Molecular Plant Advance Access published February 6, 2012

Molecular Plant • Pages 1–10, 2012 RESEARCH ARTICLE

Diurnal Dependence of Growth Responses to Shade in *Arabidopsis*: Role of Hormone, Clock, and Light Signaling

Romina Sellaro, Manuel Pacín and **Jorge J. Casal**[1]

IFEVA, Facultad de Agronomía, Universidad de Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas, Av. San Martín 4453, 1417 Buenos Aires, Argentina

ABSTRACT We investigated the diurnal dependence of the hypocotyl-growth responses to shade under sunlight–night cycles in *Arabidopsis thaliana*. Afternoon shade events promoted hypocotyl growth, while morning shade was ineffective. The *lhy-D*, *elf3*, *lux*, *pif4 pif5*, *toc1*, and quadruple *della* mutants retained the response to afternoon shade and the lack of response to morning shade while the *lhy cca1* mutant responded to both morning and afternoon shade. The *phyB* mutant, plants overexpressing the multidrug resistance-like membrane protein ABCB19, and the *iaa17/axr3* loss-of-function mutant failed to respond to shade. Transient exposure of sunlight-grown seedlings to synthetic auxin in the afternoon caused a stronger promotion of hypocotyl growth than morning treatments. The promotion of hypocotyl growth by afternoon shade or afternoon auxin required light perceived by phytochrome A or cryptochromes during the previous hours of the photoperiod. Although the ELF4–ELF3–LUX complex, PIF4, PIF5, and DELLA are key players in the generation of diurnal hypocotyl-growth patterns, they exert a minor role in the control of the diurnal pattern of growth responses to shade. We conclude that the strong diurnal dependency of hypocotyl-growth responses to shade relates to the balance between the antagonistic actions of LHY–CCA1 and a light-derived signal.

Key words: shade avoidance; hypocotyl growth; diurnal; auxin; LHY; CCA1; PIF3; PIF4; PIF5; ELF3; LUX; DELLA; circadian clock.

INTRODUCTION

Under day/night cycles, the hypocotyl of *Arabidopsis* seedlings shows maximum growth rates at dawn and a gradual decrease during the photoperiod. Growth remains slow during the first part of the night and increases towards the beginning of the following day (Nozue et al., 2007). This pattern is largely due to the combination of a circadian regulation of the expression of the *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) and *PIF5*, which increases during the night, and the negative regulation of PIF4 and PIF5 protein stability by light during the photoperiod (Nozue et al., 2007). Light transforms the Pr, inactive form, of phytochrome B (phyB) into the active, Pfr form, which binds PIF4 and PIF5 proteins and causes their phosphorylation and degradation in the proteasome (Leivar and Quail, 2011). In turn, a key component of the diurnal expression of *PIF4* and *PIF5* is the repression imposed by a complex involving EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX), which shows elevated levels during the late afternoon and early night (Nusinow et al., 2011). The effects of PIF4 and PIF5 on diurnal rhythmic hypocotyl growth involve the modulation of auxin-related pathways (Nozue et al., 2011). Many auxin-related genes oscillate with a phase similar to that of hypocotyl growth (Michael et al., 2008a; Nozue et al., 2011). In addition to its regulation of *PIF4* and *PIF5* expression, the circadian clock gates gibberellin signaling by controlling the expression of the receptor genes *GIBBERELLIN INSENSITIVE DWARF1 a* (*GID1a*) and *GID1b* (Arana et al., 2011). This results in a higher stability of DELLA proteins and a lower growth rate during the day, and a lower stability of DELLA and a higher growth rate during the night (Arana et al., 2011). DELLA proteins reduce hypocotyl growth in part by impeding PIF4 and PIF3 binding to DNA (De Lucas et al., 2008; Feng et al., 2008).

Under free-running conditions of continuous white light, the maximum rate of hypocotyl growth occurs around

[1] To whom correspondence should be addressed. E-mail casal@ifeva.edu.ar, fax 5411 4524-4040.

doi: 10.1093/mp/ssr122

Received 28 October 2011; accepted 19 December 2011

subjective dusk (Dowson-Day and Millar, 1999; Nozue et al., 2007; Michael et al., 2008a). Under these conditions, *PIF4* and *PIF5* expression levels show strong rhythmic oscillations with maximum peaks around 7 and 5 h, respectively, not very far from the time of maximum growth, whereas the expression of *GID1a*, *GID1b*, and *GID1c* shows weak oscillations (http://diurnal.cgrb.oregonstate.edu) (Mockler et al., 2007; Michael et al., 2008b). The phase of expression of phytohormone-related genes, including several auxin-related genes, correlates with growth rate and shows a shift from dawn under short days to dusk under continuous white light (Michael et al., 2008a).

Shade promotes hypocotyl growth compared to sunlight and phyB plays a major role in this response (Sellaro et al., 2010). The activity of phyB depends on the red-light irradiance and the red/far-red ratio, both of which decrease as a result of selective light absorption by photosynthetic pigments present in the foliage. Upon exposure to low red/far-red ratios, the levels of PIF5 increase rapidly (lag shorter than 15 min) and persistently (Lorrain et al., 2008). The promotion of hypocotyl growth by low red/far-red ratios is reduced in the *pif4*, *pif5*, and *pif4 pif5* mutants, which are partially epistatic to the *phyB* mutation (Lorrain et al., 2008). Low red/far-red ratios promote the synthesis of auxin by the SHADE AVOIDANCE 3 (SAV3)/TRYPTOPHAN AMINOTRANSFERASE OF *ARABIDOPSIS* 1 (TAA1) pathway in the leaves (Tao et al., 2008), enhance the expression of *PIN-FORMED 3* (*PIN3*) auxin transport gene in the hypocotyl and directs PIN3 from the basal to the lateral side of the membrane of the endodermal cells of the hypocotyls (Keuskamp et al., 2010). These changes result in increased levels of auxin in the hypocotyl and increased auxin signaling in the outer tissues of the hypocotyl that control the growth rate of the organ (Keuskamp et al., 2010). The *sav3* and *pin3* mutants show impaired hypocotyl-growth responses to low red/far-red ratios (Tao et al., 2008; Keuskamp et al., 2010). Low red/far-red ratios also reduce the abundance of DELLA proteins in the hypocotyls (Djakovic-Petrovic et al., 2007). The *gai* mutant, bearing a stable version of a DELLA protein, shows impaired hypocotyl-growth responses to the low red/far-red ratio, indicating that low red/far-red-induced degradation of DELLA is a requisite for the growth promotion (Djakovic-Petrovic et al., 2007).

