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# COP1 re-accumulates in the nucleus under shade

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## SUMMARY

**Shade-avoider plants typically respond to shade-light signals by increasing the rate of stem growth. CONSTITTUTIVE PHOTOMORPHOGENESIS 1 (COP1) is an E3 ligase involved in the ubiquitin labelling of proteins targeted for degradation. In dark-grown seedlings, COP1 accumulates in the nucleus and light exposure causes COP1 migration to the cytosol. Here, we show that in *Arabidopsis thaliana*, COP1 accumulates in the nucleus under natural or simulated shade, despite the presence of far-red light. In plants grown under white light, the transfer to shade-light conditions triggers an unexpectedly rapid re-accumulation of COP1 in the nucleus. The partial simulation of shade by lowering either blue or red light levels (maintaining far-red light) caused COP1 nuclear re-accumulation. Hypocotyl growth of wild-type seedlings is more sensitive to afternoon shade than to morning shade. A residual response to shade was observed in the *cop1* mutant background, but these seedlings showed inverted sensitivity as they responded to morning shade and not to afternoon shade. COP1 overexpression exaggerated the wild-type pattern by enhancing afternoon sensitivity and making morning shade inhibitory of growth. COP1 nuclear re-accumulation also responded more strongly to afternoon shade than to morning shade. These results are consistent with a signalling role of COP1 in shade avoidance. We propose a function of COP1 in setting the daily patterns of sensitivity to shade in the fluctuating light environments of plant canopies.**

**Keywords: CONSTITUTIVE PHOTOMORPHOGENESIS 1, shade avoidance, hypocotyl, growth, *Arabidopsis thaliana*.**

## INTRODUCTION

Shading by neighbouring plants in dense canopies leads to reduced activity of the plant photoreceptor phytochromes and cryptochromes. These photoreceptors inhibit the growth of the stem, and therefore under shade stem growth is released and plants become taller. As a result of this, the leaves are placed at higher strata within the canopy and are less likely to become shaded by the foliage of neighbours (Smith, 1982; Ballaré, 1999; Morelli and Ruberti, 2002; Franklin and Whitelam, 2005; Casal, 2013).

A signalling pathway between the photoperception of shade and the control of stem growth has recently been established. Compared with open places, the low red/far-red ratios typical of shade reduce the proportion of phytochrome B (phyB) in its active, Pfr form, which is present predominantly in the nucleus. PHYTOCHROME INTERACTING FACTOR 4 (PIF4), PIF5, PIF3 and PIF7 are basic helix-loop-helix (bHLH) transcription factors bound by Pfr (Leivar and Quail, 2011). As a result of their interaction with Pfr, PIF4 (Lorrain *et al.*, 2008), PIF5 (Shen *et al.*, 2007; Lorrain *et al.*, 2008) and PIF3 (Bauer *et al.*, 2004; Park *et al.*, 2004; Al-Sady *et al.*, 2006) are phosphorylated and degraded in the *26S* proteasome. In the presence of phyB Pfr, PIF7 becomes phosphorylated, but not significantly degraded (Leivar *et al.*, 2008a; Li *et al.*, 2012). In addition, at least for PIF7 (Li *et al.*, 2012) and PIF3 (Park *et al.*, 2012), phyB Pfr reduces the ability to bind their DNA targets. Therefore, under the low red/far-red ratios of shade, PIFs increase their abundance and/or ability to bind DNA. The targets of PIFs include genes encoding enzymes involved in the synthesis of auxin; therefore, under low red/far-red ratios the levels of auxin increase in a PIF-dependent manner, and promote stem growth (Hornitschek *et al.*, 2012; Li *et al.*, 2012).

In addition to the phyB–PIF–auxin pathway of shade-avoidance reactions, a second signalling branch could include the action of CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1) (Casal, 2013). COP1 is an E3 ligase involved in the targeting of proteins to degradation in the proteasome

(Lau and Deng, 2012). When the seedlings are grown in full darkness before the emergence of the aerial organs from the soil, COP1 is present in the nucleus, where it targets for degradation transcription factors that are required for photomorphogenesis (i.e. the developmental pattern typical of light-exposed plants; Osterlund *et al.*, 2000). As a result of this function, *cop1* mutants are unable to degrade the relevant transcription factors normally, and demonstrate constitutive photomorphogenesis (i.e. photomorphogenesis in the absence of light). Light perceived by phytochromes and cryptochromes causes COP1 migration to the cytoplasm (Osterlund and Deng, 1998). Cryptochromes also disrupt the complex between COP1 and SUPRESSOR OF PHYTOCHROME A1 (SPA1) proteins, leading to reduced COP1 activity, the accumulation of its transcription factor targets, and the progression of photomorphogenesis (Liu *et al.*, 2011; Lau and Deng, 2012). Nuclear localization of COP1 is necessary for its activity, but light-induced migration to the cytosol is too slow, suggesting that it is not sufficient for the regulation of COP1 activity (von Arnim *et al.*, 1997; Yi and Deng, 2005; Lau and Deng, 2012). The *cop1* and *spa1 spa2 spa4* mutants fail to respond with enhanced stem growth to the reduced phyB activity caused by a pulse of far-red light before the night or low daytime red/far-red ratios, and to the reduced cryptochrome and phyB activity caused by shade (McNellis *et al.*, 1994; Crocco *et al.*, 2010; Rolauffs *et al.*, 2012; Casal, 2013). The exception is the acceleration of flowering caused by low red/far-red ratios, which is present in *cop1* (Rolauffs *et al.*, 2012). However, the role of COP1 in shade avoidance is often not considered, and there are reasons for this. In fact, based on current evidence, the impaired shade-avoidance responses of the *cop1* mutant could be interpreted either as a true, direct function of COP1 in shade avoidance, or as a collateral consequence of the mutation. According to the first interpretation, COP1 activity should increase under shade, and this increase should be part of the events causing enhanced stem growth. However, whether shade enhances COP1 activity is not known.

The aim of this paper is to investigate whether shade increases the nuclear abundance of COP1, which is a requisite to reach its nuclear targets. We show an unexpectedly rapid re-accumulation of COP1 in the nucleus in response to natural or simulated shade. Under daily light–dark cycles, plants are more sensitive to shade in the afternoon than in the morning (Sellaro *et al.*, 2012). We show that COP1 plays a key role in defining this pattern of sensitivity.

