Repression of shade-avoidance reactions by sunfleck induction of \emph{HY5} expression in Arabidopsis

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SUMMARY

The light environment provides signals that play a critical role in the control of stem growth in plants. The reduced irradiance and altered spectral composition of shade light promote stem growth compared with unfiltered sunlight. However, whereas most studies have used seedlings exposed to contrasting but constant light treatments, the natural light environment may exhibit strong fluctuations. As a result of gaps in the canopy, plants shaded by neighbours may experience sunflecks, i.e. brief periods of exposure to unfiltered sunlight. Here, we show that sunflecks are perceived by phytochromes A and B, and inhibit hypocotyl growth in \emph{Arabidopsis thaliana} mainly if they occur during the final portion of the photoperiod. By using forward and reverse genetic approaches we found that \emph{ELONGATED HYPOCOTYL 5}, \emph{LATE ELONGATED HYPOCOTYL}, \emph{PHYTOCHROME KINASE SUBSTRATE 4} and auxin signalling are key players in this response.

Keywords: shade avoidance, hypocotyl, \emph{HY5}, auxin, phytochrome, Arabidopsis.

INTRODUCTION

The light environment has profound effects on plant body form and function. The presence of neighbours reduces the availability of photosynthetically active radiation for each plant because of mutual shading among individuals. In addition to this impact on the availability of resources, the presence of neighbours produces light signals that include the reduction of the red/far-red ratio perceived mainly by phytochrome B (phyB) (Holmes and Smith, 1977, Smith, 2000; Yanovsky et al., 1995), the reduction of the red plus far-red irradiance perceived by phyA and phyB (Holmes and Smith, 1977; Yanovsky et al., 1995; Smith, 2000; Franklin et al., 2007; Sellaro et al., 2010), the reduction of blue irradiance perceived mainly by cryochrome 1 (cry1) (Yanovsky et al., 1995) and the reduction of blue/green ratio also perceived by cry1 (Banerjee et al., 2007; Bouly et al., 2007; Sellaro et al., 2010). The red/far-red ratio signals may anticipate the depletion of light available for photosynthesis, providing the opportunity for adjustment before the resources become scant as a result of competition (Ballaré et al., 1987). Plant responses to shade light include enhanced stem growth, reduced branching and increased hyponasty (Franklin, 2008), which together increase the chance of capturing light for photosynthesis in crowding canopies. Therefore, these physiological outputs have been called shade-avoidance reactions (Casal and Smith, 1989; Smith, 1982, 2000).

In recent years we have significantly advanced our understanding of the molecular basis of shade-avoidance reactions (Franklin, 2008; Kami et al., 2010). \emph{PHYTOCHROME INTERACTING FACTOR 4} (PIF4) and PIF5 are basic HLH transcription factors able to promote stem extension growth (Huq and Quail, 2002; Khanna et al., 2004). Under high red/far-red ratios, the active form of phytochrome binds PIF4 and PIF5, causing their degradation in the proteasome. Upon transfer to low red/far-red ratios, the proportion of the active form of phytochrome is reduced, and this allows a higher level of PIF4 and PIF5 proteins to build up (Lorrain et al., 2008). DELLA proteins repress growth in part by impeding PIF4 and PIF3 binding to DNA (De Lucas et al., 2008; Feng et al., 2008), and become degraded both by low red/far-red ratios and low levels of blue light (Djakovic-Petrovic et al., 2007). Auxin signalling has been implicated in shade-avoidance reactions (Morelli and Ruberti, 2000, 2002; Kozuka et al., 2010). Low red/far-red ratios promote auxin synthesis (Morelli and Ruberti, 2002; Tao et al., 2008) and modify the cellular location of the PIN-FORMED 3 (PIN3) regulator of auxin efflux, thereby increasing the levels of auxin in the hypocotyl (Keuskampa et al., 2010): two changes required...
for shade-avoidance reactions. There is significant upregulation of the expression of auxin signalling genes under low red/far-red ratios (Devlin et al., 2003). The action of auxin only partially overlaps with DELLA signalling (Pierik et al., 2009). Interestingly, the bZIP transcription factor ELOGATED HYOCOTYL 5 (HY5), which is critical during de-etiolation, does not appear to be involved in the control of hypocotyl growth in response to shade (Roig-Villanova et al., 2006).

