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Title page

Nuclear phytochrome A signaling promotes phototropism in *Arabidopsis*

Chitose Kami ^a, Micha Hersch ^b, Martine Trevisan ^a, Thierry Genoud ^{a,1}, Andreas Hiltbrunner ^c, Sven Bergmann ^b and Christian Fankhauser ^{a,2}

^a Center for Integrative Genomics, Faculty of Biology and Medicine, University of Lausanne, CH-1015 Lausanne, Switzerland

^b Department of Medical Genetics, University of Lausanne, CH-1005 Lausanne, and Swiss Institute of Bioinformatics, CH-1015 Lausanne, Switzerland.

^c Centre for Plant Molecular Biology (ZMBP), University of Tübingen, D-72076 Tübingen, Germany

¹ current address: Spiritus Sanctus College, CH-3900 Brig-Glis, Switzerland

Corresponding author:

² Christian Fankhauser, Center for Integrative Genomics, University of Lausanne, Genopode Building, 1015 Lausanne, Switzerland

Phone n° ++41 21 692 3941; FAX n° ++41 21 692 3925

Email christian.fankhauser@unil.ch

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Synopsis

This work shows that the phytochrome A photoreceptor promotes re-orientation of the hypocotyl towards blue light (phototropism) by regulating the expression of nuclear genes. We also show that phytochrome A nuclear signaling events still operate in a mutant where phytochrome A does not significantly accumulate in the nucleus.

Abstract

Phototropin photoreceptors (*phot1* and *phot2* in *Arabidopsis*) enable responses to directional light cues (e.g. positive phototropism in the hypocotyl). In *Arabidopsis* *phot1* is essential for phototropism in response to low light, a response that is also modulated by phytochrome A (*phyA*), representing a classical example of photoreceptor co-action. The molecular mechanisms underlying promotion of phototropism by *phyA* remain unclear. Most *phyA* responses require nuclear accumulation of the photoreceptor but interestingly it has been proposed that cytosolic *phyA* promotes phototropism. By comparing the kinetics of phototropism in seedlings with different subcellular localizations of *phyA* we show that nuclear *phyA* accelerates the phototropic response while in the *fhy1fhl* mutant, in which *phyA* remains in the cytosol, phototropic bending is slower than in the wild type. Consistent with this data we find that transcription factors needed for a full *phyA* responses are needed for normal phototropism. Moreover, we show that *phyA* is the primary photoreceptor promoting the expression of phototropism regulators in low light (e.g. *PKS1* and *RPT2*). Although *phyA* remains cytosolic in *fhy1fhl*, induction of *PKS1* and *RPT2* expression still occurs in *fhy1fhl* indicating that a low level of nuclear *phyA* signaling is still present in *fhy1fhl*.

Introduction

Higher plants have advanced light sensing and signaling systems to control their growth and development. Seedling development is strongly influenced by the intensity, wavelength, photoperiod and direction of the light (Kami et al., 2010). In Arabidopsis, multiple photoreceptors including five phytochromes (phyA-E), two cryptochromes (cry1, 2), two phototropins (phot1,2) and UVR8 control seed germination, de-etiolation and/or phototropism (Franklin et al., 2005; Christie, 2007; Demarsy and Fankhauser, 2009; Kami et al., 2010; Rizzini et al., 2011). Later in their life cycle photoreceptors also control vegetative development (e.g. shade avoidance) and the transition to reproduction (Kami et al., 2010). Although some light responses are primarily controlled by a single photoreceptor there are numerous examples of photoreceptor co-action leading to an optimal physiological or developmental response in a changing light environment (Casal, 2000; Sellaro et al., 2009; Kami et al., 2010). Such co-action is very important during seedling establishment, a growth stage when plantlets are very vulnerable (Sellaro et al., 2009).

The phototropins control several blue light responses including phototropism, leaf flattening, chloroplast movements and opening of the stomata (Christie, 2007). phot1 and phot2 control many of these responses together, however phot1 is more sensitive to blue light than phot2 as exemplified by the essential nature of phot1 for phototropism in response to low blue light (Kagawa and Wada, 2000; Sakai et al., 2001; Christie, 2007). Upon light perception the phototropins autophosphorylate a step that is essential for all

tested physiological responses (Inoue et al., 2008; Inoue et al., 2011). How this initial step is connected to the subsequent signaling events remains to be determined. In the case of phototropism a gradient of auxin has been proposed to be a prerequisite for the asymmetric growth response allowing optimal positioning of the leaves/cotyledons (Esmon et al., 2006). Genetic studies have identified a limited number of phototropin signaling components, including NPH3 (NON PHOTOTROPIC HYPOCOTYL 3), RPT2 (ROOT PHOTO TROPISM 2), ABCB19, PKS1 (PHYTOCHROME KINASE SUBSTRATE 1) and PKS2 that interact with the phototropins and act early downstream of phototropin activation (Motchoulski and Liscum, 1999; Sakai et al., 2000; Lariguet et al., 2006; de Carbonnel et al., 2010; Christie et al., 2011). In rice it has been shown that CP1, the ortholog of NPH3, acts upstream of auxin redistribution in the coleoptile (Haga et al., 2005). The importance of auxin transport and signaling for phototropism has been confirmed genetically (Tatematsu et al., 2004; Stone et al., 2008; Moller et al., 2010; Christie et al., 2011; Ding et al., 2011).