## RESULTS

### Shade avoidance requires COP1 and SPA

Seedlings of *Arabidopsis thaliana* were grown for 3 days under either white light or simulated shade light (with lower levels of blue light, red light and red/far-red ratio to simulate all the major features of shade) under controlled conditions (photoperiod, 10 h). As expected in the wild type, shade induced a significant promotion of hypocotyl growth compared with the white-light control (Figure 1a). The reduced response to white light vs. shade observed in the *phyA phyB* and *cryptochrome 1* (*cry1*) mutants evidences the involvement of the phytochromes and the cryptochrome in the perception of shade signals. Under white light the *cop1* hypocotyl lengths relative to controls grown in the dark were greater than in the wild type because of the short *cop1* hypocotyl length when grown in darkness. The *cop1–4* and *cop1–6* mutants showed no significant growth responses to shade (Figure 1a). This result confirms and extends previous reports showing deficient *cop1* responses to either end-of-day or daytime supplementary far-red light treatments (McNellis *et al.*, 1994; Rolauffs *et al.*, 2012), which only simulate the phyB-related signals of canopy shade. The COP1-overexpressing lines (COP1OX1 and COP1OX2 in the No–0 background) also showed a reduced growth response to shade (Figure 1a). Note that the *cop1–6 phyA* and *cop1–6 phyB* double mutants partially recovered their ability to respond to shade signals.

COP1 forms complexes with SPA proteins (Zhu *et al.*, 2008). The response to shade was reduced in the simple *spa1*, *spa2* and *spa4* mutants, and was absent in the *spa1 spa2 spa4* triple mutant (Figure 1b). Neither the *spa3* nor the *spa1 spa2 spa3* mutants presented significant differences compared with the wild type. These results confirm and extend those obtained with white light supplemented with far-red light (Rolauffs *et al.*, 2012).

The *PHYTOCHROME INTERACTING FACTOR 3-LIKE 1* (*PIL1*), *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*), *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7* (*XTR7*) and *ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2* (*ATHB2*) genes are among the direct targets of PIFs, and their expression is enhanced by low red/far-red ratios (Hornitschek *et al.*, 2012). Our simulated shade conditions also enhanced the expression of these genes in the wild type, but the response was absent in the *cop1* mutants (Figure 2). Overexpression of COP1 did not affect the expression of *PIL1*, *IAA29*, *XTR7* or *ATHB2* genes under white light, but it distorted the response to shade in a direction (i.e. enhanced or reduced the response) that depended on the gene and transgenic line (Figure 2), indicating a more complex dependence on *COP1* levels.

### COP1 accumulates in the nucleus under simulated or natural shade

As shade avoidance is impaired in *cop1* mutants, we investigated whether shade signals affect COP1 localization by using *cop1–4*/$Pro_{35S}$: YFP-COP1 seedlings (Oravecz *et al.*, 2006). Wide-field fluorescence microscopy images revealed that COP1 protein is recruited into the nucleus under