The PIF4–PIF5, auxin, and gibberellin signaling pathways shape the daily progression of hypocotyl growth rate and are also involved in the promotion of hypocotyl growth by shade-light signals. Based on the latter observations, it would be reasonable to predict a diurnal sensitivity to shade caused by daily fluctuations in PIF4–PIF5, auxin, and gibberellin signaling pathways. However, this possibility remains to be tested. Actually, the scenario is not simple, even under continuous white light. The effect of the circadian clock on the promotion of hypocotyl growth by low red/far-red ratios depends on the temporal window of growth analysis. The long-term growth promotion caused by 2 h of low red/far-red treatment measured 24 h later is gated by the circadian clock and exhibits a peak during subjective dusk (Salter et al., 2003). However, the rapid promotion measured during the first 10 h of low red/far-red light is unaffected by the clock (Cole et al., 2011). In addition, while auxin signaling plays a key role in shade-avoidance responses, the circadian sensitivity of hypocotyl growth to exogenous auxin is maximum at subjective night (Covington and Harmer, 2007), namely out of phase with that of the sensitivity to low red/far-red light that shows a minimum at subjective night (Salter et al., 2003).

Due to the heterogeneous nature of plant canopies and the interaction between canopy structure and solar elevation, plants may be exposed every day to shade-light or to sunlight at given times of the photoperiod. However, we are largely ignorant about the mechanisms that plants use to cope with these dynamic fluctuations of the light environment. We have recently observed that, under natural radiation, seedling exposure to sunflecks, namely brief exposures to sunlight interrupting shade, causes a strong hypocotyl-growth response when the sunflecks occur in the afternoon (Sellaro et al., 2011). This shade-to-light response involves the enhanced expression of *ELONGATED HYPOCOTYL 5* (*HY5*), the reduced expression of *PHYTOCHROME KINASE 4* (*PKS4*), and a permissive action of the clock, likely involving down-regulation of auxin signaling in the afternoon (Sellaro et al., 2011). However, we show here that HY5 and PKS4 do not significantly alter the light-to-shade response. The aim of this paper is to investigate the diurnal dependence of the response to natural shade signals.

RESULTS

Daily Afternoon Shade Events Promote Hypocotyl Growth

To investigate whether the hypocotyl-growth response to shade-light is affected by the timing of daily shade events, seedlings of *Arabidopsis thaliana* were grown under sunlight and exposed to shade for 2 h at different times of the 10-h photoperiod (see scheme of the protocol in Figure 1A). Seedlings under uninterrupted sunlight and uninterrupted shade were included as controls. Daily shade events were effective to promote growth only when they occurred during the last 2 h of the photoperiod (Figure 1B). Compared to the sunlight control, the shade treatment caused a 90–95% reduction in the radiation between 400 and 700 nm and a reduction in the red/far-red ratio from 1.1 to 0.1–0.2. Therefore, although the shade signals were very intense at any time of the photoperiod, plants responded only to afternoon shade events.

The 2-h shade event late in the photoperiod evoked only 38% of the response elicited by shade-light during the whole photoperiod (Figure 1B). Since 2-h shade events were not effective at other times of the photoperiod, the effect of 10-h shade is more than the sum of the effects of 2-h shade events, implicating a mechanism of input signal integration. The experiments were conducted under the variable conditions of the outdoor environment, and therefore the hypocotyl growth rates changed among experiments. However, the patterns of response (e.g. the effect of afternoon

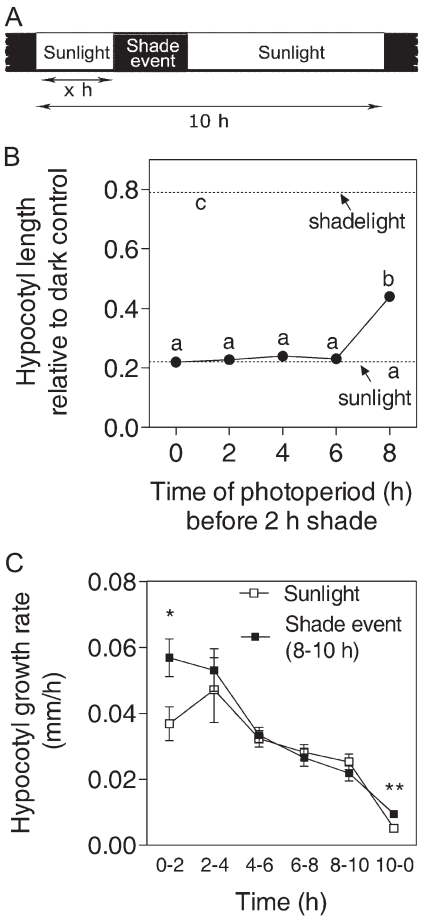

Figure 1. Afternoon Shade Events Promote Hypocotyl Growth while Morning Shade Is Not Effective.

(A) Experimental protocol: seedlings were grown under sunlight and daily exposed to 2 h of shade at different time points of the 10-h photoperiod.

(B) Hypocotyl length of wild-type seedlings grown for 3 d under sunlight daily interrupted by a shade event at the indicated times of the photoperiod. Dotted lines indicate hypocotyl length in seedlings grown under uninterrupted shade-light or under uninterrupted sunlight. Data are means and SE of nine replicate boxes. Different letters denote significant differences ($P < 0.05$) among means.

(C) Hypocotyl growth rate during Day 3 in seedlings grown either under sunlight daily interrupted by an afternoon shade event or under uninterrupted sunlight. Data are means and SE of at least seven seedlings. * $P < 0.05$; ** $P < 0.01$.

shade and not of morning shade) remained unaltered, demonstrating their robustness.

Figure 1C shows the kinetics of growth during the third day of treatment in plants grown under uninterrupted sunlight or 8 h of sunlight followed by 2 h of shade-light. In the sunlight control, the rate of hypocotyl growth was maximal at the beginning of the day and decreased during the photoperiod, following the pattern observed in previous experiments under controlled conditions (Nozue et al., 2007; Michael et al., 2008a). The average growth rate during the night was low. The promotion of hypocotyl growth by shade-light events between 8 and 10 h was not evident during the time of actual exposure to shade. Rather, shade increased the rate of growth during the subsequent night (10 to 0 h) and the first part of the morning (0 to 2 h).

Afternoon Shade Events Are Perceived by phyB

The response to shade events was absent in the *phyB* mutant (Figure 2A). The *phyA* mutant showed a slightly reduced response but more detailed experiments confirmed that afternoon shade events cause a significant promotion of hypocotyl-growth response in the absence of phyA (see below). The *cry1 cry2* double mutant had a response at least as large as that observed in the wild-type (Figure 2A). We conclude that the reduction of phyB activity by the low red/far-red ratio and red irradiance of shade-light caused the promotion of hypocotyl growth.