The studies described in the previous paragraph have been conducted under controlled conditions where plants were exposed to high or low red/far-red ratios, provided throughout the photoperiod. However, the natural environment is more complex. Plant canopies do not normally produce continuous shade. Because of the presence of gaps in the canopy, direct light can reach the soil or the lower strata of the canopy without being intercepted by the upper layers of leaves (Holmes and Smith, 1977; Pearcy, 1983; Deregibus et al., 1985). These sunflecks are transient because as solar elevation changes throughout the photoperiod, the spot that had received direct light becomes shaded. The frequency, duration and intensity of the sunflecks depend on the size and distribution of the canopy gaps, and affect the availability of understory light for photosynthesis (Packham et al., 1992). During sunflecks, the basal layers of the canopy receive higher red/far-red ratios, higher red plus far-red irradiances, higher blue/green ratios and higher blue irradiances. We are largely ignorant of the consequences of these interruptions of the shade light signals on the extent of shade-avoidance reactions. Plants might have mechanisms to either compensate for these brief interruptions of the shade light signal or, conversely, to take informational advantage of these sunflecks as a signal.

Here we characterize the effects of sunflecks on hypocotyl growth of Arabidopsis seedlings grown under dense plant canopies, and investigate the molecular mechanisms of the response to sunflecks by using both forward and reverse genetics approaches.

RESULTS

Sunflecks have large effects on growth

To investigate the effects of sunflecks, seedlings grown under shade light were daily exposed to 2 h of sunlight at different time points of the 10-h photoperiod (Figure 1). Compared with a control under uninterrupted shade light, daily sunflecks inhibited growth, and this effect was maximal at the end of the day. Compared with shade light, the late sunfleck caused an inhibition of hypocotyl growth equivalent to 83% of the inhibition caused by exposure to sunlight during the whole photoperiod (Figure 1).

As morning temperatures were typically lower than afternoon temperatures (Figure S1), we compared the effects of morning and afternoon sunflecks in plants grown on heating–cooling plates that maintained a constant growth temperature. Afternoon sunflecks were more effective than morning sunflecks, even in the absence of normal temperature fluctuations (Figure S1). The higher effectiveness of afternoon sunflecks was therefore not the result of higher temperatures at this time of the day.

Phytochromes A and B perceive sunflecks

To investigate the photoreceptors involved in the response to sunflecks, we cultivated seedlings of the wild type and of the phyA, phyB, phyA phyB and cry1 cry2 mutants under uninterrupted shade, shade interrupted by an afternoon sunfleck and uninterrupted sunlight (Figure 2). The phyA phyB double mutant failed to respond to sunflecks, whereas the phyB and phyA single mutants showed partially reduced or wild-type responses, respectively (Figure 2). Thus, sunflecks are perceived primarily by phyB and secondarily by phyA. The cry1 cry2 double mutant showed wild-type responses (Figure 2), indicating no obvious role of cryptochromes in the perception of sunflecks, despite the increased blue irradiance and blue/green ratio.

Afternoon sunflecks are intrinsically more effective than morning sunflecks

The higher effectiveness of sunflecks at the end of the day, compared with the other part of the photoperiod, could

![Figure 1. Sunflecks cause strong reductions of stem growth.](image)

(a) Hypocotyl length of wild-type seedlings grown for 4 days under shade light interrupted daily by 2 h of sunlight (simulating a sunfleck) at different times of the photoperiod (time 0, 2, 4, 6 and 8 h). Dotted lines indicate seedlings grown under uninterrupted shade light and under uninterrupted sunlight. The daily protocol is shown at the top of the figure. Data are means and SEs of between seven and nine replicate boxes. The slope ± SE and P values are indicated.