Interestingly, phyA, cry1 and cry2 also modulate the phototropic response and some studies have linked their activity to a modulation of auxin transport (Parks et al., 1996; Janoudi et al., 1997; Whippo and Hangarter, 2003; Lariguet and Fankhauser, 2004; Whippo and Hangarter, 2004; Nagashima et al., 2008; Tsuchida-Mayama et al., 2010). While some studies suggest a direct role of these photoreceptors on phototropism, others propose that they act indirectly by inhibiting the gravitropic response (Whippo and Hangarter, 2003; Lariguet and Fankhauser, 2004; Whippo and Hangarter, 2004; Iino, 2006; Nagashima et al., 2008). In addition to phototropism enhancement the

phytochromes also modulate other phototropin responses such as the control of chloroplast movements and opening of the stomata (DeBlasio et al., 2003; Wang et al., 2010). Despite the well-known importance of photoreceptor co-action particularly during early seedling establishment (Sellaro et al., 2009), the molecular mechanisms underlying phototropic enhancement by phytochromes and cryptochromes remain poorly understood.

While in higher plants phototropism is a blue light response in numerous cryptogames both red and blue trigger a phototropic response (Suetsugu et al., 2005). The fern *Adiantum* possesses a chimeric photoreceptor consisting of a phytochrome photosensory domain fused to a phototropin-type photoreceptor (Kawai et al., 2003). When expressed in *Arabidopsis* this photoreceptor triggers phototropism towards both red and blue light (Kanegae et al., 2006). This can be used as an evolutionary argument to propose that phytochromes and phototropins presumably act closely together in the control of phototropism (Marcotte et al., 1999). However, in higher plants the phytochromes and phototropins are mostly present in different subcellular compartments (Christie, 2007; Fankhauser and Chen, 2008). Phototropins are localized at the plasma membrane in the dark. In response to blue light a fraction of phot1 and phot2 is relocalized to the cytoplasm and Golgi respectively (Sakamoto and Briggs, 2002; Kong et al., 2006; Wan et al., 2008). Phytochromes are cytosolic in the dark and enter the nucleus upon light activation (Nagatani, 2004; Fankhauser and Chen, 2008). Nuclear import of phyB is triggered by the light-regulated unmasking of an NLS sequence (Chen et al., 2005). phyA nuclear import depends on its light-regulated interaction with FHY1 and FHL, a pair of

related proteins comprising an NLS sequence followed by an extended linker region and a phyA interaction domain (Hiltbrunner et al., 2006; Rosler et al., 2007; Genoud et al., 2008; Rausenberger et al., 2011). Although these two classes of photoreceptors are primarily encountered in different subcellular compartments they are both present in the cytosol when etiolated seedlings are first exposed to light.

A number of findings are consistent with the idea that phytochromes and phototropins act together in the cytosol to control phototropism (Rosler et al., 2010). The *phyA* mutant has rather severe phototropic defects in response to low blue light (Parks et al., 1996; Janoudi et al., 1997; Lariguet and Fankhauser, 2004; Whippo and Hangarter, 2004; Rosler et al., 2007). In contrast, the *phy1fhl* double mutant in which phyA cannot enter the nucleus displays a normal phototropic response (Rosler et al., 2007). This is consistent with the idea that cytosolic phyA plays a predominant function to promote phototropism. A subsequent study demonstrated that phyA plays a role in the light-regulated relocalization of phot1 to the cytosol again suggesting a possible cytosolic role for phyA (Han et al., 2008). Other studies suggest that the mechanism by which cryptochromes and phytochromes, including phyA, promote phototropism is through light-regulated induction of the expression of phototropism signaling components (Stowe-Evans et al., 2001; Lariguet et al., 2006; Tsuchida-Mayama et al., 2010). The two hypotheses are not mutually exclusive but it is currently unknown whether phytochromes primarily enhance phototropism by acting with the phototropins in the cytosol or whether phototropism enhancement depends on nuclear entry of the phytochrome.

In order to address this question we have studied phyA-mediated enhancement of phototropism. phyA is well suited for this study because the loss-of-function mutant has severe phototropic defects and because we possess genetic tools to keep phyA either in the nucleus or in the cytosol. *fhy1fhl* mutants retain normal levels of phyA but the photoreceptor is not imported into the nucleus upon light perception (Hiltbrunner et al., 2006; Rosler et al., 2007). In *phyA* mutants expressing phyA-NLS-GFP (thereafter called phyA-NLS-GFP) the photoreceptor is constitutively present in the nucleus (Genoud et al., 2008; Toledo-Ortiz et al., 2010). By comparing the WT, *phyA*, *fhy1fhl* and phyA-NLS-GFP we showed that nuclear phyA leads to accelerated phototropic bending while when phyA is present in the cytosol phototropism proceeds more slowly. Consistent with this data transcriptional regulators involved in phyA signaling are required for a normal phototropic response. Interestingly in response to blue light *fhy1fhl* retains low levels of light-induced gene expression correlating with the slow phototropic response of the mutant. Our gene expression analysis is consistent with the notion that *fhy1fhl* retains a small degree of nuclear phyA signaling particularly in response to blue light. Our study shows that phyA enhances phototropism most efficiently when localized in the nucleus, however it does not exclude a role for phyA in the cytosol.

Results

Both *fhy1fhl* and phyA-NLS-GFP seedlings show a robust phototropic response

We have previously shown that *phyA* mutants seedlings grown from the time of germination in unilateral blue light display a clear phototropic phenotype (Lariguet and Fankhauser, 2004). We thus compared the wild type, *phyA*, *fhy1fhl* and phyA-NLS-GFP using this long-term phototropic protocol. Importantly *phyA* levels in the phyA-NLS-GFP and phyA-NLS lines used here were quantified and they were not higher than in the wild type (Genoud et al., 2008). Our data confirmed previous observations that showed a reduced response in *phyA* but a similar phototropic response in *fhy1fhl* and the wild type (Rosler et al., 2007). Surprisingly however phyA-NLS-GFP seedlings also displayed a normal phototropic response suggesting that *phyA* either in the nucleus or in the cytosol is sufficient to promote phototropism (Figure 1A).

