF4 and PIF5 over-expressers, while the *pif4 pif5* double mutant maintained a short hypocotyl up to a 15 h dark period, and only reaching ~60% of wild-type length in constant darkness (Niwa *et al.* 2009 70 μ E light period). The findings demonstrating that clock mutants and PIF over-expressers remove the temporal gating of growth were confirmed, but further detailed study will be required to determine the root cause of the differences between the reported temporal growth patterns of wild-type plants.

Phytochrome-interacting factors-family transcription factors promote growth, but are degraded during the light period in a PHY-dependent manner (Al-Sady *et al.* 2006; Leivar & Quail 2011; Park *et al.* 2004; Shen *et al.* 2008). PIF3 and TOC1 interact *in vitro* (Makino *et al.* 2002). Soy *et al.* (2016) examined the functional consequences of the TOC1-PIF relationship by comparing the co-occupancy of TOC1 and PIF3 from existing ChIP-seq data sets, identifying 144 genes that are likely targets of both proteins (Huang *et al.* 2012; Pfeiffer *et al.* 2014). The high degree of functional redundancy among the PIFs suggests that this interaction may be retained across the four major PIF family members (Soy *et al.* 2016). Examination of TOC1 and PIF3 influence on target genes revealed TOC1-mediated repression in the early and mid-night and, as the circadian cycle continues, TOC1 abundance declines, relieving transcriptional repression and allowing PIFs to activate transcription of growth-promoting genes (Soy *et al.* 2016). TOC1 has also recently been shown to interact with PIF4 to suppress transcription of target genes in temperature-regulated hypocotyl elongation (Zhu *et al.* 2016). These findings combined suggest a compelling possibility of darkness activating a biological process – the degradation of TOC1 – and feeding into a key growth-promoting system (Fig. 2). It will be of particular interest to see if this or a similar mechanism has been adapted for other time-sensitive or light-sensitive processes or reactions.

While PIF4 and PIF5 are controlled by the clock at the transcript level, PIF1 and PIF3 show constant low levels of transcription across the day (Nozue *et al.* 2007; Nusinow *et al.* 2011). All four PIF proteins accumulate in the dark (Leivar *et al.* 2012; Soy *et al.* 2012; Soy *et al.* 2014). Combined, these studies provide a coherent view of circadian-gated, PIF-activated hypocotyl growth in the late night. The PHY-mediated degradation of the PIFs during the light, or early dark, provides a mechanism for relieving inhibition on a growth-promoting system. This effect is slow and might be viewed as a passive signal, occurring only after a prolonged period in the absence of light. However, the resetting properties of the clock suggest that inputs at other times of day could influence the relative coordination and gating function of TOC1 on PIF growth-promoting activity. The regulation of PIF-containing complexes is likely much more intricate given the recent characterization of HMR- and TZP-containing photobodies (Kaiserli *et al.* 2015; Van Buskirk *et al.* 2014). These complexes dissociate after ~6 h in the dark, although higher frequency sampling will be needed to determine the true kinetics. However, dissociation appears to occur on a time-scale similar to the proposed relief of inhibition by TOC1 (see preceding texts), and it will therefore be of interest to examine potential circadian contributions to photobody composition or dynamics. The EC interaction with TOC1, TZP and PIF7 suggests strong

circadian influence on this type of photobody, and shows that TOC1 can interact with multiple PIFs, although the specific effects of this complex have not yet been worked out. Direct regulation of PIFs by sucrose is another possibility, as PIF5 protein levels and PIF1, 3, 4 and 5 transcript levels increase on sucrose medium, although direct mechanisms for these increases have not been described (Liu *et al.* 2011; Stewart *et al.* 2011).