Low Pfr Levels at the Beginning of the Night Do Not Mimic the Response to Afternoon Shade

Classical experiments show that a brief pulse of far-red light given at the end of a photoperiod with high red/far-red ratios is enough to promote stem growth (Downs et al., 1957). Pfr, the active form of phytochrome, is able to persist for several hours in darkness and the end-of-day (EOD) far-red light pulse reduces the level of Pfr immediately prior to the beginning of the night. Since the low red/far ratio of shade-light (0.1–0.2) is predicted to lower the levels of stable phyB Pfr during the subsequent night, we daily treated the seedlings (exposed to uninterrupted sunlight, morning shade or afternoon shade) to a 10-min pulse of red plus far-red light with the red/far-red ratio provided by sunlight (i.e. 1.1, EOD 1.1). The EOD 1.1 treatment reduced the length of the hypocotyl only in the seedlings exposed to afternoon shade (Figure 2B). However, EOD 1.1 did not fully abolish the effect of afternoon shade. This indicates that afternoon shade is effective because (1) the seedlings are more sensitive to reductions in phyB activity in the afternoon and (2) the persistence of low Pfr levels during the night contributes to amplifying the response.

To further evaluate the contribution of the afternoon light environment compared to the Pfr levels at the beginning of the night under afternoon shade conditions, we daily exposed seedlings to afternoon shade events, a 10-min pulse with red plus far-red light with the red/far-red ratio of shade-light (i.e. 0.1, EOD 0.1) or a 10-min pulse of far-red light (the classical EOD far-red light pulse). The comparison between afternoon shade and EOD 0.1 demonstrates that afternoon shade does more that just reducing the level of Pfr at the beginning of the night (Figure 2C). The comparison between EOD 0.1 and EOD far-red light demonstrates that, in order to be effective, in plants grown under sunlight, the EOD reduction of Pfr levels has to be extremely severe (Figure 2C) (Casal et al., 1990; Sellaro et al., 2011).

The Response to Daily Shade Events in *pif*, *della*, and Auxin-Related Mutants

Since daily fluctuations in PIF4–PIF5 (and likely PIF3), DELLA, and auxin signaling control the kinetics of hypocotyl growth (Nozue et al., 2007; Michael et al., 2008a; Arana et al., 2011), we investigated whether disruptions of these signaling

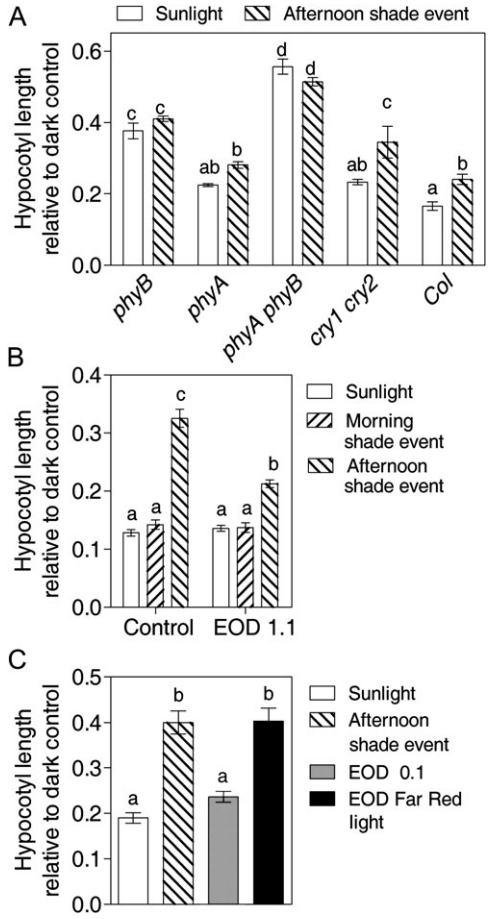

Figure 2. phyB Perceives the Shade Event.

(A) Hypocotyl length of wild-type and *phyA*, *phyB*, *phyA phyB*, and *cry1 cry2* mutant seedlings grown under sunlight daily interrupted by afternoon shade (shade during the last 2 h of the photoperiod) or uninterrupted sunlight.

(B) Hypocotyl length of wild-type seedlings grown under sunlight interrupted by shade during the first 2 h of the photoperiod (morning shade event), under sunlight interrupted by shade during the last 2 h of the photoperiod (afternoon shade event), or under uninterrupted sunlight in combination with or without (Control) a brief red plus far-red light pulse (10 min) with the red/far-red ratio of sunlight (i.e. 1.1, EOD 1.1) given immediately prior to the beginning of the night.

(C) Hypocotyl length of wild-type seedlings grown under sunlight interrupted by shade during the last 2 h of the photoperiod (afternoon shade event), under uninterrupted sunlight, or uninterrupted sunlight with a brief pulse (10 min) of either red plus far-red light with the red/far-red ratio of shade (i.e. 0.1, EOD 0.1) or pure far-red light (EOD far-red light) given immediately prior to the beginning of the night.

Data are means and SE of two to five replicate boxes. Different letters denote significant differences ($P < 0.05$) among means.

pathways alter the differential response to morning and afternoon shade events. The *pif4* mutant showed normal hypocotyl-length responses while the *pif5*, *pif3*, *pif4 pif5*, and *pif3 pif4* mutants showed some reduction of hypocotyl length in the seedlings exposed to afternoon shade (Figure 3A). Therefore, the *pif3* and *pif5* mutations limited the response to shade without altering its diurnal dependency. The quadruple *della* mutant showed longer hypocotyls than the wild-type and a larger response to afternoon shade-light (Figure 3B). The *sav3* mutant partially reduced the response to afternoon shade; the *iaa17/axr3* loss-of-function mutant and the transgenic line with ectopic/overexpression of the multidrug resistance-like membrane protein ABCB19 (*B19OE*) (Wu et al., 2010) failed to respond to either morning or afternoon shade (Figure 3C). The *iaa17/axr3* was not at its maximal growth (that could impede further promotion) as, in controls grown under shade during the whole photoperiod (not just 2 h), these seedlings were substantially taller (hypocotyl length relative to dark controls: 0.90 ± 0.04) than under the conditions of Figure 3C.

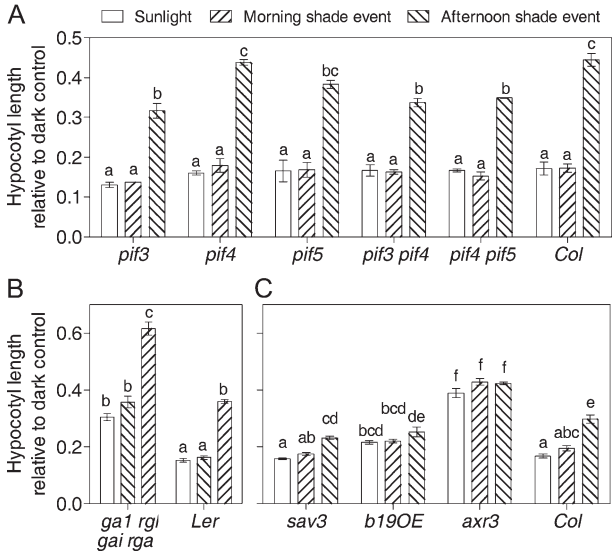

Figure 3. The Response to Shade in *pif*, *della*, and Auxin-Related Mutants.