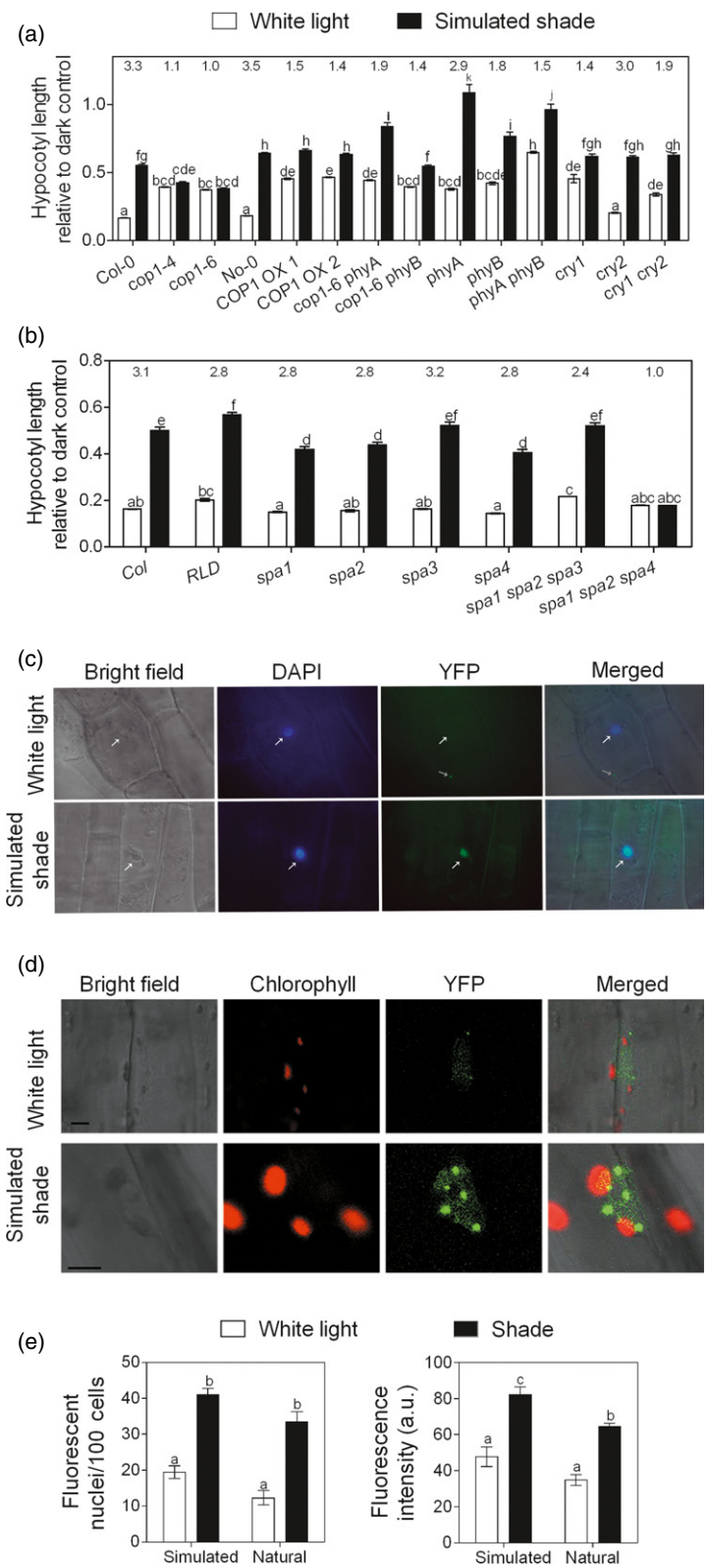


**Figure 1.** Shade avoidance requires COP1, which accumulates in the nucleus under shade.
(a) Hypocotyl length of Col–0 and No–0 wild-type seedlings, *cop1–4*, *cop1–6*, *cop1–6 phyA*, *cop1–6 phyB*, *phyA*, *phyB*, *phyA phyB*, *cry1*, *cry2* and *cry1 cry2* mutant seedlings, and the overexpressing lines COP1OX1 and COP1OX2 grown under white light or simulated shade (low red/far-red ratio, low red light and low blue light).
(b) Hypocotyl length of Col–0 and RLD wild-type seedlings, *spa1–3*, *spa2–1*, *spa3–1*, *spa4–1*, *spa1–3 spa2–1 spa4–1* and *spa1–3 spa2–1 spa4–1* mutant seedlings.
(c) Wide-field fluorescence microscopy images of representative cells of *cop1–4*/Pro35S:YFP-COP1 seedlings grown under white light or simulated shade. DAPI staining was used to confirm nuclear localization.
(d) Confocal microscopy of representative nuclei of *cop1–4*/Pro35S:YFP-COP1 seedlings grown under white light or simulated shade. Transmission images and chlorophyll fluorescence are also included. Scale bars:5 μm.
(e) Number of fluorescent nuclei and fluorescence intensity of the nuclei in seedlings expressing YFP-COP1 grown under simulated or natural sunlight and shade conditions.
Data are means and SEs of between three and nine replicate boxes (a, b) or nine replicates. Different letters denote significant differences ($P < 0.05$) among means. The simulated shade/white light hypocotyl length ratio is also shown for each genotype in (a) and (b).

simulated shade, whereas under white light COP1 is mainly observed in cytoplasmic inclusion bodies (Figure 1c). COP1 nuclear localization was studied in greater detail using confocal microscopy. COP1 protein was observed in nuclear speckles under simulated shade, whereas under white-light conditions the fluorescence of

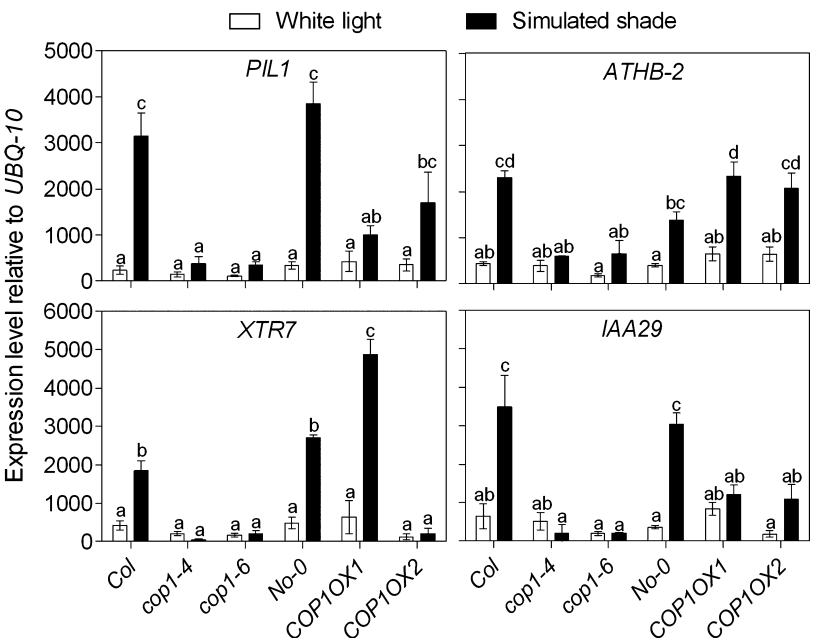


**Figure 2.** The promotion of *PIL1*, *ATHB–2*, *XTR7* and *IAA29* expression by shade requires COP1.
Col–0 and No–0 wild-type seedlings, *cop1–4* and *cop1–6* mutant seedlings, and the overexpressing lines COP1OX1 and COP1OX2 were grown under white light or simulated shade. Samples were harvested at the 10–h time point of day 3.
Data are means and SEs of three or four biological replicates.
Different letters denote significant differences ($P < 0.05$) among means.

the nuclei was significantly lower (Figure 1d). Total cellular fluorescence or GUS activity extracted from COP1OX transgenics (where COP1 is fused to GUS) was unaffected by shade, indicating that our treatment affected COP1 localization, and not total abundance (Figure S1). Both the number of fluorescent nuclei and their fluorescence intensity were remarkably higher in seedlings grown under simulated shade than in seedlings grown under white light (Figure 1e). Under natural radiation, plant canopies reduce not only blue and red light, but also the UV–B present in solar radiation, which leads to reduced phytochrome, cryptochrome and UVR8 activity. Whereas phytochrome and cryptochrome cause COP1 exclusion from the nucleus (Osterlund and Deng, 1998), UVR8 enhances COP1 nuclear accumulation (Oravecz *et al.*, 2006). To investigate the balance of these contrasting activities we investigated COP1 under sunlight compared with natural shade light. Despite the presence of UV–B, the patterns were very similar to those observed under controlled conditions (Figure 1e). This was also true for the physiological output (Figure S2).

### Diurnal pattern of nuclear COP1 abundance

To investigate the degree of association between growth and nuclear COP1 we analysed the kinetics of both variables throughout the third photoperiod under white light and simulated shade. Under white light the rate of hypocotyl growth was maximal at the beginning of the day (Figure 3a), confirming previous observations (Nozue *et al.*, 2007; Michael *et al.*, 2008). Under simulated shade, the hypocotyl growth rate was already higher than under white light at the beginning of the day (0.0–2.5 h), but the maximum peak occurred at 2.5–5.0 h. The growth rate declined towards the end of the photoperiod to the levels observed in white light-grown seedlings. The *cop1* or *spa* mutants showed differences in growth rate, but not in the daily growth pattern. The more detailed analysis revealed that the *cop1–4* retains a weak response to the shade, not observed in of the *cop1–6* or *spa1 spa2 spa4* mutants (Figure 3a).

Under white light, YFP-COP1 showed a strong diurnal pattern of nuclear accumulation (Figure 3b). At the end of the night, both the number of fluorescent nuclei and their fluorescence intensity were maximal. The number of fluorescent nuclei fell by half after 2.5 h under white light, and remained at this level until the end of the photoperiod. The fluorescence intensity of nuclear COP1 showed a more gradual decrease. These results indicate that COP1 can be rapidly excluded from the nucleus after the beginning of the day. Interestingly, at the end of the night, the levels of nuclear COP1 were similar in white-light or simulated shade-treated seedlings, but under shade the levels remained high during the photoperiod (Figure 3b).