Many of the plant's photoreceptor proteins provide light input signals to the circadian clock (reviewed in Christie *et al.* 2015; Demarsy & Fankhauser 2009; Jones 2009; Millar 2004). The blue-light photoreceptor and F-box protein ZTL provides an opportunity to examine the effects of darkness on photoreceptor signaling in the clock. It is rapidly degraded during the light phase but can be stabilized in a blue light-dependent manner when GI levels increase in the late day (Fujiwara *et al.* 2008; Kim *et al.* 2007; Kim *et al.* 2003). In the absence of blue light, ZTL can form a SCF (SKP1-CULLIN-F-BOX) complex to regulate ubiquitination and degradation of target proteins (Han *et al.* 2004; Kiba *et al.* 2007; Kim *et al.* 2003). The core clock protein TOC1 is degraded in the early-mid night, targeted to the proteasome by the ZTL-CUL1 complex (Harmon *et al.* 2008; Kiba *et al.* 2007; Kim *et al.* 2007; Mas *et al.* 2003). The interaction between GI and ZTL may partially protect TOC1 from degradation in the late day and early night, perhaps by keeping the ZTL-GI complex in the cytoplasm (Kim *et al.* 2013). As GI levels decrease later in the night, TOC1 degradation can proceed (David *et al.* 2006; Kim *et al.* 2007). ZTL provides an important example of how a photoreceptive system can be activated in the dark and fine-tuned by the circadian clock to converge on a key growth regulator in parallel with other inputs, providing functional redundancy and robustness to the system. Together, these studies implicate photobody dynamics in regulating the linked clock-PIF growth module. Thus, we have a network of sensors and GRNs that both predicts and responds to multiple internal and external stimuli, creating a redundant temporal gate regulating plant growth.

TIME WILL TELL: CLOCK INFLUENCE IN CONSTANT VERSUS OSCILLATING ENVIRONMENTS

A similar suite of transcription factors directs growth in light–dark cycles, during skotomorphogenesis, and in growth deceleration during de-etiolation. However, there are important differences between these growth modes. The circadian clock plays key roles in coordinating many processes in light–dark cycles but clock function in etiolated seedlings (i.e. in darkness prior to light exposure) has been a matter of some debate. Zhong *et al.* (1998) observed an oscillation in the light-mediated induction of a cationic amino acid transporter 2 (CAT2):luciferase reporter, suggesting a low amplitude clock running in dark-grown seedlings. A subsequent study aimed at more directly testing clock function in the dark, measured transcript levels of core clock genes in etiolated seedlings and found no detectable oscillation (Kikis *et al.* 2005). However, when examining a population of asynchronous oscillators, the combined measurement may appear to be arrhythmic as the individual waveforms are averaged out. Examination of clock gene luciferase reporters allowed examination of potential oscillations at single seedling resolution, removing this potentially confounding population effect (Salome *et al.* 2008). This revealed that the clock starts within 24–30 h after imbibition, and that parts of the clock may be spatially restricted

by organ at this stage. LHY, a core circadian transcription factor and binding partner of CCA1, appears to be expressed exclusively in the cotyledons of etiolated seedlings (Salome *et al.* 2008). While the core clock starts running with some synchrony in etiolated seedlings, they lose rhythmicity by days 8–9. However, it is important to note that luciferase reporters for two output genes (LHCB and CAT3) did not become rhythmic until about day 7 and lost rhythmicity by day 9 of constant darkness (Salome *et al.* 2008). These output reporters fail to synchronize as a population when grown in constant light but, in contrast to constant darkness, the individual plants maintain strong oscillations. This indicates that light is required for the circadian clock to establish its regulatory role over some, if not all, output functions (Salome *et al.* 2008). Thus, the initial appearance of light serves not only to synchronize the clock to the outside environment but also establishes important governing mechanisms of the GRN hierarchy.