Seedlings of the *pif3*, *pif4*, *pif5*, *pif3 pif4*, *pif4 pif5* **(A)**, *ga1 rgl2 gai rga* quadruple *della* **(B)**, *sav3* and *axr3* mutants and of the *B19OE* line **(C)** were grown with their respective wild-types under sunlight interrupted by shade during the first 2 h of the photoperiod (morning shade event), under sunlight interrupted by shade during the last 2 h of the photoperiod (afternoon shade event), or under uninterrupted sunlight. Data are means and SE of three to six replicate boxes. Different letters denote significant differences ($P < 0.05$) among means.

The *hy5* and *pks4* mutants that affected the response of the hypocotyls when the seedlings were grown under shade-light and exposed to sunflecks in the afternoon (Sellaro et al., 2011) showed normal responses to afternoon shade events in seedlings grown under sunlight (hypocotyl length relative to dark controls, mean ± SE; sunlight controls: Col = 0.17 ± 0.01; *hy5* = 0.48 ± 0.04; *pks4* = 0.15 ± 0.01; afternoon shade: Col = 0.22 ± 0.01; *hy5* = 0.61 ± 0.03; *pks4* = 0.19 ± 0.01; light condition by genotype interaction: not significant).

Exogenous Auxin Is More Effective to Promote Growth in the Afternoon

Since mutations that affect auxin-related genes severely impaired normal hypocotyl growth responses to afternoon

shade, we used a pharmacological approach to investigate the diurnal dependence of auxin-induced hypocotyl-growth responses. Seedlings of *Arabidopsis thaliana* were grown on paper placed on top of a water bath with continuous air bubbling and grown under sunlight photoperiods of 10 h (i.e. the conditions used for the shade-event experiments). Every day, at the indicated times of the photoperiod (Figure 4A), the seedlings were transferred to a similar solution containing 5 μM picloram (a synthetic auxin) and returned to the water control conditions 6 h later. Hypocotyl length was recorded at the end of the experiment. The choice of 6 h for the duration of the daily exposure to auxin is based on preliminary experiments conducted under controlled conditions, which demonstrate that short exposures are not effective to increase final hypocotyl length (Supplemental Figure 1). The promotion of hypocotyl growth was significantly higher when picloram was applied during the final part of the photoperiod than during the morning (Figure 4B). This result demonstrates a correlation between the diurnal dependence of the hypocotyl growth response to shade and synthetic auxin.

Morning and Afternoon Shade Promote the Expression of Auxin-Related Genes

Since afternoon shade and not morning shade is effective to promote growth and intact auxin signaling is required for a normal response, we investigated whether the control of auxin-related gene expression by shade-light follows the same trend. We selected five auxin-related genes from our database of genes promoted by shade compared to sunlight (Sellaro et al., 2011). Four of these genes showed a significant promotion by shade independently of its occurrence in the morning or the afternoon (significant effect of shade event and no interaction with the time of harvest; Figure 5). One gene (*YDK1*) showed a significantly higher effect when the shade event occurred in the afternoon, but, even in this case, morning shade was effective (Figure 5).

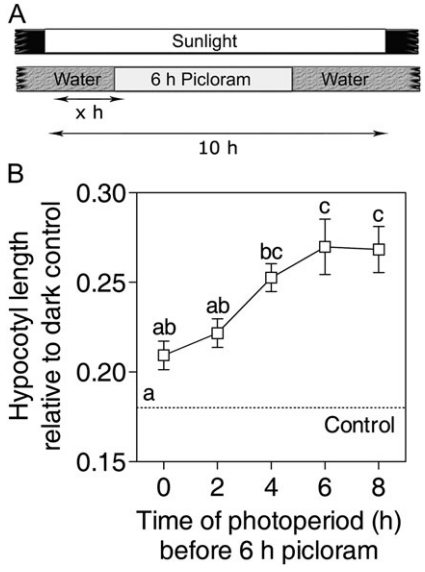

Figure 4. Diurnal Dependence of the Promotion of Hypocotyl Growth by Picloram.

(A) Daily protocol: seedlings were grown for 3 d under sunlight and daily exposed for 6 h to picloram at different times of the photoperiod.

(B) Hypocotyl length. Dotted line indicates hypocotyl length in controls without picloram. Data are means and SE of seven replicates. Different letters denote significant differences ($P < 0.05$) among means.

The Sensitivity to Shade and Auxin in Clock-Related Mutants

Previous experiments had shown that, under free-running conditions of continuous white light, hypocotyl-growth

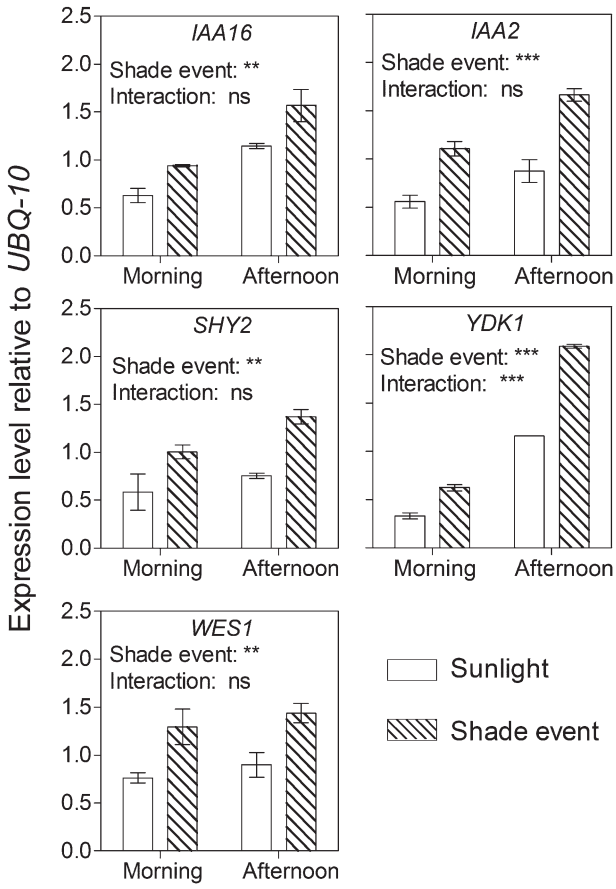

Figure 5. Expression of Auxin-Related Genes as Affected by Morning or Afternoon Shade Events.