### Rapid re-accumulation of nuclear COP1 in response to shade

To investigate the kinetics of the shade response, the seedlings were grown under white light and then transferred to simulated shade 1 h after the beginning of the third day (the controls remained under white light). A rapid growth response to shade was observed in most genotypes. The response was reduced in *cop1–4* and *cop1–6* mutants, and was completely absent in the *spa1 spa2 spa4* triple mutant (Figure 4a).

Nuclear COP1 showed a rapid re-accumulation upon transfer from white light to simulated shade. Simulated shade induced the rapid formation of well-defined nuclear speckles. More diffuse fluorescence was observed later,

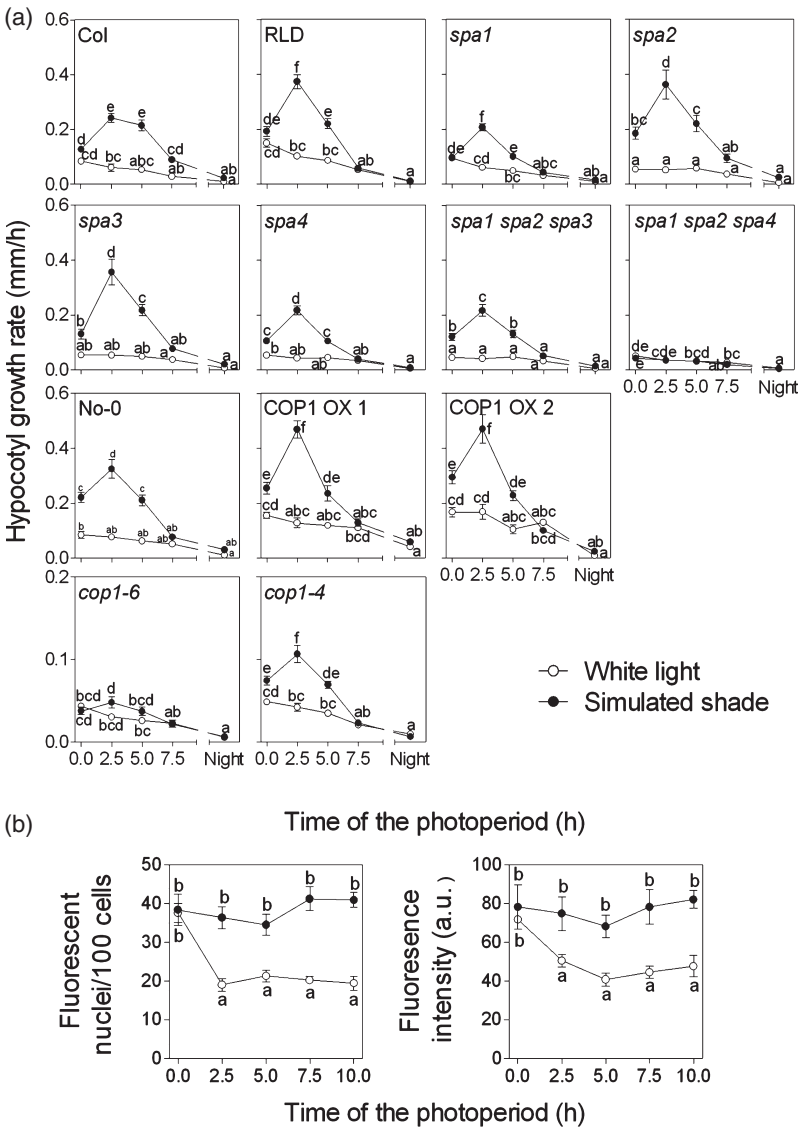


**Figure 3.** Diurnal pattern of nuclear COP1 abundance.
(a) Time course of hypocotyl growth rate during day 3 in Col–0, No–0 and RLD wild-type seedlings, *spa1–3*, *spa2–1*, *spa3–1*, *spa4–1*, *spa1–3 spa2–1 spa4–1*, *spa1–3 spa2–1 spa4–1*, *cop1–4* and *cop1–6* mutant seedlings, and COP1OX1 and COP1OX2 overexpressing lines grown under white light or simulated shade.
(b) Time course of number of fluorescent nuclei and fluorescence intensity of the nuclei during day 3 in seedlings expressing YFP-COP1 grown under white light or simulated shade.
Data are means and SEs of eight (a) replicate boxes or between five and 12 (b) replicates.
Different letters denote significant differences ($P < 0.05$) among means.

and particularly in the seedlings grown for 3 days under simulated shade (Figure 4b). The number of fluorescent nuclei increased 1 h after the beginning of shade, and showed a peak after 3 h of shade (Figure 4c). The fluorescence intensity of the nuclei also increased rapidly, showed a smooth rise between 1 and 6 h of shade, and then slightly declined towards the end of the photoperiod (Figure 4c).

### Both blue and red light reduction induce COP1 accumulation in the nucleus

Natural shade involves a stronger reduction in red and blue light than in far-red light; therefore, the red/far-red ratio is also reduced as a result of the selective effects. To investigate the contribution of these signals to the overall effect of shade, COP1 nuclear accumulation was studied in seedlings expressing YFP-COP1 grown under blue, red and far-red light (with a red/far-red ratio of 1.1) and transferred to conditions simulating selective features of shade: reduced blue light (with no change in red or far-red light), reduced red light (with no change in blue or far-red light, and with a red/far-red ratio of 0.3) or reduced blue and red light (with no change in far-red light) 1 h after the beginning of the third day. Both the reduction of blue light and the reduction of red light induced a significant increase in the number of fluorescent nuclei compared with the control that remained under the initial levels of blue, red and far-red light (Figure 5). The effects were additive, and the highest number of fluorescent nuclei was observed in seedlings transferred to reduced blue and red light. The reduction of blue, red or both blue and red light induced a similar increase of nuclear fluorescence (Figure 5).