The weak influence of the clock in dark-grown seedling is reinforced by examination of etiolated clock mutants. Etiolated clock mutant seedlings have only modest defects, maintaining similar hypocotyl length as wild-type seedlings (Kato *et al.* 2007; Niwa *et al.* 2009). However, the observation that the LHY:luc reporter is expressed in cotyledons and excluded from the hypocotyl of etiolated seedlings suggests other tissue-specific functions during that stage of development (Salome *et al.* 2008). Mutations disrupting the clock modify the rate of elongation and the rate of chloroplast development upon exposure to light, but do not alter hypocotyl length in dark-grown seedlings (Kato *et al.* 2007; Niwa *et al.* 2009). While etiolated wild-type plants cease elongating upon red light exposure, the *prp9*, 7, 5 triple mutant fails to respond to this signal, resembling dark-grown seedlings. The *prp* triple mutant and *CCA1ox* plants both maintain the ability to green after long-term (6 d) dark growth, at which point wild-type has lost this ability, indicating that the clock plays a role in chloroplast signaling during de-etiolation (Kato *et al.* 2007). Interestingly, a study of Norway spruce showed that, while the clock genes are highly similar to those of angiosperms, the clock genes did not oscillate in constant light or dark conditions as they do in angiosperms. The authors suggested that a strong clock and high-precision day length measurement may not be required in gymnosperms because of the nature of the DPOR enzyme, which reduces the risk of a catastrophic ROS burst if protochlorophyllide is allowed to accumulate in the dark (Gyllenstrand *et al.* 2014). While growth in the dark is largely clock-independent, the links between light and growth are processed through the clock during the de-etiolation transition. Thus, the regulatory role of the clock is established and solidified at some point during this transition. The specific mechanisms by which light allows the clock to initiate links with other GRNs, the signaling and network-scale properties of those connections, and potential information transfer between systems through the clock itself are ripe for exploration.

The rhythmic nature of photosynthesis provides another input that plants can use to sense the transition from light to dark. The circadian clock and light combine to control activation of genes in the photosynthetic apparatus in the late night and early morning, preparing the plant for maximum photon capture during the light phase (Covington *et al.* 2008; Millar *et al.* 1992). Haydon *et al.* (2013) examined the possibility that light capture from the photosynthetic apparatus could feed into the clock in parallel with the other photoreceptors. Inhibiting photosynthesis using low CO₂ treatment or pharmacological inhibition of

Photosystem II caused defects in the clock period and rhythmicity that could be rescued by the addition of sugar to the growth media. Classical phase-response analysis showed that acute sugar addition can phase-advance the clock in the morning and mid-day, and induce phase-delays when introduced in the evening (Haydon *et al.* 2013). These effects required the clock components CCA1 and PRR7. Interestingly, *prp7* (and to a lesser degree *prp5*) mutants also showed general defects in glucose homeostasis similar to those observed in *gin2* mutants that lack hexokinase, which has been characterized as a sugar sensor (Jang *et al.* 1997; Moore *et al.* 2003). Sugar was still able to shift the clock in *gin2* mutants, however, indicating a distinct point of direct sensing, possibly upstream of PRR7 transcription (Haydon *et al.* 2013). Transcription of CCA1 increases around dawn, and PRR7 rises shortly after, suggesting that the sucrose-sensitive transcriptional control of these genes may occur in the late night or early morning under normal conditions. A more recent study observed similar effects of sucrose on the oscillation pattern of several clock gene:luciferase reporters and suggested that GI is important for sucrose input to the clock, although this hypothesis was primarily based on mathematical modeling. The effect of sucrose observed in *gi* mutants was small, and no tests on other clock mutants were reported (Dalchau *et al.* 2011). This suggests a feedback relationship between sugar and clock function. Kato *et al.* (2007) observed that the CCA1:luc reporter in dark-grown wild-type seedlings completes two full cycles without sucrose, but maintains rhythmicity for over 5 d on 1% sucrose, indicating that sucrose promotes the oscillation of the clock in etiolated seedlings. This is in agreement with the finding that sucrose can regulate the phase and amplitude of clock gene expression in light-grown seedlings (Haydon *et al.* 2013). Interestingly, this relationship puts sugar in the category of a retrograde signal, in that it can transmit information from the chloroplast to the nucleus. The observation that the clock progression is enhanced by sucrose in both etiolated and light-grown seedlings raises questions about the initiation of the clock in etiolated seedlings. Determining the true magnitude of sucrose influence on the clock will require considerably more detailed analysis of endogenous circulating sucrose and identification of the genetic or biochemical link between that and the clock.