Most phototropism studies are performed with etiolated seedlings treated with a unilateral light source, which prompted us to further test this hypothesis using a more conventional protocol. We used two-day-old etiolated seedlings (4-6 mm) and exposed them for 24 hours to different fluence rates of blue light before measuring the deviation from vertical growth. *phyA* showed a reduced phototropic response at all tested fluence rates but the phenotype was strongest at the lowest fluence rate (Figure 1B). Interestingly at low fluence rates *fhy1fhl* showed a significantly reduced phototropic response, while phyA-NLS-GFP showed enhanced bending (WT; n=239, *fhy1fhl*; n=204, $P < 0.05$, *t*-test) (WT; n=239, *phyA-NLS-GFP*; n=272, $P < 0.01$, *t*-test) (Figure 1B).

Previous studies have shown that phyA-GFP enters the nucleus in response to white, red, blue and far-red light (Kircher et al., 1999; Kim et al., 2000). In order to verify that the same occurs under our experimental conditions we analyzed the subcellular localization of phyA-GFP and phyA-NLS-GFP in etiolated seedlings treated with low blue light by confocal microscopy. These light conditions triggered entry of phyA-GFP into the nucleus and formation of nuclear bodies (Figure S1 A-D). A prolonged light treatment led to a reduced phyA-GFP signal consistent with the light-regulated degradation of phyA (Figure S1 A-D). Consistent with a previous report phyA-NLS-GFP was constitutively nuclear, the levels of GFP fluorescence decreased upon light treatment and nuclear bodies only appeared in response to light (Figure S1 E, F) (Genoud et al., 2008; Toledo-Ortiz et al., 2010). Collectively these experiments indicate that phyA entered the nucleus in response to a light treatment triggering phototropism and that phyA-NLS-GFP was present in the nucleus from the beginning of the experiment.

Nuclear phyA accelerates phototropic bending

Our experiment indicated that phyA enters the nucleus rapidly in response to a phototropism-stimulating light treatment and that nuclear phyA may be more effective than cytosolic phyA to promote phototropism (Figures 1, S1). In order to test this hypothesis more carefully we performed phototropism time-course experiments as classical experiments have shown that phytochrome accelerates phototropic bending (Parks et al., 1996; Janoudi et al., 1997; Iino, 2006). Our analysis of time-lapse images showed that the kinetics of hypocotyl bending was influenced by the length of the etiolated hypocotyl when the light treatment started and confirmed previous findings

showing that the position of the cotyledon relative to the unilateral light source strongly influences the response (Figure S2) (Khurana et al., 1989). In order to account for those developmental effects on phototropic bending we size selected seedlings (4-5.9 mm long hypocotyls) and used 20 seedlings with the cotyledons on each side for each time point. By following the phototropic bending response we observed a strong phenotype in *fhy1fhl* that was much more striking than in end-point experiments (compare Figures 1 and 2A). The bending response was very slow in *phyA* and much slower in *fhy1fhl* than in the wild type (Figure 2A). Interestingly bending occurred more rapidly in *phyA-NLS-GFP* than in the wild type (Figure 2A). These results indicate that nuclear *phyA* is more efficient than cytosolic *phyA* to promote phototropism. A classic way to demonstrate the promoting effect of *phyA* on phototropism is to pre-treat etiolated seedlings with a red light pulse prior to subjecting them to unilateral blue light (Parks et al., 1996; Janoudi et al., 1997). Such a light treatment leads to nuclear accumulation of *phyA* which may explain why *phyA-NLS-GFP* re-oriented hypocotyl growth direction faster than the wild type (Figure 2A). To test this idea we pretreated etiolated seedlings with red light 1 hour prior to unilateral blue light irradiation and followed bending of the hypocotyls over time. Interestingly this treatment enhanced the speed of bending in the wild type that showed an initial speed of re-orientation very similar to *phyA-NLS-GFP* (Figure 2B). *phyA-NLS-GFP* also responded to the red light pretreatment indicating that translocation of *phyA* into the nucleus is not sufficient for *phyA* activation, this is consistent with previous studies using these lines (Genoud et al., 2008; Toledo-Ortiz et al., 2010). Importantly however the red light pulse had a lower impact on *phyA-NLS-GFP* than the wild type (compare 2 A and 2B). As anticipated *phyA* did not respond to this red light treatment

while interestingly bending in *fhy1fhl* reoriented more rapidly after a red light pretreatment (Figure 2B).

In order to confirm that plants with constitutively nuclear phyA have a more rapid phototropic response than plants where phyA only enters the nucleus in response to light we compared the kinetics of phototropic bending of the WT, phyA-GFP and phyA-NLS seedlings (Genoud et al., 2008) (Figure 3). The experiment was performed as in Figure 2 and measurements were either done manually or with a semi-automatic analysis software designed to this end (HypoPhen) (Figures 3 and S3). The HypoPhen software takes as input time-lapsed images of growing hypocotyls and measures bending angles and hypocotyl growth (Methods). Bending values are then computed as the direction of the upper tip of the hypocotyl. The software is open-source and based on the OpenCV library (Bradski, 2008). It is documented and freely available at <http://www.unil.ch/cbg/index.php?title=HypoPhen>. HypoPhen allowed us to obtain higher temporal resolution and greater reproducibility (no user-induced bias) than manual measurements. During the first hours of phototropism phyA-NLS reoriented faster than the WT and phyA-GFP while in *phyA* and *fhy1fhl* the response was much slower (Figure 3 and S3). These data confirm that in plants where phyA is constitutively nuclear the early bending response is more rapid than in the WT (Figures 2 and 3).