(b) Photographs of representative seedlings.
result from their effects on the status of phyB during the subsequent night (Downs et al., 1957). To test this hypothesis, we provided a brief red plus far-red light pulse (15 min) with the red/far-red ratio of shade light, i.e. 0.1 (end-of-day [EOD] 0.1), immediately prior to the start of the night. The brief EOD 0.1 pulse is predicted to establish the same level of active phyB at the beginning of the night in all seedlings. Afternoon sunflecks continued to be more effective than morning sunflecks in EOD 0.1-treated seedlings (Figure 3). This indicates that the higher effectiveness of afternoon sunflecks is not the result of elevated levels of the active, far-red-absorbing form of phytochrome during the night.

Transcriptome responses to sunflecks

To investigate changes in the transcriptome induced by sunflecks, wild-type seedlings were grown under uninterrupted shade, shade interrupted by an afternoon sunfleck and uninterrupted sunlight, and were then harvested at the 9-h point of day 3. Among the 793 genes with expression promoted by sunflecks, compared with shade light (Table S1, \( q < 0.05 \); Storey and Tibshirani, 2003), the over-represented gene ontology terms (Table S2) include fatty acid metabolism (mainly fatty acid biosynthesis), response to red light or far-red light, which is consistent with the observed role of phytochromes (see Figure 2), pigmentation metabolism (mainly pigment biosynthesis and chlorophyll metabolism) and response to UV-B, despite the fact that our sunfleck conditions did not increase UV-B irradiance (achieved by filtering through the box lid). Among the 1594 genes with expression repressed by sunflecks compared with shade light (Table S1, \( q < 0.05 \); Storey and Tibshirani, 2003), the overrepresented gene ontology terms were dominated by hormone-related functions such as response to auxin stimulus, response to ethylene stimulus, response to brassinosteroid stimulus and the jasmonic acid-mediated signalling pathway (Table S2). Another over-represented function was nitrogen compound catabolism (mainly amino acid and amine catabolism). It is noteworthy that the CACGTG motif, which is the most frequent binding site of HY5 (Lee et al., 2007), was highly enriched \((P < 10^{-10})\) among the promoters of the genes with expression repressed by sunflecks.

The growth response to sunflecks requires HY5, HYH and PKS4

To investigate the genes involved in the response to sunflecks, we followed both reverse genetics and forward genetics approaches. In the reverse genetics approach, we searched for genes with known function in photomorphogenesis that responded to sunflecks in a direction that could account for the observed growth response to sunflecks (i.e. enhanced expression of growth inhibitor genes or reduced expression of growth promoter genes under sunflecks). The list of genes that fulfill this criterion includes HY5, HOMOLOG OF HY5 (HYH; Holm et al., 2002), PHYTOCHROME KINASE SUBSTRATE 4 (PKS4; Schepens et al., 2008) and PHYTOCHROME INTERACTING FACTOR 3 (PIF3; Ni et al., 1998) (Figure 4a; Table S1). Some genes involved in the inhibition of growth, such as LONG HYOCOTYL IN FAR-RED (HFR1; Fankhauser and Chory, 2000; Sessa et al., 2005) and PHYTOCHROME RAPIDLY REGULATED 1 (PART; Roig-Villanova et al., 2007) showed a reduction of expression under sunfleck conditions, which is consistent with their role as repressors of shade avoidance induced by shade. We selected HFR1 for further studies (Figure 4a).

The hy5 mutant showed reduced response to sunflecks, the hyh mutant showed a normal response and the hy5 hyh double mutant completely failed to respond to sunflecks (Figures 4b and S2). This indicates that HY5 and HYH are redundantly required for the hypocotyl growth response,
with a more important role for HY5. Compared with the wild type, the pks4 mutant showed a reduced response to sunflecks (Figure 4b). This indicates that PKS4 is required for a full response to sunflecks. The pif3 and hfr1 mutants showed wild-type hypocotyl length under sunflecks (mean hypocotyl length relative to dark controls, SE < 0.03: wild type, 0.2; pif3, 0.2; hfr1, 0.2) or shade light conditions (wild type, 0.4; pif3, 0.4; hfr1, 0.4).