In addition to feeding into the clock, sucrose may participate in determining the time of peak hypocotyl elongation. The Niwa *et al.* (2009) and Nozue *et al.* (2007) studies, both showing more growth in the late night, were done on sucrose media. However, Stewart *et al.* (2011) showed that sucrose can shift the time of peak elongation. They showed that the peak elongation rate in wild-type plants without sucrose occurred near dusk, while on sucrose the peak shifted to late night, matching the aforementioned studies. However, Soy *et al.* (2012) examined hypocotyl elongation rate in short days without sucrose at ~85 μ E and observed peak growth in the late night. It therefore appears that sucrose can have a strong effect on the timing of hypocotyl elongation, but may not be a singular determining factor. The combination of a key transition in the degree of clock control over growth with sucrose influence on the clock requires that this variable be strictly controlled and accounted for in future examination of de-etiolation and early seedling growth dynamics, as well as clock function. Energy drawn from the seed in etiolated seedlings may not be sufficient to promote clock function as strongly as exogenous sucrose can, but the finding that sucrose derived from photosynthesis can set the clock via PRR7 suggests that light, carbon fixation and

clock function might work together in the switch from the skoto- to photomorphogenic growth mode.

DARK ENERGY

Time-resolved studies of rhythms in carbon metabolism, from CO₂ capture, to starch accumulation, to rhythms in abundance of free sugars, provide a possible new angle on dark signaling. Several studies in adult plants have demonstrated that starch synthesis and degradation were tightly linked to the photoperiod, with synthesis occurring through the light period, and acute degradation starting at the onset of the dark period (Graf *et al.* 2010; Lu *et al.* 2005). The rate of degradation in the dark is regulated by the circadian clock, such that a minimum of starch is reached at the end of the night, when the clock predicts the lights will come on and begin the accumulation phase once again (Graf *et al.* 2010). Starch is still degraded in constant light and is weakly rhythmic, indicating some circadian control.

While transcript levels for genes involved in starch mobilization are rhythmic as well, protein levels appear to stay at constant levels across the day, suggesting post-transcriptional control (Lu *et al.* 2005; Smith *et al.* 2004). Metabolite analysis demonstrated that maltose, an intermediate sugar species that is freed from long-chain starch, shows a sharp increase within the first hour in the dark (Chia *et al.* 2004; Fulton *et al.* 2008; Pal *et al.* 2013). This spike in maltose levels suggests a possible control point for dark-induced starch breakdown, and the rapid onset is consistent with a post-transcriptional mechanism of activation. Thus, an early step in starch breakdown, prior to maltose production, may be a logical place to look for an acute dark signal (Fig. 3a).

Starch degradation depends on both kinase and phosphatase activity, including the phosphatases Starch EXcess 4 (SEX4) and Like Sex Four 2 (LSF2) (Comparot-Moss *et al.* 2010; Santelia *et al.* 2011). These phosphatases are required for the breakdown of linear and branched chain starches to simpler sugars that can then be transported out of the chloroplast to participate in downstream processes. Interestingly, SEX4 activity is redox-sensitive, with an internal disulfide bond that forms under hydrogen peroxide (H₂O₂) stress, inhibiting its phosphatase activity (Silver *et al.* 2013; Sokolov *et al.* 2006). Chloroplast thioredoxins can reduce this bond *in vitro*, activating the protein (Silver *et al.* 2013). H₂O₂ levels exhibit daily, partially clock-dependent, oscillations, with peak levels at mid-day, dropping off to a trough early/mid-night (Lai *et al.* 2012). It is tempting to speculate about the potential physiological consequences of this system under natural environmental oscillations: During the light period, H₂O₂ generated by in the photosynthetic apparatus accumulates to high levels, saturating the thioredoxin/ferridoxin/peroxiredoxin system and oxidizing the cysteines in SEX4. The disulfide bond formed under these conditions inhibits phosphatase activity, which in conjunction with a high carbon fixation rate, allows a shift in the equilibrium toward long chain starch synthesis over breakdown. At night, photosystem activity is low, the ROS scavenging systems are no longer saturated, and the SEX4 disulfide can be reduced, activating the protein. Carbon fixation is lower, and this, combined with newly activated SEX4, can promote starch degradation to release sugars for energy during the night. The true pathway is likely more complex, however, as starch synthesis is also inhibited by redox stress on the rate-limiting enzyme ADP-glucose pyrophosphorylase (AGPase), and the