Our data indicate that phyA in the nucleus is very efficient to promote phototropism (Figures 2 and 3). To test this hypothesis further we analyzed phototropism using mutants deficient in nuclear phyA signaling events. We selected HFR1 (LONG HYPOCOTYL IN

FR LIGHT 1) and HY5 (HYPOCOTYL 5), which code for a bHLH and a bZIP transcription factor respectively (Kami et al., 2010). A phototropic time course showed that *hfr1* displays a significantly slower phototropic response (Figure 4). Moreover this phenotype is further enhanced in an *hfr1hy5* double mutant consistent with the enhanced de-etiolation phenotype of such a double mutant in FR light conditions (Figure 4) (Kim et al., 2002). These data are consistent with the importance of nuclear phyA events in the promotion of phototropism.

In blue light FHY1 and FHL are required for nuclear import of phyA but phyA nuclear signaling still works partially in *fhy1fhl*.

Our time course experiment showed that nuclear phyA was more efficient than cytosolic phyA to promote phototropism. However our results also confirmed a previous study that demonstrated that phototropism is more effective in *fhy1fhl* than in *phyA* (Rosler et al., 2007). A possible explanation for this result is that phyA may still enter the nucleus in *fhy1fhl* when seedlings are exposed to blue light. We thus compared the subcellular localization of phyA-YFP in *FHY1FHL* and *fhy1fhl* mutant backgrounds both in FR light where FHY1 and FHL are known to be required for phyA responses and nuclear import and also in blue light (Figure 5 and S4) (Hiltbrunner et al., 2006; Rosler et al., 2007). Our results confirmed previous observations as rapid nuclear import of phyA-YFP depended on FHY1 and FHL in FR light (Figure S4). Similarly in response to blue light nuclear accumulation of phyA was not observed in *fhy1fhl* indicating that phyA nuclear import depends on FHY1 and FHL both in FR and B light (Figure 5 and S4). The reduced

overall signal observed after several hours in blue light correlates with the light-regulated degradation of phyA that occurs in response to light (Figure 5 and S4). As expected the decrease in signal was more rapid in blue than FR light given that blue light leads to a greater Pfr/Ptot ratio (active phytochrome/total phytochrome), however phyA-YFP was still clearly visible in the nucleus after 4 hours of blue light (compare figures 5 and S4).

These microscopic observations cannot exclude the possibility that a small fraction of phyA still enters the nucleus in *fhy1fhl*. We thus decided to test a rapid nuclear phyA response in seedlings exposed either to FR or B light in order to determine whether phyA-dependent nuclear responses still operate in *fhy1fhl*. Etiolated wild type, *phyA*, *fhy1fhl* and phyA-NLS-GFP seedlings were thus either kept in darkness or exposed to 1 hour FR or low blue light and gene expression was analyzed by RT-Q-PCR in all experimental conditions. We chose these two light conditions because FR light-regulated gene expression exclusively depends on phyA and in blue light phyA plays an important role but *fhy1fhl* has a clearly distinct phenotype than *phyA* (Lariguet et al., 2006; Rosler et al., 2007; Peschke and Kretsch, 2011) (Figures 2 and 3). We analyzed the expression of *RPT2* and *PKS1*, which are known components of phototropism signaling and are early light-induced genes (Lariguet et al., 2006; Tsuchida-Mayama et al., 2010). Both genes did not respond to FR light in *phyA*, while the response was very similar to the wild type in phyA-NLS-GFP and very similar to *phyA* in *fhy1fhl* (Figure 6A and B). In response to low blue light the expression of those two genes very strongly depended on phyA (Figure 6A and B). Interestingly and in contrast to the situation in FR light *fhy1fhl* showed a very distinct gene expression phenotype from *phyA* in blue light. Indeed both *PKS1* and *RPT2*

showed a significantly more robust induction in *fhy1fhl* than in *phyA* (Figure 6A and B). Importantly the blue light-regulated gene expression in *fhy1fhl* was dependent on phyA as gene expression in *phyA**fhy1fhl* and *phyA* were not significantly different (Figure 6C and D). Consistent with this gene expression data phototropic bending in *phyA**fhy1fhl* was not more impaired than in *phyA* (Figure 2). This data indicates that in blue light FHY1 and FHL largely control phototropic bending and gene expression through phyA and that early nuclear signaling events still take place in *fhy1fhl* grown in blue light.

Cryptochromes 1 and 2 are the major photoreceptors mediating de-etiolation in response to blue light (Kami et al., 2010). We tested their implication in low blue light induced gene expression and phototropism by analyzing *cry1cry2*. The expression of *PKS1* and *RPT2* were only marginally affected in the cryptochrome mutant (Figure 6C and D). Moreover a kinetic analysis of phototropism showed that under these conditions *fhy1fhl* was more impaired than *cry1cry2* (Figure S5). To test whether FHY1 and FHL act in a different pathway than the cryptochromes we analyzed phototropism and gene expression in the *cry1cry2**fhy1fhl*. Interestingly these experiment showed that the phenotype of *cry1cry2* was strongly enhanced in *fhy1fhl**cry1cry2* thus strongly suggesting that FHY1 and FHL do not act in cryptochrome signaling in blue light (Figure 6 and S5). Moreover gene expression in *fhy1fhl**cry1cry2* was more strongly impaired than in *fhy1fhl* indicating that the blue-light regulated gene expression in *fhy1fhl* may also partially depend on the cryptochromes (Figure 6C and D).