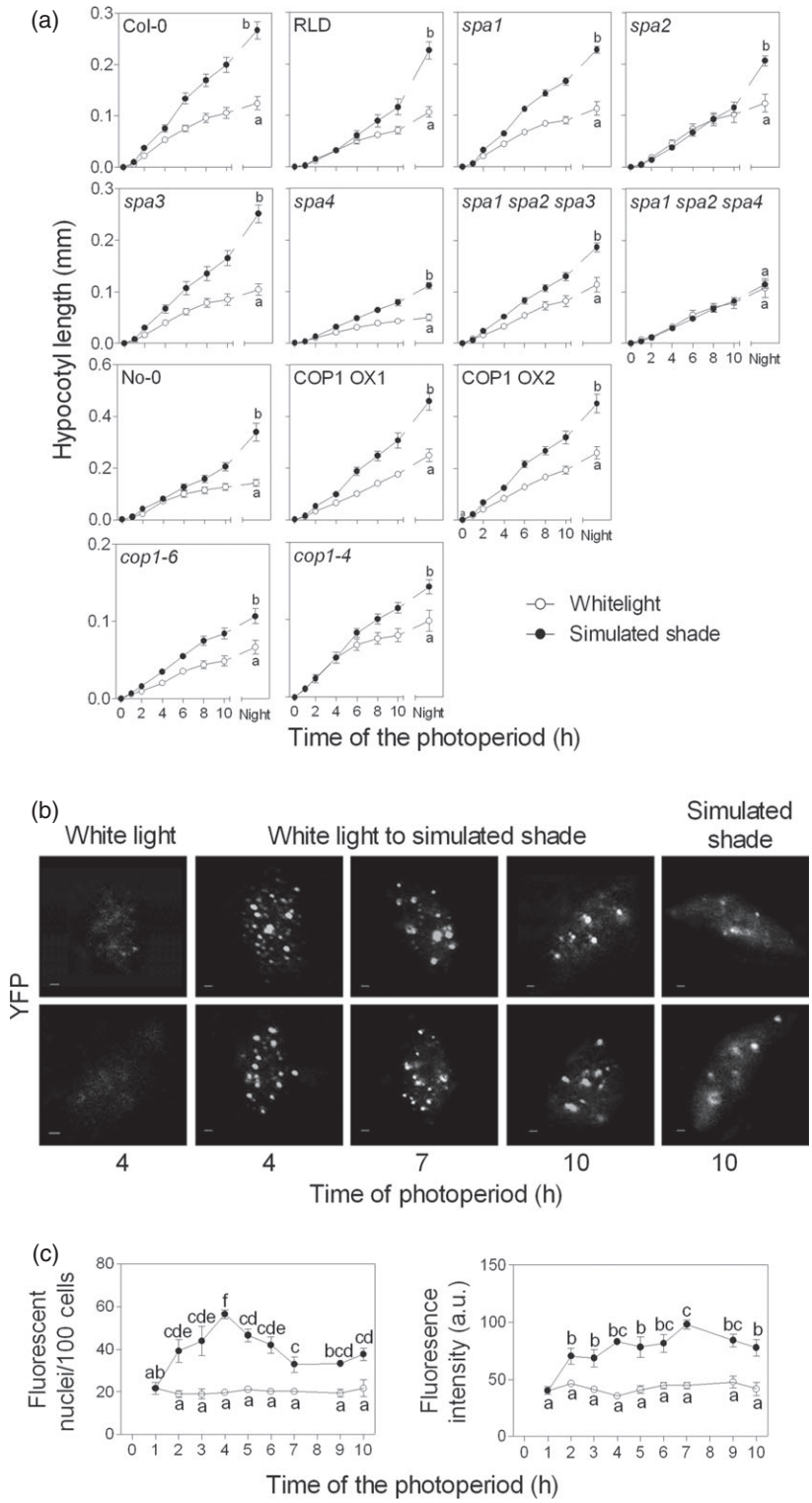


**Figure 4.** Rapid re-accumulation of nuclear COP1 in response to shade.
(a) Hypocotyl growth accumulated during day 3 in Col–0, No–0 and RLD wild-type seedlings, *spa1–3*, *spa2–1*, *spa3–1*, *spa4–1*, *spa1–3 spa2–1 spa4–1*, *spa1–3 spa2–1 spa4–1*, *cop1–4* and *cop1–6* mutant seedlings, and COP1OX1 and COP1OX2 overexpressing lines grown under white light, and either transferred to simulated shade 1 h after the beginning of the photoperiod or left as a control under white light.
(b) Representative nuclei in seedlings expressing YFP-COP1 grown under white light and either transferred to simulated shade 1 h after the beginning of day 3 or left as a control under white light (the control for 9 h is similar to that at 4 h, and is not included). A control grown under simulated shade photoperiods for the 3 days is also included. Scale bars:1 μm.
(c) Time course of number of fluorescent nuclei and fluorescence intensity of the nuclei during day 3 in seedlings expressing YFP-COP1 grown under white light, and either transferred to simulated shade 1 h after the beginning of the photoperiod or left as a control under white light.
Data are means and SEs of between six and eight (a) replicate boxes or between four and 12 (c) replicates. Different letters denote significant differences ($P < 0.05$) between end-point means (a) or among means (c).

### The diurnal pattern of sensitivity to shade requires normal levels of COP1

When sunlight-grown plants are exposed daily to brief periods (2 h) of shade, afternoon shade promotes stem growth but morning shade is not effective in this way (Sellaro *et al.*, 2012). Here we report a similar pattern of sensitivity to shade under controlled conditions (Figure 6a). The partial recovery of the ability to respond to simulated shade in the *cop1–6 phyA* and *cop1–6 phyB* double

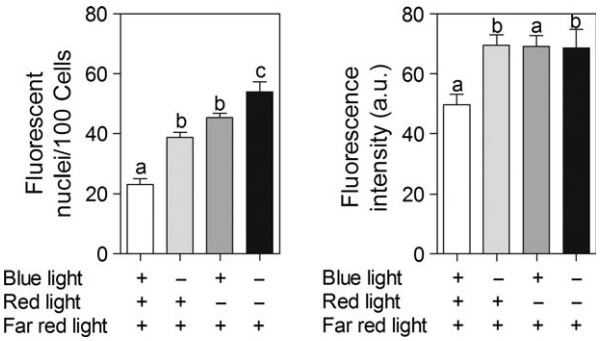


**Figure 5.** Selective blue or red light signals of shade induce COP1 accumulation in the nucleus.
The number of fluorescent nuclei and fluorescence intensity of the nuclei of seedlings expressing YFP-COP1 grown under white light (blue plus red plus far-red light), and transferred to reduced blue light, reduced red light or reduced blue and red light 1 h after the beginning of the third photoperiod, or left as a control under white light. Confocal images were taken 10 h after the beginning of the third photoperiod (i.e. after 9 h of differential treatment). Data are means and SEs of 15–19 replicates. Different letters denote significant differences ($P < 0.05$) among means.