IAA16, *IAA2*, *SHY2*, *YDK1*, and *WES1* expression level in wild-type seedling grown under sunlight interrupted by shade during the first 2 h of the photoperiod (morning shade event), under sunlight interrupted by shade during the last 2 h of the photoperiod (afternoon shade event), or under uninterrupted sunlight. Samples were harvested at 2 h of Day 3 for morning sunlight and morning shade event and at 10 h of Day 3 for afternoon sunlight and afternoon shade event. Data are means and SE of three to nine replicate boxes (biological replicates). The significance of shade-event effect and of its interaction with time of the day is indicated. ** $P < 0.01$, *** $P < 0.001$; ns, not significant.

responses to either low red/far-red (Salter et al., 2003) or exogenously applied auxin (Covington and Harmer, 2007) are controlled by the clock. To investigate whether this is also the case under day–night cycles, we tested the effects of morning and afternoon shade events and morning and afternoon picloram compared to sunlight controls on the growth of the hypocotyl in clock-related mutants. The *lhy-D* mutant with elevated levels of *LHY* expression and the *lux* and *elf3* mutants, affecting members of the ELF3–ELF4–LUX complex, showed quantitatively normal responses to afternoon shade and no response to morning shade (wild-type behavior) (Figure 6A), despite the fact that these mutations increase hypocotyl growth. The *lhy cca1* double mutant responded not only to afternoon, but also to morning shade (a treatment that is ineffective in the wild-type) (Figure 6A). The *toc1* mutant also had a wild-type response (hypocotyl length relative to dark controls, mean ± SE; sunlight control: 0.18 ± 0.01; morning shade event: 0.18 ± 0.01; afternoon shade event: 0.26 ± 0.01).

In contrast to their restricted effect on the response to shade events, the *lhy-D*, *elf3*, and *lhy cca1* mutants were completely unable to respond to picloram and the *lux* mutant showed similar responses to morning and afternoon picloram (afternoon picloram is more efficient in the wild-type) (Figure 6B). These results indicate that, despite the temporal overlap of the phase of maximum response to shade and picloram under day–night cycles, the impact of mutations affecting clock function diverges between both stimuli.

Light Control of the Response to Shade and Auxin

Although both shade events and exogenous auxin are more effective when applied during the afternoon, mutations related to clock function have divergent effects on the responses to shade and auxin, suggesting that other processes might be involved in setting the sensitive phase to these treatments under day–night cycles. Previous experiments have shown that the phyB-mediated hypocotyl-growth response to a pulse of far-red light requires light absorbed by phyA or blue light (presumably perceived by cryptochromes) during the hours preceding the far-red light pulse (Casal, 1996). Based on the later observations, we reasoned that the promotion of hypocotyl growth caused by a shade event perceived by phyB could also require exposure to light perceived by phyA, cry1, or cry2 during the previous hours. If both shade and exogenous auxin require light absorbed by phyA, cry1, or cry2 during the previous hours, such requirement would at least in principle explain why morning treatments are not effective (as they are preceded by the night) and both treatments have a stronger effect in the afternoon.

To test the null hypothesis that the response to shade and the response to auxin do not require light absorbed by phyA, cry1, or cry2 during the previous hours, we conducted two types of complementary experiments. For these experiments, we shifted from treatments applied daily and final measurements of hypocotyl length to treatments applied only during the third photoperiod and measurements of subsequent hypocotyl length increment. The reason for this change is that we wanted to modify the light environment in the second experiment only after allowing 2 d for de-etiolation (see below). In the first experiment, we cultivated plants of the wild-type (Landsberg *erecta*) and of the *phyA cry1 cry2* mutant under sunlight. Two hours before the end of the third photoperiod,

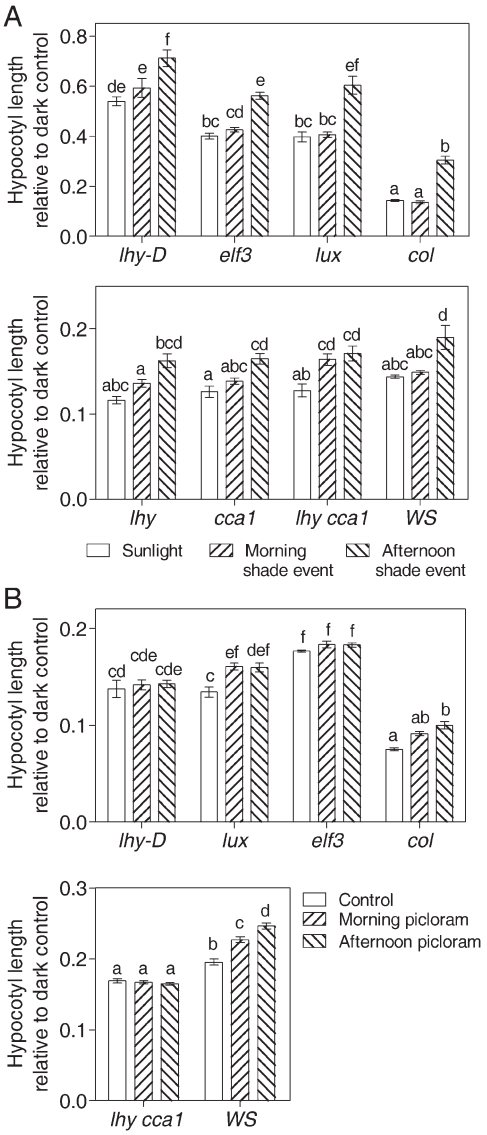

Figure 6. The Response to Morning and Afternoon Shade Events and Morning or Afternoon Picloram in Clock-Related Mutants.

(A) Seedlings of the *lhy-D*, *elf3*, *lux*, *lhy*, *cca1*, and *lhy cca1* mutants were grown with their respective wild-types under sunlight interrupted by shade during the first 2 h of the photoperiod (morning shade event), under sunlight interrupted by shade during the last 2 h of the photoperiod (afternoon shade event), or under uninterrupted sunlight.

(B) Seedlings of the same genotypes included in (A) grown under sunlight and daily exposure to picloram in the morning (0–6 h), exposed to picloram in the afternoon (4–10 h), or left as controls. Data are means and SE of 4–10 replicates. Different letters denote significant differences ($P < 0.05$) among means.

the seedlings were exposed to shade, exposed to picloram, or left as controls and the hypocotyl-length increment between the beginning of the treatments and the end of the night was recorded (Figure 7A). Both a shade event and the exposure to picloram caused a significant promotion of hypocotyl growth in the wild-type. None of these treatments was effective in the *phyA cry1 cry2* mutant (Figure 7B).

The above results are consistent with two interpretations. One is that immediately previous exposure to light absorbed by phyA, cry1, or cry2 defines the difference between morning and afternoon. The other is that the seedlings have to reach a certain de-etiolation status before becoming responsive to shade and this stage is not reached in the *phyA cry1 cry2* mutant. Therefore, the second experimental protocol was designed to test whether light absorbed by phyA, cry1, or cry2 is still required for the hypocotyl-growth responses to picloram and shade in seedlings that are already de-etiolated. For this purpose, the *phyA* mutant was exposed for 2 d to sunlight, as this mutant has no serious de-etiolation problems under white light (Whitelam et al., 1993). At the beginning of the third photoperiod, the seedlings were exposed to sunlight or sunlight minus blue light. Two hours before the end of the photoperiod, the seedlings were exposed to shade, to picloram, or remained as control (Figure 7A). The blue-light component of sunlight still activates cry1 and cry2 in the *phyA* mutant. However, in the minus blue light condition, no active phyA, cry1, or cry2 would be present during the hours preceding the shade event or the exposure to picloram. When exposed to sunlight during the hours preceding the treatments, the *phyA* mutant showed a significant response to shade and a significant response to auxin. In the absence of blue light during the hours preceding the treatments, the *phyA* mutant showed no response to subsequent shade or picloram (Figure 7C).