As the CACGTG motif is also bound by bHLH transcription factors, and PIF4 and PIF5 are important for hypocotyl growth (Nozue et al., 2007) and shade avoidance responses (Lorrain et al., 2008), we investigated sunfleck responses in the pif4, pif5 and pif4 pif5 mutants. Although these mutations affected growth, they had little effect on the response to sunflecks (Figure S3).

**HY5 represses the expression of PKS4 and auxin-related genes**

To investigate the mechanism of action of HY5, we compared the transcriptome of wild-type and hy5 mutant seedlings grown under afternoon sunfleck conditions, and harvested at the 9-h point of day 3. We classified the genes in three groups according to their enhanced, reduced or unaffected expression in hy5 compared with the wild type (Table S1). We then compared these groups with the groups defined by the enhanced, reduced or unaffected expression in wild-type seedlings grown under sunflecks compared with uninterrupted shade light conditions (Table S1). The observed number of genes with expression simultaneously enhanced by HY5 compared to hy5 and by sunflecks compared to shade light, or simultaneously reduced by HY5 compared to hy5 and by sunflecks compared to shade light (Table S1), was significantly higher than expected by chance ($\chi^2 < 0.0001$). The latter is consistent with the idea that HY5 mediated a significant proportion of gene-expression responses to sunflecks. Auxin-related genes are over-represented among the genes with expression reduced both by HY5 compared with hy5 and by sunflecks compared with shade light conditions ($\chi^2 < 0.0001$) (Figure 5). This group also included PKS4 (Table S1).

**Dysfunction of the circadian clock impairs the response to sunflecks**

Following the forward genetics approach we searched for seedlings bearing either long or short hypocotyls in a screening based on pools of the T-DNA activation tagging lines grown under sunfleck conditions. The 277F mutant line selected by this procedure shows long hypocotyls and failed to respond to sunflecks occurring either in the morning or in the afternoon (Figure 6a). By using thermal asymmetric interlaced PCR technology (Liu et al., 1995) we placed the T-DNA insertion in the intergenic region between At1g01060 and At1g01070. The insert co-segregated with the 277F phenotype ($\chi^2 < 0.0001$). The mutant
Compared with the wild type, the lhy cca1 showed a higher response to morning sunflecks (note the lhy cca1, even in double mutant, the prr7 prr9 effective than morning sunflecks (Figure 6c). The CCA1 transgenics overexpressing lhy cca1 result of diurnal fluctuations in LHY diurnal fluctuations in sensitivity to sunflecks could be the afternoon than in the morning, we speculated that the response to sunflecks and sunflecks are more effective in afternoon (Figure S4). As high levels in the morning and at low levels in the afternoon (at 8 h) sunflecks.

277F showed overexpression of the At1g01060 gene, which encodes the LHY protein (Figure 6b). LHY is a component of the circadian clock together with its homologue CCA1, and its overexpression has been reported to yield long hypocotyls (Schaffer et al., 1998).

As expected (Kim et al., 2003), LHY was expressed at high levels in the morning and at low levels in the afternoon (Figure S4). As high LHY levels impair the response to sunflecks and sunflecks are more effective in the afternoon than in the morning, we speculated that the diurnal fluctuations in sensitivity to sunflecks could be the result of diurnal fluctuations in LHY expression. To test this possibility we analysed the double mutant lhy cca1. Compared with the wild type, the lhy cca1 double mutant showed a higher response to morning sunflecks (note the significant light x genotype interaction in Figure 6c). However, even in lhy cca1 afternoon sunflecks were more effective than morning sunflecks (Figure 6c). The prr7 prr9 double mutant, the lux, elf3 and elf4 mutants, and the transgenics overexpressing CCA1 showed a reduced response to afternoon sunflecks, whereas the prr5, prr7, prr9, gi and toe1 single mutants showed normal responses (Figure 6c). We conclude that severe clock dysfunction impairs the response to sunflecks.