Discussion

Nuclear phyA accelerates phototropism

By comparing phototropism in the wild type, *fhy1fhl*, *phyA* and phyA-NLS-GFP seedlings we could show that nuclear phyA accelerates phototropism while in *fhy1fhl* re-orientation towards blue light occurred more slowly than in the wild type (Figures 2 and 3). The fact that the transcription factors HY5 and HFR1 are needed for a normal phototropic response is also consistent with the importance of nuclear signaling events in the promotion of phototropism (Figure 4). Although the phototropic phenotype of *hy5* and *hfr1* could result from altered gene expression in the etiolated mutants affecting cytosolic events during phototropism, collectively our data show the importance of nuclear phyA in the promotion of phototropism (Figures 1-4). Under the light conditions we used to trigger phototropism, phyA plays the primary role in inducing the expression of *PKSI* and *RPT2* and light regulation of those genes was similar to the wild type in phyA-NLS-GFP but not in *fhy1fhl* (Figure 6). Interestingly this correlates with the prevalent function of phyA rather than the cryptochromes in the promotion of low blue light-mediated phototropism (Lariguet and Fankhauser, 2004; Tsuchida-Mayama et al., 2010) (Figure S5). Considering that over-expression of *RPT2* can complement the phototropic phenotype of *phyAcry1cry2* our results strongly support the notion that nuclear phyA promotes phototropism by regulating gene expression (Figures 2, 3 and 6) (Tsuchida-Mayama et al., 2010). This is also consistent with the previously shown function of phyA in the promotion of *PKSI* expression (Lariguet et al., 2006).

Phytochrome-mediated promotion of phototropism is traditionally demonstrated by pretreating etiolated seedlings with a red light pulse, returning seedlings into darkness for 1-2 hours prior to applying unilateral blue light (Han et al., 2008). Such a treatment will lead to nuclear import of phyA which is consistent with our findings that nuclear phyA efficiently promotes phototropism (Kircher et al., 1999; Fankhauser and Chen, 2008) (Figures 1-3). Moreover our phyA-NLS and phyA-NLS-GFP lines showed a faster phototropic response than the wild type and a red light treatment accelerated phototropism significantly in the wild type while the red light promotion effect was not as strong in phyA-NLS-GFP (Figure 2B, 3 and S3). However even in phyA-NLS-GFP plants a red light pretreatment further accelerated phototropism which is consistent with the finding that translocating phyA into the nucleus is not sufficient to induce phyA responses because in such lines light activation of the photoreceptor is still required (Genoud et al., 2008; Toledo-Ortiz et al., 2010). Collectively our data indicate that phyA promotes phototropism by controlling nuclear gene expression.

Our data also confirm that phototropism in *phy1fhl* is more effective than in *phyA* (Figures 1 and 2) (Rosler et al., 2007). This suggests that phyA may also promote phototropism in the cytosol. Indeed cytosolic functions of phyA have previously been reported (Rosler et al., 2010). In particular it has been shown that phyA has an effect on the light-regulated localization of phot1. This effect might be due to cytosolic phyA, however the fact that a dark period is required between the red light treatment and an efficient effect on phototropism is also compatible with phyA leading to changes in gene expression leading to changes in phot1 localization (Han et al., 2008). In summary although our studies

demonstrate a more potent phototropism promotion effect of nuclear than cytosolic phyA, our studies do not rule out cytosolic effects of phyA on the regulation of hypocotyl phototropism.

Nuclear signaling is still present in *fhy1fhl* particularly in blue light

The analysis of rapid light-regulated gene expression in response to FR light shows that *fhy1fhl* and *phyA* have a very similar phenotype (Figure 6). Consistent with previous reports these mutants are essentially blind to a one-hour FR treatment (Figure 6) (Tepperman et al., 2001; Peschke and Kretsch, 2011). The general tendency is that in *fhy1fhl* there is slightly more expression of light-induced genes than in *phyA* but for most genes this is not statistically significant (Figure 6) (data not shown). This gene expression phenotype of *fhy1fhl* correlates well with the morphological phenotype of *fhy1fhl*, which is indistinguishable from *phyA* when grown in FR light (Zhou et al., 2005; Hiltbrunner et al., 2006; Rosler et al., 2007).

In contrast, light-regulated gene expression in response to low blue light is still partially functional in *fhy1fhl* (Figure 6). Importantly the gene expression phenotype in *phyA* was not further enhanced in *phyA**fhy1fhl* indicating that in blue light as well FHY1 and FHL act in the phyA pathway (Figure 6). This is also consistent with the phototropism phenotype of the *phyA**fhy1fhl* that is not more severe than the *phyA* phenotype (Figure 2). Importantly, the analysis of phyA-YFP subcellular localization in *fhy1fhl* reveals no difference in the pattern of phyA-YFP subcellular localization in FR or blue light

(Figures 5 and S4), showing that in both light conditions FHY1 and FHL are essential for robust nuclear import of phyA.

Under low blue light conditions phyA plays a more prominent role in the regulation of gene expression than the cryptochromes (Figures 6). Importantly in the *cry1cry2fhy1fhl* quadruple mutant blue-light regulation of *PKS1* and *RPT2* was attenuated compared to *fhy1fhl*, (Figure 6) indicating that the residual light-regulated gene expression in *fhy1fhl* depends at least in part on the cryptochromes. Our data thus suggest that in low blue light residual nuclear phyA signaling in *fhy1fhl* contributes to light-regulated gene expression. The remaining blue-light regulated gene expression in *fhy1fhl* may thus either be due to a small amount of phyA still entering the nucleus in this mutant or cytosolic phyA initiating a signaling cascade in the cytosol that results in regulated gene expression (Neuhaus et al., 1993). An interesting question that our study helps addressing is why is *fhy1fhl* more similar to *phyA* in far-red than in blue light (Rosler et al., 2007) (Figures 2, 3 and 6). Light-regulated gene expression in etiolated seedlings transferred into FR light suggests that the residual phyA activity in *fhy1fhl* is insufficient to lead significant changes in gene expression when the light response is exclusively controlled by phyA (Figure 6). In contrast in blue light the low levels of phyA signaling still present in *fhy1fhl* are revealed because the combined action of multiple photoreceptors - i.e. phyA, cry1 and cry2 - mediates changes in gene expression (Figure 6) (Sellaro et al., 2009).