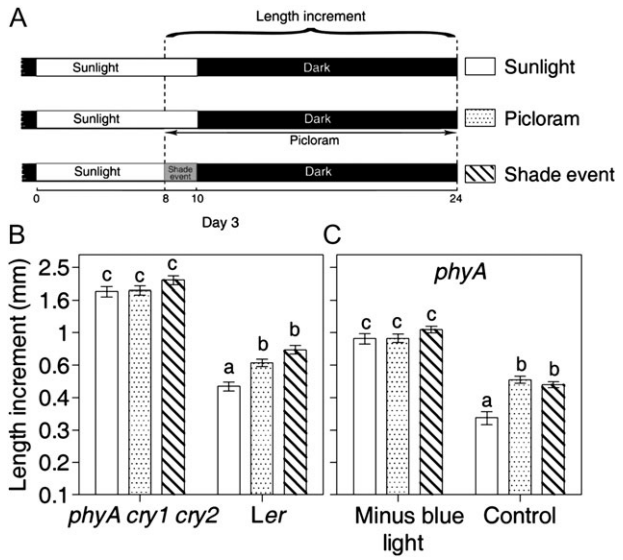

Figure 7. The Response to Afternoon Shade or Picloram Requires Previous Activation of phyA, cry1, or cry2 during the Photoperiod.

(A) Protocol for Day 3: the seedlings were grown for 2 d under sunlight and exposed to shade (between 8 and 10 h) or auxin (between 8 and 24 h) at the end of Day 3. Hypocotyl length increments were measured between 8 and 24 h.

(B) Wild-type and *phyA cry1 cry2* seedlings.

(C) *phyA* seedlings grown with (control) or without blue light during Day 3.

Data are means and SE of 16–47 seedlings. Different letters denote significant differences ($P < 0.05$) among means.

DISCUSSION

Under free-running conditions, the circadian clock gates the hypocotyl-growth response to the low red/far-red ratio, setting the phase of maximum effectiveness at dusk (Salter et al., 2003). Here, we show that, under short photoperiods (10 h) of natural radiation, shade events (reduced red/far-red ratio and irradiance) are also effective close to the end of the photoperiod and not in the morning (Figure 1). Despite this coincidence between the phases of maximum response to shade-light, the mechanisms that set the timing are not necessarily shared, as the *toc1* mutation attenuates and phase-shifts the response under free-running conditions (Salter et al., 2003) and has no obvious effects under sunlight–night conditions (this report).

Under sunlight–night cycles, morning and afternoon shade induced a largely similar promotion of expression of auxin-related genes (Figure 5) and, under free-running conditions, maximal de-repression of *PIL1* expression occurs at subjective dawn (Salter et al., 2003). Therefore, growth and gene expression can have divergent phases of maximum response to shade-light signals. In other words, the diurnal dependence of the growth response to shade does not reflect a general diurnal dependence of shade-light signaling.

The phyB–PIF4–PIF5 module has a well-defined function in the regulation of diurnal fluctuations in hypocotyl growth rate (Nozue et al., 2007, 2011). The results presented here confirm the importance of PIF5 in hypocotyl shade-avoidance reactions (Lorrain et al., 2008) and extend the function to PIF3 (Figure 3A). The expression of *PIF5* is higher in the morning than in the afternoon but the activation of phyB by morning light with a high red/far-red ratio causes PIF5 degradation and the subsequent drop in hypocotyl growth rate (Nozue et al., 2007). A priori, we thought that morning shade would be particularly effective to promote hypocotyl growth by reducing PIF4 and PIF5 destruction compared to sunlight (Lorrain et al., 2008) at a time when *PIF4* and *PIF5* expression levels are high. Conversely, afternoon shade would increase PIF4 and PIF5 stability when *PIF4* and *PIF5* transcript levels are low and impose a limit on the enhancement of PIF4 and PIF5 by shade. However, the results fully contradict this prediction by showing that afternoon shade is more effective than morning shade and not vice versa. Furthermore, although *pif5* and *pif3* mutations reduced the shade-avoidance response, these mutants retained a higher effectiveness in the afternoon (Figure 3A). Similarly, the *lux* and *elf3* mutants affecting the ELF4–ELF3–LUX complex involved in the control

of *PIF4* and *PIF5* expression (Nusinow et al., 2011) showed a wild-type pattern of diurnal sensitivity (i.e. they responded to afternoon shade and not to morning shade) despite their large effects on basal hypocotyl growth (Figure 6A). Noteworthy, *lux* and *elf3* did impair the hypocotyl-growth response (inhibition) when the seedlings were gown under shade and daily exposed to sunlight (sunflecks) in the afternoon, indicating a partial divergence of the processes involved in light-to-shade and shade-to-light responses (Sellaro et al., 2011).

The GID1–DELLA module also plays a role in the regulation of the daily patterns of hypocotyl growth (Arana et al., 2011). As reported, the quadruple *della* mutant was significantly taller than the wild-type (Djakovic-Petrovic et al., 2007). As the wild-type, the quadruple *della* mutant responded to afternoon and not to morning shade (Figure 3B). Therefore, DELLA negatively regulate shade-avoidance reactions but daily fluctuations of DELLA function would not account for the daily fluctuations in hypocotyl response to shade, as it persists in the quadruple *della* mutant.

The high rate of hypocotyl growth at dawn correlates with a more intense phytohormone signaling status (including auxin signaling) at this time of the photoperiod (Michael et al., 2008a). Auxin signaling is a critical component of the hypocotyl shade-avoidance response, and the *sav3* mutant showed reduced response to afternoon shade (Figure 3C). Noteworthy, the *iaa17/axr3* mutant and the *B19OE* transgenic line overexpressing the multidrug resistance-like membrane protein ABCB19 (Wu et al., 2010) failed to respond to either morning or afternoon shade (Figure 3C), indicating that misregulation of auxin signaling alters the responsivity to shade. The response to picloram was maximal in the afternoon (Figure 4). On the contrary, under free-running conditions of continuous white light, there is little promotion of hypocotyl growth by exogenous auxin applied at subjective afternoon (Covington and Harmer, 2007). The temporal overlap of maximum sensitivity to shade and exogenous auxin during the photoperiod (cf. Figures 1 and 4) indicates that both responses are controlled either by the same pathway or by temporally correlated pathways. Clock-related mutations had very different effects on the responses to shade and exogenous auxin (Figure 6), indicating that a direct action of the clock is not likely to account for the shared phase of maximum response.