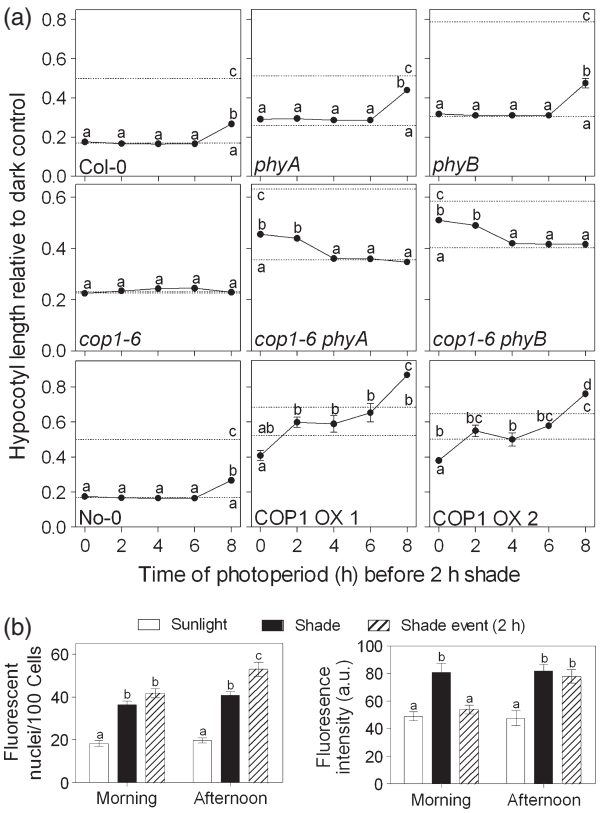


**Figure 6.** The diurnal pattern of sensitivity to shade requires normal levels of COP1.
(a) Hypocotyl length of Col–0 and No–0 wild-type seedlings, *phyA*, *phyB*, *cop1–6*, *cop1–6 phyA* and *cop1–6 phyA* mutant seedlings, and COP1OX1 and COP1OX2 overexpressing lines, grown for 3 days under white light interrupted daily by a 2–h shade event initiated at the indicated times of the photoperiod. Dotted lines indicate hypocotyl length in seedlings grown for the whole photoperiod under simulated shade (above) or under uninterrupted white light (below).
(b) The nuclear accumulation of COP1 is more intense in response to afternoon shade than in response to morning shade. Number of fluorescent nuclei and fluorescence intensity of the nuclei of seedlings expressing YFP-COP1 grown for 3 days under white light, interrupted daily by a 2–h shade event in the morning (beginning at 0 h) or in the afternoon (beginning at 8 h of the photoperiod). Fluorescence was analysed immediately after the relevant shade event. Control seedlings grown for the whole photoperiod under simulated shade or under uninterrupted white light are included.
Data are means and SEs of three (a) replicate boxes or between nine and 19 (b) replicates. Different letters denote significant differences ($P < 0.05$) among means.

mutants, compared with the *cop1* single mutants, revealed that the *cop1* mutation inverts the pattern of sensitivity (note that a normal pattern is preserved in *phyA* and *phyB* mutant seedlings, indicating that these mutations are not the cause of altered sensitivity). In essence, in *cop1–6 phyA* and *cop1–6 phyB* double mutants morning shade was effective and afternoon shade was not effective in promoting hypocotyl growth (Figure 6a) . This suggests that COP1 is necessary to repress the response to morning shade, and to promote the response to afternoon shade. In agreement with this interpretation, in COP1OX1 and COP1OX2 lines morning shade actually reduced stem growth, and afternoon shade caused a promotion of growth that was higher than that observed in the wild type (Figure 6a).

### Sensitivity of COP1 nuclear accumulation in response to shade

As altered levels of COP1 disrupt the normal sensitivity to morning shade, compared with afternoon shade, we investigated the sensitivity of COP1 accumulation in the nucleus in response to morning compared with afternoon shade. Seedlings expressing YFP-COP1 were exposed daily to 2 h of shade, either in the morning or in the afternoon. Controls were grown either under white light or under simulated shade. Under stable white light or shade conditions the number of nuclei with COP1 and the fluorescence intensity of these nuclei were similar in the morning, compared with the afternoon; however, nuclear COP1 accumulation was significantly more intense in response to afternoon shade than in response to morning shade (Figure 6b).

### Diurnal sensitivity of growth to shade requires normal patterns of *CSN1/FUS6* expression

CSN1/FUS6, a subunit of the COP9 signalosome, is required for the nuclear localization of COP1 (Wang *et al.*, 2009). The expression of *CSN1/FUS6* increases during the photoperiod, reaching higher levels during the afternoon than during the morning (Mockler *et al.*, 2007). As the normal pattern of growth sensitivity to shade requires normal COP1 levels (Figure 6a), and this correlates with a more intense accumulation of nuclear COP1 in response to

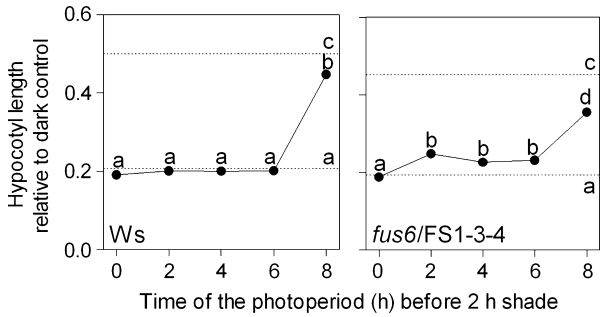


**Figure 7.** Diurnal sensitivity of growth to shade requires normal patterns of *CSN1/FUS6* expression.
Hypocotyl length of Ws wild-type seedlings and of the *fus6*/FS1-3-4 line grown for 3 days under white light, interrupted daily by a 2–h shade event initiated at the indicated times of the photoperiod. Dotted lines indicate hypocotyl length in seedlings grown the whole photoperiod under simulated shade (above) or under uninterrupted white light (below).
Data are means and SEs of between three and six replicate boxes. Different letters denote significant differences ($P < 0.05$) among means.

afternoon shade (Figure 6b), we reasoned that altering the patterns of expression of *CSN1/FUS6* could disrupt the diurnal pattern of sensitivity to shade events. To test this prediction we used the *fus6*/FS1-3-4 line that expresses the full-length sequence of *CSN1/FUS6* under the control of a constitutive promoter in the *fus6* background (Wang *et al.*, 2009). In contrast to the wild type, *fus6*/FS1-3-4 showed a significant response to shade events at 2, 4 and 6 h, whereas the response at the end of the photoperiod (at 8 h) was partially reduced (Figure 7).