Development of a new software for hypocotyl growth measurements.

In order to distinguish the phenotype of *fhy1fhl*, phyA-NLS-GFP and the wild type it was essential to perform time course experiments of phototropic bending (Figures 1-3, S3). In

the course of this work we developed the HypoPhen software to accelerate and standardize hypocotyl-bending measurements. In order to validate this semi-automatic measurement system we have compared the output of HypoPhen with manual measurements of the same dataset (Figure S3). Although the data is not exactly identical there is very large agreement between the two measurement methods and all our conclusions are supported by both measurement methods (Figures 2, 3 and S3). Importantly using a semi-automatic measurement system diminishes the user bias that occurs inevitably when different experimenters measure datasets. The setup used for time-lapsed imaging was inspired by previous publications (Miller et al., 2007; Wang et al., 2009; Cole et al., 2011). However we have chosen to follow a larger number of seedlings (typically 21-24 per camera) in order to increase the throughput. Although this results in reduced image resolution of the seedlings analyzed, the compromise that we selected is still sufficient to perform tasks such as measurements of phototropic bending or growth rates. Indeed this lower image resolution is compensated by more sophisticated image processing algorithms, which make the phenotyping more robust to poor image quality. For example, in contrast to HypoTrace (Wang et al., 2009) and HyDe (Cole et al., 2011) where each time point image is analyzed independently, the HypoPhen software compares successive images to assess the shape of the hypocotyl. Thus, it does not make any a priori assumption on the developmental stage of the hypocotyl and handles hypocotyls with closed cotyledons (assumed by HypoTrace), as well as open cotyledons (assumed by HyDe). It can also deal with perturbing elements such as seed caps, leaves, uneven illumination that make image processing more difficult. Furthermore, our software provides the user the possibility to manually adjust the

detection and tracing of the hypocotyls and to save them as images for later data inspection. Perhaps more importantly, in contrast to the two afore-mentioned pieces of software, HypoPhen is not a “black box”, but is totally open-source and can be adapted and redistributed by anybody with the adequate skills. This will enable researchers to adapt it to their specific needs, for example for the selection of hypocotyls or the computation of bending angles and other measures. We believe that this software will be useful to the community as it become clear that real-time measurements are important to unravel the rapid effects of light on growth responses (Figures 1-3) (Cole et al., 2011).

Methods

Plant materials and growth conditions

The following genotypes of *Arabidopsis thaliana* were used: WT (Columbia-0), *phyA-211* (Reed et al., 1994), *fhy1-3fhl-1* (Rosler et al., 2007), *PHYApro:PHYA-GFP phyA-211*, *PHYApro:PHYA-NLS phyA-211*, *PHYApro:PHYA-NLS-GFP phyA-211* (Genoud et al., 2008), *cry1cry2*, *phyAcry1cry2* (Duek and Fankhauser, 2003). The *fhy1-3 fhl-1 cry1-304 cry2-1* quadruple mutant was obtained by crossing the *fhy1-3 fhl-1* (Rosler et al., 2007) and *cry1-304 cry2-1* double mutants; *hy5-215* (Oyama et al., 1997), a T-DNA insertion line disrupting the *HFR1* open reading frame (SALK_037727) was used as *hfr1* allele, the *hfr1hy5* mutant was obtained by crossing. Transgenic lines expressing *PHYApro:PHYA-YFP* in *phyA-211* and *fhy1-3 fhl-1* background were generated by *Agrobacterium* mediated transformation of *phyA-211* and *fhy1-3 fhl-1* plants. The T-DNA vector containing the *PHYApro:PHYA-YFP* construct (pPPO30A-phyA; contains a selection marker conferring resistance to Butafenacil) and the selection of transgenic plants using the herbicide Butafenacil/Inspire has been described (Rausenberger et al., 2011).

Surface sterilized seeds were plated on half strength MS plates with 0.8% agar, kept at 4°C in the dark for 3 days. Plates were transferred to 21 ± 1°C and exposed to 100 μmoles m⁻² s⁻¹ cool white light for 6 hours to induce germination and incubated (vertically) in the dark at 21°C ± 1°C until hypocotyls had the appropriate hypocotyl

length (SANYO incubator, Osaka, Japan). The blue light source was a light emitting diode (blue-LED, λ_{\max} 470nm; CLF PlantClimatics GmbH, Emersacker, Germany).

Microscopy.

To examine the subcellular relocalization of phyA-GFP, phyA-NLS-GFP, phyA-YFP, 3-day-old dark-grown seedlings or light treated as described in the figure legends were placed on slides in a drop of half strength MS medium with 0.01% agar. For confocal microscopy, Arabidopsis seedlings were observed with an inverted Zeiss confocal microscope (LSM 510 Meta INVERTED, Zeiss AXIO Vert 200 M ; x40 objective). Images were processed with Zeiss software (LSM Software Rel. 3.5).