inhibitory disulfide is reduced by the thioredoxin system upon light exposure (Fig. 3b) (Hendriks *et al.* 2003; Michalska *et al.* 2009; Thormahlen *et al.* 2013).

The SEX4 disulfide is efficiently reduced by several thioredoxins in-vitro, but mutating one of the cysteines to serine resulted in activity that is more sensitive to H₂O₂-mediated inhibition than the wild-type, with less activity returning after treatment with reducing agent – the opposite of the expected results (Silver *et al.* 2013). The combination of a redox-activated enzyme early in the starch breakdown pathway with acute and clock-controlled ROS production suggests possible pathways for dark-mediated and clock-mediated degradation. However, the specific mechanisms, complexes and interactions remain to be discovered. The diversity within the redox regulatory system may provide capacity for specific regulation of starch metabolism by time of day or other signaling pathways. The process of controlling starch accessibility at the surface of granules involves many factors, any of which may be regulated by acute environmental or circadian inputs. Clock-regulated transcription rhythms, and regulated protein accumulation and degradation may play a role in setting up internal and/or external coincidence scenarios for activating photosynthesis-derived carbohydrate reserves under light–dark cycles. We suggest that, based on the rapid increase in maltose, darkness may exert acute influence upstream of this step, and that regulation of SEX4 activity may be a logical place to start.

Starch degradation at night leads to sequential waves of simpler sugars being produced in the late night and early morning, with sucrose and glucose peaking 2–4 h after lights-on in adult plants (Graf *et al.* 2010; Pal *et al.* 2013; Sulpice *et al.* 2014). Carbon fixation and leaf expansion occur at higher rates during the day, with polysome formation increasing in the morning concurrent with increasing free sucrose (Pal *et al.* 2013; Sulpice *et al.* 2014). However, the growth measurements in adult plants are not entirely consistent with measurements of hypocotyl elongation in seedlings. The rate of carbon fixation in adult plants is higher during the day than in the night in all photoperiods tested (Pal *et al.* 2013; Sulpice *et al.* 2014). Adult leaf expansion rate measurements are quite variable, with some studies showing faster expansion during the day, some in the late night or early morning, some with fairly consistent expansion rates across the day–night cycle, and one report showing that the peak time of expansion switched from day in new leaves to night in 4 to 5-day-old leaves (Apelt *et al.* 2015; Poiré *et al.* 2010; Wiese *et al.* 2007; Pantin *et al.* 2011). Interestingly, this switch could be modulated by both water stress and starch. The starch-less pgm mutant maintained a day-phased peak expansion rate for several days longer than wild-type before switching to night-phased growth (Pantin *et al.* 2011). Meanwhile, hypocotyl elongation occurs (in most studies) primarily during the end of the night (Chang *et al.* 2011; Nozue *et al.* 2007; Soy *et al.* 2014; Stewart *et al.* 2011). There are three important points to make regarding the differences between these measurements. Firstly, carbon fixation will necessarily occur at a higher rate during the light period and would likely show a similar pattern if this measurement were taken in seedlings. Secondly, the adult plants were grown on soil rather than agar medium, meaning a difference in available nutrients. As previously mentioned, nutrients could make a difference in behaviour in the time domain (Stewart *et al.* 2011). Thirdly, elongation growths in the seedling hypocotyl and leaf expansion in adult plants are fundamentally different modes of growth. Hypocotyl elongation growth occurs with only limited cell division and is dominated by cell elongation, while cell division plays