## DISCUSSION

The *cop1* mutants were amongst the first to show a severe shade-avoidance phenotype (McNellis *et al.*, 1994; Crocco *et al.*, 2010; Rolauffs *et al.*, 2012; Casal, 2013). In fact, *cop1* is probably the most severe single mutant in terms of lacking shade-avoidance responses under natural or simulated shade. In addition, the *spa1 spa2 spa4* triple mutant, deficient in proteins that form a complex with COP1, shows no growth response to natural or simulated shade (Rolauffs *et al.*, 2012 and this report). However, COP1 is not normally considered to operate within the mechanisms of shade avoidance. A key piece of evidence required to support a direct role of COP1 is to demonstrate that shade can positively affect COP1 activity. Here, we show that COP1 nuclear abundance increases under shade. As at least some COP1 targets are nuclear (Lau and Deng, 2012; Rolauffs *et al.*, 2012), COP1 nuclear localization is important for its activity.

Arabidopsis seedlings grown under simulated shade (low blue light, low red light and low red/far-red ratio) showed more nuclei with YFP-COP1 and increased fluorescence of nuclear COP1 (Figure 1c,d,e). During de-etiolation (i.e. when the seedlings are exposed to light for the first time), blue, red and far-red light acting via cry1, phyB and phyA induce the migration of COP1 from the nucleus to the cytosol (Osterlund and Deng, 1998). Here, we show that the transfer from white light to simulated shade caused a rapid accumulation of nuclear COP1 (Figure 4b,c). This indicates that COP1 migration to the cytosol is a reversible process. Selective reduction of the blue or red light (and hence also the red/far-red ratio) were effective to increase COP1 nuclear signals (Figure 5). These observations suggest that continued cry1 and phyB activity would be required to maintain COP1 outside the nucleus, but that activation of phyA by far-red light would not be enough. Under natural radiation, nuclear COP1 increased under a grass canopy compared with unfiltered sunlight (Figure 1e). This is important because natural shade reduces not only blue and red light levels (which reduce COP1 nuclear abundance), but also UV–B (which is perceived by UVR8, and in turn increases COP1 nuclear abundance). The similar quantitative results under natural and simulated conditions suggest that the drop of UV–B under natural shade does not create a strong conflicting signal.

In seedlings grown under white light COP1 rapidly re-accumulated in the nucleus in response to shade (Figure 4b). This rapid response is surprising because COP1 exclusion from the nucleus during de-etiolation is slow (von Arnim *et al.*, 1997; Yi and Deng, 2005; Lau and Deng, 2012). These kinetics are consistent with the rapid hypocotyl growth response to shade (Figure 4a) . Under day–night cycles the levels of nuclear COP1 were high at the end of the night and white light rapidly reduced nuclear COP1 during the first hours of the photoperiod (Figure 3b). We are currently investigating whether COP1 nucleo-cytoplasmic partitioning becomes more dynamic during the transition between skotomorphogenesis and photomorphogenesis, in order to cope with the more dynamic environment the shoot has to face upon emergence from the soil.

Previous studies have concluded that sensitivity to shade is under the control of the circadian clock under continuous light (Salter *et al.*, 2003), and under the control of the circadian clock and light-derived signals under day–night cycles (Sellaro *et al.*, 2012). Daily natural shade events are more effective to promote hypocotyl growth when they occur in the afternoon than when they take place in the morning (Sellaro *et al.*, 2012). We obtained three pieces of evidence in favour of a significant role of COP1 in setting this pattern of sensitivity to shade. First, a normal pattern of diurnal growth sensitivity to shade requires normal levels of COP1. The weak *cop1* mutant alleles retained some response to shade, particularly in the *phyA* or *phyB* mutant backgrounds. However, in these mutants the sensitivity was reversed, i.e. high sensitivity in the morning and low sensitivity in the afternoon (Figure 6a). Conversely, the COP1-overexpressing lines showed enhanced sensitivity to afternoon shade, and inhibition (instead of promotion) of hypocotyl growth in

response to morning shade (Figure 6a). In other words, COP1 promotes afternoon sensitivity and reduces morning sensitivity to shade events. Second, nuclear COP1 accumulation is also more sensitive to afternoon shade than to morning shade (Figure 6b). Third, CSN1/FUS6 is a component of the COP9 signolosome, which physically interacts with COP1 and regulates its localization (Wang *et al.*, 2009). Under day–night cycles the expression of *CSN1/FUS6* shows a diurnal rhythm reaching higher levels in the afternoon (Mockler *et al.*, 2007). A *csn1/fus6* mutant complemented with the *CSN1/FUS6* gene, under the control of a constitutive promoter, showed a distorted diurnal pattern of sensitivity to shade (Figure 7), despite its normal seedling morphology.

The results presented here are consistent with a scenario where the promotion of stem growth by shade would be mediated by two major signalling branches: one pathway involving the enhanced activity of PIFs promoting auxin synthesis genes; and another pathway likely to involve COP1. The promotion of *PIL1*, *IAA29*, *XTR7* and *ATHB2* expression by shade signals requires both binding PIFs (Hornitschek *et al.*, 2012) and the presence of COP1 (Figure 2), indicating at least a partial convergence of these pathways. Putative direct or indirect targets of COP1 activity in shade avoidance include the two B–box-containing zinc-finger transcription factors BBX21 and BBX22 (Datta *et al.*, 2007; Crocco *et al.*, 2010), HFR1 (Rolauffs *et al.*, 2012) and PIFs (Bauer *et al.*, 2004; Leivar *et al.*, 2008b). PIFs have a positive role in shade avoidance and, at least in some contexts, their abundance is positively affected by COP1 (Bauer *et al.*, 2004; Leivar *et al.*, 2008b), providing one possible mechanism of convergence. Both BBX and HFR1 have negative effects on shade avoidance, and their abundance is negatively regulated by COP1. The negative action of HFR1 on PIFs (Hornitschek *et al.*, 2009) provides another point of convergence. HY5 is a key target of COP1 during de-etiolation (Osterlund *et al.*, 2000), but not during shade avoidance (Rolauffs *et al.*, 2012), and HY5 is important to terminate shade signalling in response to daily sunflecks (Sellaro *et al.*, 2011), but has little effect on the generation of shade-avoidance responses. Note that the activity of PIFs is important to control the daily growth kinetics that peak at dawn (De Lucas *et al.*, 2008; Soy *et al.*, 2012) , but not the daily pattern of sensibility to shade signals (Sellaro *et al.*, 2012), whereas COP1 plays a key role in defining the pattern of daily sensitivity to shade, peaking in the afternoon (Figure 6a), but is not in control of the daily pattern of growth (Figure 3a).