Diurnal dependence of sunfleck promotion of HY5 expression

We analysed in further detail the response of HY5 expression to light to investigate the link with the growth response. At the end of the night, HY5 expression was low, but 2 h after the beginning of the day the levels were high, irrespective of beginning the day either under shade or sunfleck conditions (Figure 7a). This promotion induced by the dark-to-light transition was mediated by phytochromes (Figure 7a). Under shade, the levels of expression of HY5 decreased during the day, but the afternoon sunfleck was able to re-establish high levels (Figure 7a). The difference between afternoon shade or sunfleck conditions was perceived by phytochromes (Figure 7a). Therefore, afternoon sunflecks are more effective than morning sunflecks to inhibit growth and to promote HY5 expression. This was also the case for PKS4 expression (Figure S5), which is consistent with a control of PKS4 expression by HY5 (Table S1).

Two results indicate that the diurnal dependence of HY5 responses to sunflecks is not the main point of action of clock genes. First, when the seedlings were incubated in full light, wild-type seedlings showed overexpression of the At1g01060 gene, which encodes the LHY protein (Figure 6b). LHY is a component of the circadian clock together with its homologue CCA1, and its overexpression has been reported to yield long hypocotyls (Schaffer et al., 1998).

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darkness during the first 8 h of the subjective photoperiod, both shade and sunfleck conditions were able to establish high HY5 expression in the afternoon (Figure 7a), resembling the effect of the morning dark-to-light transition. Second, the lhy cca1 mutation increased the effect of morning sunflecks on growth, and actually reduced the HY5 response to morning sunflecks (Figure 7b).

Enhanced auxin signalling reduces the response to sunflecks

Auxin-related genes tend to show high expression at dawn (Michael et al., 2008a), when sunflecks are less effective. An lhy mutant overexpressing LHY and the lux mutant tend to show high levels of expression of the genes, with reduced expression in response to sunflecks and HY5 (Michael et al., 2008a) (Figure S6). This suggests that the impaired response to sunflecks in these mutants could result from elevated auxin signalling. We elevated auxin signalling either by exogenously adding a synthetic auxin (Picloram) or by using the axr3 mutant, impaired in a gene that represses auxin signalling (Rouse et al., 1998). The results of both approaches indicate that enhanced auxin signalling reduces the response to sunflecks (Figure 8).

DISCUSSION

Compared with open places, the light environment of plant canopies is characterized by reduced irradiance, and reduced red/far-red and blue/green ratios. These signals, perceived by phytochromes and cryptochromes, promote stem extension growth, among other shade-avoidance reactions. However, plant canopies are heterogeneous and generate gaps where sunlight temporarily penetrates with higher irradiance, and red/far-red and blue/green ratios. Here, we have shown that these sunflecks significantly reduce long-term hypocotyl growth in A. thaliana (Figure 1). The occurrence of the sunflecks is perceived primarily by phyB and secondarily by phyA (Figure 2). It is of note that cryptochromes, which sense the degree of shade (Yanovsky et al., 1995; Sellaro et al., 2010), are dispensable for the perception of sunflecks.

The occurrence of sunflecks depends on the interaction between the position of the gap and solar elevation (Holmes and Smith, 1977; Pearcy, 1983; Dereibus et al., 1985). Therefore, sunflecks are repeated daily, approximately at the same time of the photoperiod. Here we show that daily sunflecks occurring late in the photoperiod are much more effective to inhibit hypocotyl growth than those occurring in the morning (Figure 1). This differential sensitivity does not result from the fact that late sunflecks establish high levels of active phyB throughout the night, because re-establishing low levels of active phyB by means of a brief end-of-day light pulse did not affect the magnitude of the effect of afternoon sunflecks (Figure 3). The reduced response to morning than to afternoon sunflecks was observed even under stabilized temperature conditions (Figure S1), indicating that differences in temperature were not the cause of the differential sensitivity.