Physiological analysis for phototropism:

For long-term phototropism experiment, following induction of germination seeds were incubated at $21 \pm 1^\circ\text{C}$ with unilateral blue light for 3 days. For short-term phototropism experiment, seedlings were grown in darkness typically for 54-60 h prior to irradiation with unilateral blue light (0.1, 1 or 10 $\mu\text{moles m}^{-2} \text{s}^{-1}$). The angles relative to vertical of seedlings with a hypocotyl length of 4 to 5.9 mm at the time of the beginning of the light treatment was determined after 24 hours irradiation. For time-lapse monitoring of hypocotyl orientation, we also used etiolated seedlings with a hypocotyl length of 4 to 5.9 mm at the time of the beginning of the light treatment. Given that the speed of phototropic bending depends on the position of the cotyledons relative to the light source (Figure S2) we always used the same number of seedlings with each orientation for a

measurement. Time-lapse images were acquired by using a monochrome CCD camera (CV-M50IR; JAI, Kanagawa, Japan), and infrared light-emitting diodes (FQ15603; peak emission at 940 nm, half-bandwidth 50 nm; Adlos AG, Vaduz, Liechtenstein) placed in an incubator (floraLED^S; CLF PlantClimatics GmbH, Emersacker, Germany). The MetaMorph software (Molecular Devices, CA, USA) was used to control the CCD camera system and to process images. Hypocotyl length and angles were measured by using stacked images (using National Institutes of Health ImageJ software version 1.38 [<http://rsb.info.nih.gov/ij/>]) as described by (Folta et al., 2003).

Automatic measurement system

The HypoPhen software was developed for the semi-automatic measurement of hypocotyl bending. It takes as input time-lapsed images of growing hypocotyls and outputs bending angles. Using the first image, the user marks with the computer mouse the apical hook of the hypocotyls that need to be measured and sets a value for the image thresholding used for background removal. For the remaining images, optical flow computations are then used to track the apical hook and a snake image processing algorithm tracks the hypocotyls. Bending values are then computed as the direction of the upper tip of the hypocotyl. The software is open-source and based on the OpenCV library (Bradski). It is documented and freely available at [HYPERLINK "http://www.unil.ch/cbg/index.php?title=HypoPhen"](http://www.unil.ch/cbg/index.php?title=HypoPhen) <http://www.unil.ch/cbg/index.php?title=HypoPhen>. Batches of infrared images displaying up to 21 hypocotyls were taken at 30 minutes intervals up to 24 hours after continuous lateral blue light irradiation and analyzed with HypoPhen.

To reduce the measurement noise, outliers were then removed and the data of each hypocotyl was smoothed while ensuring a monotonous bending function (Silverman, 2002). This resulted in a final data set containing, for each genotype, at least 60 hypocotyls and the same number of left and right- positioned cotyledons. Differences in kinetics between two genotypes were assessed using a t-test at each time points. The significance level was set to 5%, corrected for multiple testing by the method described in (Gao et al., 2008).

RNA extraction and quantitative RT-PCR

RNA isolation and RT-PCR was performed as previously described (Lorrain et al., 2009). After inducing germination seedlings were grown for 3 days in the dark at 22°C and either kept in darkness for 1 hour, transferred to FR light ($5 \mu\text{mol m}^{-2} \text{sec}^{-1}$) or blue light ($0.5 \mu\text{mol m}^{-2} \text{sec}^{-1}$) for 1 hour. First-strand cDNA synthesis was performed with 1 μg of RNA. 1 μl of 20-fold diluted cDNA was used for quantitative RT-PCR.

UBC (*At5g25760*) and *HMG1* (*AT1g76490*) were used as housekeeping genes for normalization of the experiments. The primers that were not previously described in (Lorrain et al., 2009) are described below.

HMG1

HMG1_F: AAC TTT GAT ACT TTG GCA GTA GTC TTC A

HMG1_R: CGC GAT TGT GCA TTT AAC ACT T

RPT2

RPT2_F: TGC AAG AAC CGG TCA ATG

RPT2_R: TCT TGT CAC GTC GCT ATC

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Author contribution

C.K.: designed the research; performed research; analyzed data, contributed to paper writing.

M.H.: contributed new analytic/computational tool, designed the research; performed research; analyzed data.

M.T.: performed research; analyzed data.

T.G.: designed the research; performed research; analyzed data.

A.H.: contributed new tools.

S.B.: analyzed data.

C.F.: designed the research; analyzed data; wrote the paper.

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Figure legends

Figure 1. Phototropic response in WT, *phyA*, *fhy1fhl* and *phyA-NLS-GFP*.

(A) Long-term phototropism experiment. Seedlings were grown for 3 days in unilateral blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$). Final growth direction relative to vertical was measured (0° represents vertical growth). Data are average angles relative to vertical $\pm 2 \times \text{SE}$ ($n > 120$).

(B) Short-term phototropism experiment. Hypocotyl curvatures of WT, *phyA*, *fhy1fhl* and *phyA-NLS-GFP*. Three-day-old etiolated seedlings were exposed to blue light (0.1 , 1 or $10 \mu \text{mol m}^{-2} \text{s}^{-1}$) for 24 hours. Data are average angles relative to vertical $\pm 2 \times \text{SE}$ of hypocotyl ($n > 180$).

Asterisks indicate the P-value for statistical difference with the WT in each condition with *: $P < 0.1$, **: $P < 0.05$, ***: $P < 0.01$ and n.s. not significant.

Figure 2. Kinetics of the phototropic response in WT, *phyA*, *fhy1fhl*, *phyA fhy1fhl* and *phyA-NLS-GFP*.

(A) Phototropism kinetics from time-laps images of seedlings grown under unidirectional blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$).

(B) Phototropism kinetics from time-laps images under blue light with a red light pre-treatment. For the red light pre-treatment, etiolated seedlings were exposed with R light ($1 \mu \text{mol m}^{-2} \text{s}^{-1}$, 10 seconds) and incubated 1 hour in darkness before exposing them to unilateral blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$).