## EXPERIMENTAL PROCEDURES

### Plant material

The mutants *cop1–4*, *cop1–6* (McNellis *et al.*, 1994), *cop1–6 phyB–9*, *cop1–6 phyA–211* (Boccalandro *et al.*, 2004), *phyB–9* (Reed *et al.*, 1993), *phyA–211*, *phyA–211 phyB–9* (Reed *et al.*, 1994), *cry1–304*, *cry2–1* and *cry1–304 cry2–1* (Guo *et al.*, 1999) were compared with their Columbia (Col–0) wild type. Transgenic lines overexpressing COP1 (Boccalandro *et al.*, 2004) were compared with their Nossen (No–0) wild type. The transgenic line *cop1-4*/Pro35S:YFP-COP1 (Oravecz *et al.*, 2006) is in the Columbia background. The mutant *spa1–3* (Hoecker *et al.*, 1998) is in the RLD background, whereas *spa2–1*, *spa3–1* and *spa4–1* (Laubinger *et al.*, 2004) are in the Columbia background. The *fus6*/FS1-3-4 line (Wang *et al.*, 2009) is in the Columbia background.

Fifteen seeds per genotype were sown on 3 ml of 0.8% agar in each clear plastic box (4 × 3.5 × 1.5 cm height). The boxes were incubated in darkness at 5°C for 5 days and given 8 h of red light followed by 16 h of darkness (22°C) before treatments.

### Light treatments

The seedlings were grown either under white light, provided by a mixture of fluorescent and incandescent lamps, with a red/far-red ratio typical of sunlight (1.1), or under simulated shade light provided by the same light sources in combination with two green acetate filters (#089; LEE Filters, http://www.leefilters.com) to reduce the blue and red light and the red/far-red ratio. The spectral distribution of the light was measured with an USB4000-UV-VIS spectrometer, pre-configured with a DET4-200-850 detector and a QP600-2-SR optical fibre (Figure S3; Ocean Optics Inc., http://www.oceanoptics.com). Blue light, red light and far-red light were reduced from 7.2, 5.1 and 4.9 μmol $m^{-2}$ $s^{-1}$ under white light to 0.4, 0.1 and 1.4 μmol $m^{-2}$ $s^{-1}$, respectively, under simulated shade. The red/far-red ratio was reduced from 1.1 to 0.1. The temperature was held at 22°C.

Selected experiments were conducted in the field, where the boxes were exposed daily to a photoperiod of 10 h, either under sunlight (photosynthetically active radiation 600 μmol $m^{-2}$ $s^{-1}$ and a red/far-red ratio of 1.1 at midday) or under the shade of a *Lolium multiflorum* canopy (photosynthetically active radiation 40 μmol $m^{-2}$ $s^{-1}$ and a red/far-red ratio of 0.1 at midday) (Figure S3). Dark controls were placed under sunlight conditions wrapped with black plastic (inner cover) and aluminium foil (outer cover).

To investigate the contribution of selected shade-light signals, the seedlings were grown under a mixture of blue (7.4 μmol $m^{-2}$ $s^{-1}$), red (7.1 μmol $m^{-2}$ $s^{-1}$) and far-red light (6.5 μmol $m^{-2}$ $s^{-1}$, with a red/far-red ratio of 1.1) and transferred to conditions simulating selective features of shade: reduced blue light (3.8 μmol $m^{-2}$ $s^{-1}$, no change in red or far-red light), reduced red light (2 μmol $m^{-2}$ $s^{-1}$, no change in blue or far-red light and with a red/far-red ratio of 0.3) or reduced blue and red light (no change in far-red light). Red and blue light were provided by alternate rows of red (maximum emission, 623 nm) and blue (maximum emission, 465 nm) light-emitting diodes. Far-red light was provided through the space between the rows of diodes by incandescent lamps in combination with a blue acetate filter (Paolini 2031, La Casa del Acetato, Buenos Aires, Argentina) placed above the panel of diodes.

### Hypocotyl growth

The final hypocotyl length was measured to the nearest 0.1 mm with a ruler, and the length of the 10 tallest seedlings per genotype and per box were averaged (one replicate). To calculate the growth rate or accumulated growth, the seedlings were photographed using a digital camera (PowerShot; Canon, http://www.canon.com) and hypocotyl length was determined using image processing software (Sellaro *et al.*, 2009).

### Microscopy

Wide-field fluorescence microscopy images were taken with an Olympus BX60F5 microscope (http://www.olympus-global.com), with an oil-immersion objective lens (UplanF1 100×/1.0). For nuclei staining, seedlings were soaked in DAPI solution (2 μg $ml^{-1}$ 4′,6-diamidino-2-phenylindole; Invitrogen, http://www.invitrogen.com). Excitation of fluorophores was performed with a 100–W high-pressure mercury burner (Olympus). Detection of DAPI fluorescence was performed with a U–MNU cube (Olympus), and detection of YFP fluorescence was performed with a YFP filter cube (Olympus).

Confocal fluorescence images were taken with an LSM5 Pascal (Zeiss, http://www.zeiss.com) laser scanning microscope with a water-immersion objective lens (C–Apochromat 40×/1.2; Zeiss). For chloroplast visualization, probes were excited with a He-Ne laser and fluorescence was detected using an LP560 filter. For COP1-YFP fusion protein visualization, probes were excited with an Argon laser and fluorescence was detected using a BP 505-530 filter. A transmitted light channel was also configured. Fluorescent nuclei were defined as regions of interest (ROIs) and fluorescence intensity was measured using ImageJ from the National Institutes of Health (Abràmoff *et al.*, 2004). Representative cells of the hypocotyl parenchyma (first layers beneath the epidermis) were documented by photography during the first 15 min of microscopical analysis.

### Quantitative RT-PCR

Seedlings were harvested in liquid nitrogen, total RNA was extracted with the Trizol Reagent (Invitrogen) and subjected to a DNAse treatment with RQ1 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, available from Invitrogen) cycler. The Polyubiquitin 10 (UBQ–10) gene was used as the normalization control (Staneloni *et al.*, 2009). The primers used for *PIL1*, *ATHB–2*, *XTR7*, *IAA29* and *UBQ–10* are described in Table S1.

### Statistics

Data were analysed by either two-way or one-way ANOVA (Figures 5 and S1), and the differences among means were evaluated by using Bonferroni's *post-hoc* tests.

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## SUPPORTING INFORMATION

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

**Figure S1.** Simulated shade does not affect COP1 abundance.

**Figure S2.** Hypocotyl growth response to natural shade requires COP1.

**Figure S3.** Spectral distribution of the light fields.

**Table S1.** Oligonucleotide sequences of the primer pairs used in qRT-PCR.

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