To investigate the molecular mechanisms underlying the response to sunflecks, and its dependence on the time of day, we used both reverse and forward genetics approaches. Microarray experiments pointed to HY5, HYH and PKS4 as key players in the response to sunflecks, because their expression levels responded specifically to these light conditions (Figure 4a). Afternoon expression levels of HY5 and HYH are low in seedlings grown under uninterrupted shade or uninterrupted sunlight, and exhibit a significant increase in seedlings exposed daily to afternoon sunflecks. Conversely, the afternoon expression level of PKS4 is high in seedlings grown under uninterrupted shade, and is significantly reduced in seedlings exposed daily to afternoon sunflecks (Figure 4a). The hy5 and pks4 mutants showed severely impaired hypocotyl growth responses to sunflecks, which were further reduced in the hy5 hyh double mutant (Figure 4b). Based on the comparison of simulated sunlight and shade light under stable conditions, HY5 and HYH had been considered to play no role in shade-avoidance responses (Roig-Villanova et al., 2006). By introducing fluctuations in the light environment that are characteristic of most natural conditions, we conclude that HY5 and HYH do play a role in the repression of shade-avoidance responses when a sunfleck interrupts shade light on a daily basis.

In the microarray experiment we observed a significant overlap between the effects of sunflecks compared with uninterrupted shade and the effect of HY5 compared with hy5 under sunfleck conditions. Furthermore, the most frequent binding site of HY5 (Lee et al., 2007) is over-represented among the genes that responded to sunflecks. This indicates that (as observed for growth responses) gene
expression responses to sunflecks are to a large degree mediated by HY5. Auxin-related genes were over-represented among the genes with expression reduced by both sunflecks and HY5. Some of these genes are direct targets of HY5 (Lee et al., 2007). The action of HY5 and HYH had been connected to auxin signalling in the root (Cluis et al., 2004; Sibout et al., 2006). Therefore, HY5 would inhibit hypocotyl growth in part by reducing auxin signalling.

Compared with shade light, only afternoon sunflecks increased HY5 expression (Figure 7a) and reduced PKS4 expression (Figure S5), consistent with the control of PKS4 expression by HY5 (Table S1). The diurnal dependency of HY5 and PKS4 expression can therefore account for the diurnal dependency of the growth response. The increased HY2 response to afternoon sunflecks, compared with morning sunflecks, stems from the fact that afternoon sunflecks occur after several hours of exposure to shade light, and is not indicative of circadian control of the HY5 response. If on the day of harvest for HY5 expression analysis the seedlings remained in darkness before exposure to afternoon shade or sunfleck environments, these conditions did not result in different HY5 expression levels, resembling the case of morning sunfleck versus shade conditions (Figure 7a). During the morning, both shade and sunfleck conditions perceived by phytochromes elevate HY5 expression above the levels observed at the end of the night (Figure 7a). However, even under continued shade conditions, which promote HY5 expression in the morning, HY5 expression decreases in the afternoon (Figure 7a). Then, if the seedlings are transferred to sunfleck conditions HY5 expression shows a second promotion (Figure 7a) to levels that are not observed even in seedlings exposed to sunlight for all of the photoperiod (Figure 4a). In other words, HY5 expression responds to the changes in the light environment (i.e. from darkness to either shade or sunfleck conditions, and from shade to sunfleck conditions) rather than reflecting the current light conditions itself. This pattern resembles the process termed adaptation or desensitization, where the response to a stimulus returns to its prestimulus value even in the continued presence of the signal (Yi et al., 2000). A new change in signalling strength elicits a new spike of response. This pattern is typical, for instance, of bacterial chemotaxis (Yi et al., 2000).

We isolated a mutant with elevated expression of LHY that showed reduced responses to sunflecks. Other mutants and transgenics with impaired clock function also exhibited reduced responses to sunflecks (Figure 6c). The clock appears mainly to establish a permissive state for the sunfleck response in the afternoon, because none of the clock-defective plants exhibited a strong gain in morning response. Only lhy cca1 showed a modest increment of the growth responses to morning sunflecks, but afternoon sunflecks still remained more effective than morning sunflecks in this mutant (Figure 6c).