No response to shade or picloram was observed in *phyA* mutant seedlings exposed to sunlight minus blue light during the hours previous to the treatments (Figure 7). This indicates that the responses to afternoon shade or auxin require light absorbed by phyA, cry1, or cry2 during the preceding hours. The *lhy cca1* mutant responded not only to afternoon shade (as the wild-type), but also to morning shade (not effective in the wild-type) and an intermediate morning response was observed in the *lhy* and *cca1* single mutants (Figure 6A). Since the *toc1*, *lhy-D*, *lux*, and *elf3* mutants showed a wild-type pattern of diurnal sensitivity (Figure 6A), a likely interpretation of the *lhy cca1* phenotype is that the high morning levels of *LHY* and *CCA1* impair the morning response to shade-light via an action on the growth response itself rather than by disrupting clock function. The observation that *lhy cca1* responds to morning shade while wild-type seedlings require previous exposure to light to respond in the afternoon suggests that the response to shade depends on a balance between the antagonistic actions of LHY–CCA1 and a light-derived signal. The accumulation of the light-derived signal in the afternoon would allow the response to shade even in the *lhy-D* mutant (with elevated *LHY* expression; Sellaro et al., 2011).

In conclusion, the experiments reported here indicate that shade-light signals are more effective to promote hypocotyl growth when they occur close to the end of the photoperiod. High afternoon sensitivity would result from the accumulation of a light-derived signal and low morning sensitivity would be caused by high LHY–CCA1 levels and reduced levels of the light-derived signal after the night. The diurnal sensitivity is not caused by daily fluctuations in PIF, DELLA, or the ELF4–ELF3–LUX complex with known function in the diurnal pattern of hypocotyl growth.

METHODS

Plant Material

The mutants *phyB-9* (Reed et al., 1993), *phyA-211*, *phyA-211 phyB-9* (Reed et al., 1994), *cry1-304 cry2-1* (Guo et al., 1999), *hy5-221* (Shin et al., 2007), *pks4-1* (Schepens et al., 2008), *axr3-1* (Rouse et al., 1998), *toc1-101* (Kikis et al., 2005), *elf3-1* (Zagotta et al., 1996), *lux-4* (Hazen et al., 2005), *lhy-100D* formerly 277F (Sellaro et al., 2011), and the transgenic *B19OE* seedlings (Wu et al., 2010) were compared to their Columbia (Col) wild-type. The quadruple *della ga1-3 rgl2-1 gai-t6 rga-t2* (Achard et al., 2006), *phyA-201* formerly *fre-1* (Nagatani et al., 1993), and the *phyA-201 cry1-1 cry2* (Mazzella and Casal, 2001) mutants were compared to their Landsberg *erecta* (L*er*) wild-type. The *lhy-21*, *cca1-11*, and *lhy-21 cca1-11* (Hall et al., 2003) mutants were compared to their Wassilewskija (WS) wild-type.

Experiments Involving Shade Treatments

For the experiments involving shade treatments, 15 seeds per genotype were sown on 3 ml of 0.8% agar in clear plastic boxes (4 × 3.5 cm). The boxes were incubated in the dark at 5°C for 5 d, given 8 h of red light followed by 16 h of darkness (22°C), and transferred to the treatment conditions in the field (located at Faculty of Agronomy, University of Buenos Aires, latitude 34° 35′ S, longitude 58° 28′ W). In the field, the boxes were daily exposed to a photoperiod of 10 h under unfiltered sunlight (photosynthetically active radiation 600 μmol m^{-2} s^{-1} and a red/far-red ratio of 1.1 at midday) or under unfiltered sunlight interrupted during 2 h by the shade of a 3-m tall canopy of *Viburnum tinus* (Eve Price) (photosynthetically active radiation 40 μmol m^{-2} s^{-1} and a red/far-red ratio of 0.1–0.2 at midday). Dark controls were placed under sunlight conditions wrapped with black plastic (inner cover) and

aluminum foil (outer cover). After the night of the third day of treatment, hypocotyl length was measured to the nearest 0.5 mm with a ruler and the length of the 10 tallest seedlings per genotype and per box were averaged (one replicate box). Hypocotyl-length data are presented relative to the length of dark controls to increase accuracy, and the length in darkness is shown in Supplemental Table 1.

Experiments Involving Picloram Treatments

For the experiments involving the application of the synthetic auxin picloram (Tordon 24K), the seeds were sown on paper placed on top of the agar and induced to germinate as described for shade experiments. One-day-old seedlings were transferred to sunlight and grown on the filter paper used for germination but laid on the surface of aerated distilled water. At the indicated times, the seedlings were transferred on their filter paper from distilled water to a distilled water solution containing 5 μM of picloram for 6 h and then transferred back to distilled water. Before transfer from picloram to water, the seedlings and filter were extensively washed with distilled water. After the night of the third day of treatment, hypocotyl length was measured to the nearest 0.5 mm as described for shade experiments.

Experiments Involving Shade and Picloram Treatments

The seedlings were grown as described for shade treatments but on vertically oriented agar. Sunlight and shade conditions were as described. For picloram treatments, the boxes (with their lids removed) were immersed in a picloram solution bath covering the roots of the seedling contained in a bigger clear box with lid. Hypocotyl length was recorded with a Canon Power Shot A520 camera under sunlight before exposure to shade-light or auxin treatments (8 h of Day 3) and again at the end of the subsequent night (24 h of Day 3) (Figure 7A). Seedling images of the different time points were aligned using Photoshop 7.0 to record hypocotyl length increments. In some experiments, during Day 3, the seedlings were exposed to the indicated treatments but under yellow plus orange filters (Lee filters 101 and 105, respectively) to avoid seedling exposure to blue light.

Quantitative RT–PCR

Seedlings were harvested in liquid nitrogen; total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen). cDNA derived from this RNA was synthesized using Invitrogen Super-Script III and an oligo-dT primer. The synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche, www.roche.com) using the 7500 Real Time PCR System (Applied Biosystems, www.appliedbiosystems.com) cycler. The Polyubiquitin 10 (UBQ-10) gene was used as normalization control (Staneloni et al., 2009). The primers used for *IAA2*, *IAA16*, *SHY2*, *YDK1*, *WES1*, and *UBQ10* are described in Supplemental Table 2.

Statistics

Data were analyzed by two-way ANOVA and the differences between means were evaluated by using Bonferroni post-tests. However, in the kinetics reported in Figure 1C, each time point was analyzed separately (*t*-test) because the variances were homogeneous between treatments but not among time points.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

FUNDING

This work was supported by grants from the University of Buenos Aires (grant no. G044 to J.J.C.), the International Centre for Genetic Engineering and Technology (grant no. CRP/ARG07-02 to J.J.C.), and the Agencia Nacional de Promoción Científica y Tecnológica (grant no. PICT 1748 to J.J.C.).

ACKNOWLEDGMENTS

We thank Jason Reed (University of North Carolina) and Edgard Spalding (University of Wisconsin) for their kind provision of *axr3* and *B19OE* lines, respectively. No conflict of interest declared.