a larger role in leaf expansion (Azpiroz *et al.* 1998; Gonzalez *et al.* 2012). Growth in both tissues requires considerable remodeling of the cell wall (see preceding texts) and protein translation. Isotopic measurements of cell wall and protein synthesis (taken in short day conditions) indicate that both occur primarily during the light phase (Ishihara *et al.* 2015). There is a positive relationship between sucrose levels (which peak in the morning) and polysome formation in adult plants (Pal *et al.* 2013). It therefore seems that new synthesis and expansion growth are temporally decoupled in adults; however, this relationship is not known in seedling hypocotyl. In the interest of establishing tighter correlations between metabolic and circadian inputs, it will be important to examine synthesis of cellular components across various photoperiods.

A final, more technical point involves how these oscillations are sampled over multiple days. Two of the studies in the adult leaf only sampled the plants once during the day and once during the night. When Pantin *et al.* (2011) observed the peak leaf expansion change from day to night, it is possible that this was a function of a slightly longer or shorter than 24 h process shifting what they describe as a metabolic oscillation to a hydrostatic pressure-driven process in slightly older leaves. It is possible that they have documented a form of entrainment in the new organ. Interestingly, this phase-switching phenomenon can be modulated by both light intensity and carbon metabolism. Higher sampling frequency during the day–night cycle will be crucial for picking apart this discrepancy and further understanding the physical, chemical and genetic forces at work in controlling tissue growth.

Interestingly, recent analysis in maize showed a similar pattern of diurnally regulated starch accumulation and degradation, suggesting this may be a generally conserved pattern of carbon utilization in plants. The starch utilization pattern varied within a leaf, matching the photoperiod in the mature zones, but degrading more slowly in elongation and division zones (Czedik-Eysenberg *et al.* 2016). While the existing live imaging techniques are informative, it will be of interest to further examine these patterns at a higher level of detail in *Arabidopsis* using novel imaging strategies or with tissue specific genetic manipulations. Existing data average out effects from many tissues, and thus may obscure more subtle and specific patterns of starch utilization and downstream energy use, such as translation. In the end, it is clear that, in both juvenile and adult structures, the temporal organization of growth is highly sensitive to the local conditions including carbon and water.

The observation that starch is acutely degraded upon the onset of dark, combined with the increasingly detailed descriptions of the enzymatic functions guiding this process, the possibility of a ‘dark sensor’, a molecule that can be acutely activated by darkness to carry out light-sensitive or otherwise restricted reactions, is experimentally accessible. It is possible that an acute change in the activity of a single enzyme upon the onset of darkness might signal to other processes and cause general changes in the plant. However, plants have a tendency toward parallel sensing and signaling, as demonstrated by the functional diversity of photoreceptor proteins. We therefore predict a scenario in which sensitive steps in multiple discrete pathways may be rapidly modified by reduced photon flux, the integrated results of which change on-going physiological processes to ‘dark mode’.

Energy balance is key to hypocotyl growth in etiolated seedlings, as they are drawing exclusively on energy stores from the seed. In oilseed species such as *Arabidopsis*, the main energy source for etiolated growth come from fatty acids stored in the embryo and endosperm. This system provides an interesting access point to the links between metabolism and growth in the dark. Mutations in two enzymes involved in mobilizing energy stores in the seed, phosphoenolpyruvate carboxykinase 1 (pck1) and isocitrate lyase (icl), showed inhibition of hypocotyl growth mainly in the dark. Both are important for catabolizing fatty acids to produce sugars by the glyoxylate cycle and gluconeogenesis (Eastmond *et al.* 2000; Penfield *et al.* 2004; Rylott *et al.* 2003). Removing of the endosperm of *Arabidopsis* seeds result in similar phenotype, underscoring the importance of energy derivation by this pathway (Penfield *et al.* 2004).