Figure 9 provides a summary that integrates the findings reported here. The presence of gaps in the canopy allows direct sunlight to reach the lower strata of the vegetation stand at certain times of the day. This causes a transient elevation of the red/far-red ratio and the red plus far-red irradiance perceived by phyA and phyB. During the late hours of the photoperiod, after several hours of exposure to shade light, phyA and phyB perception of the sunflecks elevates HY5 expression, which inhibits stem growth. However, if the sunflecks occur early in the photoperiod they have no significant effects on HY5 expression (already elevated under shade by the dark-to-light transition) or hypocotyl growth. The action of HY5 occurs in part via a...
reduction of the auxin signalling status and a reduction in PKS4 expression levels. Correct clock function would be necessary to establish a permissive low-auxin signalling state, particularly in the afternoon.

**EXPERIMENTAL PROCEDURES**

**Plant material and growth conditions**

We used 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 (Lariguet et al., 2006), axr3-1 (Rouse et al., 1998), elf3-1 (Zagotta et al., 1996), elf4-101 (Khanna et al., 2003), gi-2 (Fowler et al., 1999), toc1-101 (Kikis et al., 2005), prr5 (salk 006280), prr7-3 prr9-1 (Farre et al., 2005), lux-4 (Hazen et al., 2005) and CCA1-35¢ in the Columbia background. We used hy5-KS50, hyh, hyhKS50-hyh (Holm et al., 2002) and lhy-21 cca1-11 (Hall et al., 2003) in the Wassilewskija (WS) background. The stock CS31100 of ABRC donated by Wolf Scheible and Chris Somerville was used for the mutant screening. In physiological experiments, 15 seeds per genotype were sown on 3 mL of 0.8% agar in clear plastic boxes (4 × 3.5 cm). In some experiments, picloram (Tordon 24K) was added to the agar solution before melting. In microarray experiments, 200 seeds were sown on 25 mL of 0.8% agar in Petri dishes. The boxes or dishes were incubated in the dark at 5°C for 5 days, given 8 h of red light (to induce seed germination) followed by 16 h of darkness (22°C) and transferred to the treatment conditions in the field.

In the field, the boxes were exposed daily to a photoperiod of 10 h either under the shade of a 3-m tall canopy of Viburnum tinus (Eve Price) (photosynthetically active radiation, PAR, of 40 μmol m\(^{-2}\) s\(^{-1}\)) with red/far-red ratio of 0.1–0.2 at midday) or under unfiltered sunlight (PAR 600 μmol m\(^{-2}\) s\(^{-1}\)) and a red/far-red ratio of 1.1 at midday). The experimental field was located at the Faculty of Agriculture, University of Buenos Aires, 34°35’S, 58°28’W. To simulate sunfleck conditions the seedlings grown under shade light were transferred daily for 2 h to sunlight conditions. This treatment elevated PAR between 10-fold with a midday sunfleck and 30-fold at the extremes of the photoperiod, and elevated the red/far-red ratio to 1.1. Dark controls were placed under sunlight conditions wrapped with black plastic (inner cover) and aluminum foil (outer cover).

**Measurements of hypocotyl length**

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). Data were analysed by a two-way ANOVA and Bonferroni’s post hoc test.