REFERENCES

Achard, P., et al. (2006). Integration of plant responses to environmentally activated phytohormonal signals. Science. **311,** 91–94.

Arana, M.V., Marín-De La Rosa, N., Maloof, J.N., Blázquez, M.A., and Alabadí, D. (2011). Circadian oscillation of gibberellin signaling in *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **108,** 9292–9297.

Casal, J.J. (1996). Phytochrome A enhances the promotion of hypocotyl growth caused by reductions of phytochrome B Pfr levels in light-grown *Arabidopsis thaliana*. Plant Physiol. **112,** 965–973.

Casal, J.J., Sánchez, R.A., and Gibson, D. (1990). The significance of changes in the red/far-red ratio, associated with either neighbour plants or twilight, for tillering in *Lolium multiflorum* Lam. New Phytol. **116,** 565–572.

Cole, B., Kay, S.A., and Chory, J. (2011). Automated analysis of hypocotyl growth dynamics during shade avoidance in *Arabidopsis*. Plant J. **65,** 991–1000.

Covington, M.F., and Harmer, S.L. (2007). The circadian clock regulates auxin signaling and responses in *Arabidopsis*. Plos Biol. **5,** 1773–1784.

De Lucas, M., et al. (2008). A molecular framework for light and gibberellin control of cell elongation. Nature. **451,** 480–484.

Djakovic-Petrovic, T., Wit, M.D., Voesenek, L.A.C.J., and Pierik, R. (2007). DELLA protein function in growth responses to canopy signals. Plant J. **51,** 117–126.

Downs, R.J., Hendricks, S.B., and Borthwick, H.A. (1957). Photoreversible control of elongation of pinto beans and other plants under normal conditions of growth. Bot. Gaz. **118,** 199–208.

Dowson-Day, M.J., and Millar, A.J. (1999). Circadian dysfunction causes aberrant hypocotyl elongation patterns in *Arabidopsis*. Plant J. **17,** 63–71.

Feng, S., et al. (2008). Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. Nature. **451,** 475–479.

Guo, H., Duong, H., Ma, N., and Lin, C. (1999). The *Arabidopsis* blue-light receptor cryptochrome 2 is a nuclear protein regulated by a blue-light dependent post-transcriptional mechanism. Plant J. **19,** 279–289.

Hall, A., et al. (2003). The time for coffee gene maintains the amplitude and timing of *Arabidopsis* circadian clocks. Plant Cell. **15,** 2719–2729.

Hazen, S.P., Schultz, T.F., Pruneda-Paz, J.L., Borevitz, J.O., Ecker, J.R., and Kay, S.A. (2005). LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. Proc. Natl Acad. Sci. U S A. **102,** 10387–10392.

Keuskamp, D.H., Pollmann, S., Voesenek, L.A.C.J., Peeters, A.J.M., and Pierik, R. (2010). Auxin transport through PIN-FORMED 3 (PIN3) controls shade avoidance and fitness during competition. Proc. Natl Acad. Sci. U S A. **107,** 22740–22744.

Kikis, E.A., Khanna, R., and Quail, P.H. (2005). ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY. Plant J. **44,** 300–313.

Leivar, P., and Quail, P.H. (2011). PIFs: pivotal components in a cellular signaling hub. Trend Plant Sci. **16,** 19–28.

Lorrain, S., Allen, T., Duek, P.D., Whitelam, G.C., and Fankhauser, C. (2008). Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. Plant J. **53,** 312–323.

Mazzella, M.A., and Casal, J.J. (2001). Interactive signalling by phytochromes and cryptochromes generates de-etiolation homeostasis in *Arabidopsis thaliana*. Plant Cell Environ. **24,** 155–162.

Michael, T.P., et al. (2008a). A morning-specific phytohormone gene expression program underlying rhythmic plant growth. Plos Biol. **6,** 1887–1898.

Michael, T.P., et al. (2008b). Network discovery pipeline elucidates conserved time-of-day-specific *cis*-regulatory modules. PLoS Gen. **4,** e14. doi:10.1371/journal.pgen.0040014

Mockler, T.C., et al. (2007). The diurnal project: diurnal and circadian expression profiling, model-based pattern matching, and promoter analysis. Cold Spring Harb. Symp. Quant. Biol. **72,** 353–363.

Nagatani, A., Reed, J.W., and Chory, J. (1993). Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. Plant Physiol. **102,** 269–277.

Nozue, K., et al. (2007). Rhythmic growth explained by coincidence between internal and external cues. Nature. **448,** 358–361.

Nozue, K., Harmer, S.L., and Maloof, J.N. (2011). Genomic analysis of circadian clock-, light-, and growth-correlated genes reveals PHYTOCHROME-INTERACTING FACTOR5 as a modulator of auxin signaling in *Arabidopsis*. Plant Physiol. **156,** 357–372.

Nusinow, D.A., et al. (2011). The ELF4–ELF3–LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature. **475,** 398–404.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M., and Chory, J. (1994). Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. Plant Physiol. **104,** 1139–1149.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the Red/Far-Red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. Plant Cell. **5,** 147–157.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M., and Leyser, O. (1998). Changes in auxin response from mutations in an AUX/IAA gene. Science. **279,** 1371–1373.

Salter, M.G., Franklin, K.A., and Whitelam, G.C. (2003). Gating of the rapid shade-avoidance response by the circadian clock in plants. Nature. **426,** 680–683.

Schepens, I., Boccalandro, H.E., Kami, C., Casal, J.J., and Fankhauser, C. (2008). Phytochrome Kinase Substrate4 modulates phytochrome-mediated control of hypocotyl growth orientation. Plant Physiol. **147,** 661–671.

Sellaro, R., et al. (2010). Cryptochrome as a sensor of the blue/green ratio of natural radiation in *Arabidopsis*. Plant Physiol. **154,** 401–409.

Sellaro, R., Yanovsky, M.J., and Casal, J.J. (2011). Repression of shade-avoidance reactions by sunfleck induction of *HY5* expression in *Arabidopsis*. Plant J. **68,** 919–928.

Shin, J., Park, E., and Choi, G. (2007). PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in *Arabidopsis*. Plant J. **49,** 981–994.

Staneloni, R.J., et al. (2009). Bell-like homeodomain selectively regulates the high-irradiance response of phytochrome. A. Proc. Natl Acad. Sci. U S A. **106,** 13624–13629.

Tao, Y., et al. (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell. **133,** 164–176.

Whitelam, G.C., et al. (1993). Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. Plant Cell. **5,** 757–768.

Wu, G., Cameron, J.N., Ljung, K., and Spalding, E.P. (2010). A role for ABCB19-mediated polar auxin transport in seedling photomorphogenesis mediated by cryptochrome 1 and phytochrome. B. Plant J. **62,** 179–191.

Zagotta, M.T., Hicks, K.A., Jacobs, C.I., Young, J.C., Hangarter, R.P., and Meeks-Wagner, D.R. (1996). The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. Plant J. **10,** 691–702.