Metabolic control of growth and derivation of energy is an important determinant of the growth program activated in the seedling. The weak clock in freshly imbibed seeds, combined with enhanced clock cycling of imbibed seeds on sucrose medium, suggests that the amount of available sucrose may regulate the degree of clock influence on downstream and parallel processes (Kato *et al.* 2007). Once the clock is entrained to light–dark cycles, it will run for several cycles when shifted to either constant light or constant darkness, but sugar stores are exhausted by the end of the nighttime duration and are not replenished in continued darkness (Salome *et al.* 2008; Lu *et al.* 2005). Thus, it is unlikely that rhythmic sucrose release is the sole mechanism keeping the clock running. However, some genes that are heavily influenced by the clock can maintain rhythmic transcription in constant light but fail to do so in constant darkness (e.g. LHCB – Salome *et al.* 2008). Some of these ‘output’ genes may in fact require rhythmic sugar as well as the clock itself to maintain their temporal profiles. Upon light exposure, chloroplast maturation and the onset of photosynthesis may provide a rhythmic sugar pulse, in addition to the oscillating light cycle, that might create or enhance links between the clock and metabolic systems, as well as other GRNs (Haydon *et al.* 2013). It will therefore be of interest to examine the rates of sucrose derivation from the fatty acids in the seed during etiolated and de-etiolated growth, and the influence of metabolic oscillations on setting up the larger scale gene expression oscillators that link to the master clock. Additionally, given that the typical experimental regime of square-wave light cycles may contribute to artifacts in photosynthetic sugar metabolism, we may be approaching a time to transition to closer approximation of natural dawn and dusk light gradients.

The highly dynamic nature of sugar and starch metabolism described in the above-ground tissues across the day–night cycle suggests that it will be important to examine temporal control of carbohydrate metabolism in roots. The photosynthetic tissues above ground are likely to generate large carbohydrate fluxes, influencing transport and storage below ground (in the dark). The circadian clock does run in the roots, but requires signaling from the shoot and can be promoted by sucrose (Takahashi *et al.* 2015). This is consistent with the observed influence of sugar on clock oscillations in both dark and light grown seedlings, and that daily sugar pulses from photosynthesis can set and maintain the clock (Haydon *et al.* 2013).

SLIPPING INTO DARKNESS

Plants pay special attention to the dark, so we must take darkness into account if we wish to understand plants. They detect the onset of dark and use it to coordinate growth programmes. They measure the duration of night, calculate energy stores, and direct that energy toward growth in a window just before dawn, demonstrating the importance of darkness. We have examined several interacting systems that are particularly sensitive to darkness, or use the light to dark transition to guide their growth and metabolism (Fig. 4). The circadian clock acts as a guide, organizing these processes in time, but even the clock takes darkness as a cue to reset itself. The direct perception of darkness might seem like an oxymoron; at least, we typically frame it as a passive function of merely sensing fewer photons than were sensed earlier in the day, but that information is acutely transmitted and integrated into the decisions that the plant must make to grow and survive. There may not be a 'dark receptor', but the signaling functions of photoreceptors can be activated or inhibited by light, facilitating information flow in either condition. The diversity of photoreceptor functions shows this to be true at the biochemical level. We therefore propose that darkness is a true signal, and that there is much to be learned when we turn off the lights.