**Microarray experiments**

Two microarray experiments were conducted. In the first experiment, seedlings of the wild type were grown under conditions of uninterrupted shade, sunfleck (afternoon) or uninterrupted sunlight. In the second experiment, seedlings of the hy5-221 mutant and the wild type were grown under sunfleck (afternoon) conditions. Two biological replicates per light/genotype condition were harvested in liquid nitrogen after 9 h of the beginning of the photoperiod of the third day of treatment. Total RNA was extracted with the RNeasy Plant mini kit (Qiagen, http://www.qiagen.com) following the manufacturer’s protocols. cDNA and cRNA synthesis and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were performed in accordance with Affymetrix instructions. Expression data were normalized to the sum of each microarray (Clarke and Zhu, 2006), restricted by presence criteria (two presence flags in at least one condition). To identify the genes with expression significantly affected by sunfleck versus uninterrupted shade in the wild type, and by hy5 versus HYS under sunfleck conditions, we conducted an ANOVA, including the wild type under shade light and the wild type under sunfleck conditions from the first experiment, and the wild type under sunfleck conditions and hy5 under sunfleck conditions from the second experiment. We selected the genes showing significant effects of treatment (q < 0.05; Storey and Tibshirani, 2003). For these genes we performed a Student’s t-test (q < 0.05) comparing the wild type under shade light versus the wild type under sunfleck conditions from the first experiment, and the wild type under sunfleck conditions versus hy5 under sunfleck conditions from the second experiment. The use of independent samples of wild type grown under sunfleck conditions for each Student’s t-test precludes the spurious assignment of coincidence of light and HYS effects. The normalized data of all genes is presented in Table S1.

Over-represented gene ontology terms and transcription factor binding sites were investigated by using the ATCOESIS homepage (Vandepoele et al., 2009) and Athena homepage (O’Connor et al., 2005), respectively.

**Quantitative RT-PCR**

Seedlings were harvested in liquid nitrogen, total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) and subjected to a DNase treatment with RN1 RNase-Free DNase (Promega, http://www.promega.com). cDNA derived from this RNA was synthesized using Invitrogen SuperScript III and an oligo-dT primer. The synthesized cDNAs were amplified with FastStart Universal SYBR Green Master (Roche, http://www.roche.com) using the 7500 Real Time PCR System (Applied Biosystems, http://www.appliedbiosystems.com) cycler. The Protein Phosphatase 2A Subunit A3 (PP2A) gene was used as normalization control (Czechowski et al., 2005). The primers used for HYS and LHY are described elsewhere (Hazen et al., 2005; Sibout et al., 2006), and for PKS4 were: PKS4-FW, 5’-GGCTCTGCTTCCAGTATAAACCCG-3’; and PKS4-RV, 5’-CGCTTTGTCCTTCTTGCTCATG-3’. Data were analysed by two-way ANOVA and Bonferroni’s post hoc tests.

**Cloning of the 277F mutant**

The flanking genomic sequence from the T-DNA insertion site was recovered using the thermal asymmetric interlaced-PCR protocol (Liu et al., 1995). The T-DNA insertion was confirmed by PCR with specific primers. Perfect co-segregation (in 100 chromosomes analysed) between the T-DNA insertion and the mutant phenotype was observed in F₂ seedlings derived from a cross between the 277F mutant and wild-type Col-2. To investigate the LHY expression levels in the 277F mutant, the synthesized cDNA was amplified by PCR using ACTIN2 as a loading control in the exponential range of amplification (26 cycles for LHY and 20 cycles for ACTIN2). The primers used for LHY were: LHY-FW, 5’-AATTCGCCCTCTTCCTGTA-3’; and LHY-RV, 5’-CCTGTTAACTGAGATCCT-3’. The primers for ACTIN2 were: ACT2RTF, 5’-AGTTGCCTGACAACCGGATTTGGT-3’; and ACT2RTR, 5’-CGCAGCTCCAGACTGTACTTCTCTT-3’.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The response to sunflecks does not depend on natural temperature fluctuations.

Figure S2. HY5 is required for a full response to sunflecks.

Figure S3. The response to sunflecks in pif4, pif5 and pif4 pif5.

Figure S4. LHY expression level at different times of photoperiod.

Figure S5. PKS4 expression levels.

Figure S6. Expression of the auxin-related genes in lhy and lux.

Table S1. Normalized gene expression in microarray experiments.

Table S2. Gene ontology terms over-represented among the genes with expression promoted or reduced by sunflecks compared with shade light.

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