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Figure 1

. The Arabidopsis Circadian Clock. The circadian clock is diagrammed clockwise according to approximate time of maximal transcript level. The ring around the gene network diagram indicates the approximate light–dark cycle. late elongated hypocotyl (*LHY*) and circadian clock associated 1 (*CCA1*) transcripts peak in the morning, reveille (*RVE*) and pseudo-response regulator (*PRR*) transcripts rise sequentially from the late morning through afternoon, timing of cab expression 1 (*TOC1*) peaks in the early night, and gigantea (*GI*) and the members of the evening complex rise in the early-mid night. Internal cross-regulation of clock gene transcripts is indicated by arrowheads (positive regulation) or bars (negative regulation). For detailed description of clock function and input mechanisms, we direct the reader to Hsu & Harmer (2014); Jones (2009); McClung (2011) and Sanchez & Kay (2016). ELF, early flowering.

Figure 2.

Temporal gating of phytochrome-interacting factor (PIF) function by phytochromes, photobodies, and the circadian clock. During the day, active PHY-Pfr (green) suppress PIF levels and activity. In the dark, PHY-Pfr reverts to the inactive Pr form (grey). The rate of dark reversion likely depends on membership in photobodies (pink circles), with free PHY reverting more quickly than photobody associated PHY. In the late day and early night, PIF (blue) activity is repressed by timing of cab expression 1 (TOC1) and by photobody-associated PHY-Pfr. Later in the night, TOC1 is degraded, free PHY has reverted to Pr or is degraded, and the photobodies dissociate, thus relieving the repression of PIF abundance and activity. This coordinated derepression of PIF activity promotes hypocotyl growth at the end of the night.

Figure 3.

Oscillations of metabolites in light–dark cycles suggest dark-activated starch breakdown. **(a)** Model profiles of starch (solid black), maltose (solid blue), sucrose (solid green) and hydrogen peroxide (H_2O_2 – dashed black) across a 12 h light, 12 h dark cycle.

Photosynthesis keeps sucrose levels high during the day and simultaneously builds up starch granules. Reactive oxygen species, including H_2O_2 , are high through the day, and begin to drop in the late day. At lights-off, starch degradation is rapidly initiated and there is a rapid increase in maltose. Sucrose levels drop quickly at lights-off but rebound as the starch reserves are freed, providing energy during the night. **(b)** A prospective model of a carbohydrate homeostasis control system. H_2O_2 inhibits the activity of key enzymes in both starch synthesis (AGPase) and degradation (SEX4), but light increases synthesis and AGPase activity, while starch breakdown to an early product, maltose, only occurs rapidly in the dark. Starch degradation occurs in several parallel pathways that may also be subject to light- or dark-specific regulation (some important factors are noted in gray). Starch synthesis and breakdown has been thoroughly reviewed (Ruan 2014, Stitt & Zeeman 2012, Streb & Zeeman 2012).

Growth control systems in constant darkness and oscillating light–dark cycles. During etiolated growth (Left panel) the COP/DET/FUS complexes, together with the phytochrome-interacting factors (PIFs) promote hypocotyl growth, in part by repressing the photomorphogenesis-promoting factors such as hypocotyl 5 (HY5). The energy for the etiolated growth comes mainly from the seed reserves. During this stage, the influence of the etioplast is very limited, as it is not photosynthetically active. The clock runs weakly, but can be enhanced by added sugar. Clock cycling damps quickly due to lack of entraining stimuli and does not strongly regulate expression of output genes. During the day/night cycle (right panel), the chloroplasts have matured and, together with the clock, add additional control over hypocotyl growth. The clock is reset by light through photoreceptor proteins and via photosynthesis-derived sugars. It then begins to control genes involved in photosynthesis, carbon metabolism and growth, including at least two of the PIFs. The light to dark transition causes several acute signals: **(1)** photosynthesis stops and redox stress from photosystem activity is alleviated; **(2)** starch granules in the chloroplasts begin to break down to simpler sugars; **(3)** the clock protein TOC1 is destabilized in the dark in a ZTL-dependent process, relieving repression on the next phase of clock gene expression and on PIF activity; **(4)** PIF levels begin to increase because of less PHY activity, and fine-tune the timing and rate of hypocotyl growth.