Data shows average hypocotyl angles (n= 40, 20 with cotyledons facing blue light and 20 with cotyledons in the opposite direction, see methods) +/- 2 X SE.

Figure 3. phyA-NLS seedlings have a faster phototropic response than the WT and phyA-GFP seedlings

(A) Phototropism kinetics of seedlings grown as in Figures 2A (black; WT, green; phyA-GFP, red; phyA-NLS) analyzed by using semi-automatic measurements (HypoPhen). The data are average values of both cotyledon position +/- 2 X SE, n > 60 with the same number of cotyledons for each position.

(B) Analysis of a difference in phototropism kinetics for the data shown in (A). Data are represented as pairwise comparisons between WT vs phyA-GFP (a), WT vs phyA-NLS (b) and phyA-GFP vs phyA-NLS (c) for each time point. The p-values resulting from a t-test are represented. The red line represents the 5% threshold corrected for multiple testing.

Figure 4. Nuclear components of phyA signaling are required for a fast phototropic response.

Phototropism kinetics of seedlings grown under unilateral blue light ($0.1 \mu \text{ mol m}^{-2} \text{ s}^{-1}$). Data show average hypocotyl angles (n=40, 20 with cotyledons facing blue light and 20 with cotyledons in the opposite direction, except for *hfr1hy5* where n=34 with 17 seedlings in each orientation see methods) +/- 2 X SE.

Figure 5. phyA-YFP nuclear import depends on FHY1 and FHL in blue light

Three-day-old dark-grown *FHY1FHL* or *fhy1fhl* seedlings transformed with PHYA-YFP were analyzed by confocal laser scanning microscopy. The seedlings were analyzed directly (dark), and after 2 or 4 hours irradiation with blue light ($0.1 \mu \text{ mol m}^{-2} \text{ s}^{-1}$). D; Dark, B2 and B4; 2 or 4 hours blue light treatment. Bar, 50 μm .

Figure 6. A blue light treatment leads to significant induction of *PKSI* and *RPT2* expression in *fhy1fhl*.

(A) Expression of *PKSI* was measured by RT-qPCR in WT, *phyA*, *fhy1fhl* and *phyA-NLS-GFP*. Three-day-old etiolated seedlings were either kept in the dark or exposed for 1 hour to far-red light (FR; $5 \mu \text{ mol m}^{-2} \text{ s}^{-1}$) or exposed for 1 hour to blue light (B; $0.5 \mu \text{ mol m}^{-2} \text{ s}^{-1}$). Data are average expression of *PKSI* normalized to two control genes and expressed relative to the wild type in the dark $\pm 2 \times \text{SE}$. Average from three biological replicas with three technical replicates for each are shown.

(B) Expression of *RPT2* was performed as in panel (A).

(C) Expression of *PKSI* was performed as in panel (A) but in WT, *phyA*, *fhy1fhl*, *phyA-fhy1fhl*, *cry1cry2* and *cry1cry2fhy1fhl*.

(D) Expression of *RPT2* was performed as in panel (C).

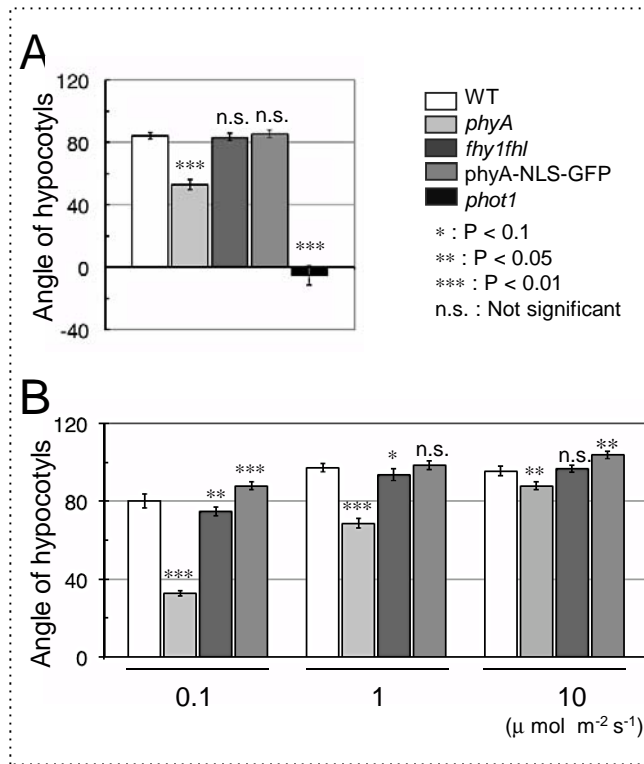


Figure 1. Phototropic response in WT, *phyA*, *fhy1fhl* and *phyA-NLS-GFP*.

(A) Long-term phototropism experiment. Seedlings were grown for 3 days in unilateral blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$). Final growth direction relative to vertical was measured (0° represents vertical growth). Data are average angles relative to vertical $\pm 2 \times \text{SE}$ ($n > 120$).

(B) Short-term phototropism experiment. Hypocotyl curvatures of WT, *phyA*, *fhy1fhl* and *phyA-NLS-GFP*. Three-day-old etiolated seedlings were exposed to blue light (0.1 , 1 or $10 \mu \text{mol m}^{-2} \text{s}^{-1}$) for 24 hours. Data are average angles relative to vertical $\pm 2 \times \text{SE}$ of hypocotyl ($n > 180$).

Asterisks indicate the P-value for statistical difference with the WT in each condition with *: $P < 0.1$, ** $P < 0.05$, ***: $P < 0.01$ and n.s. not significant.

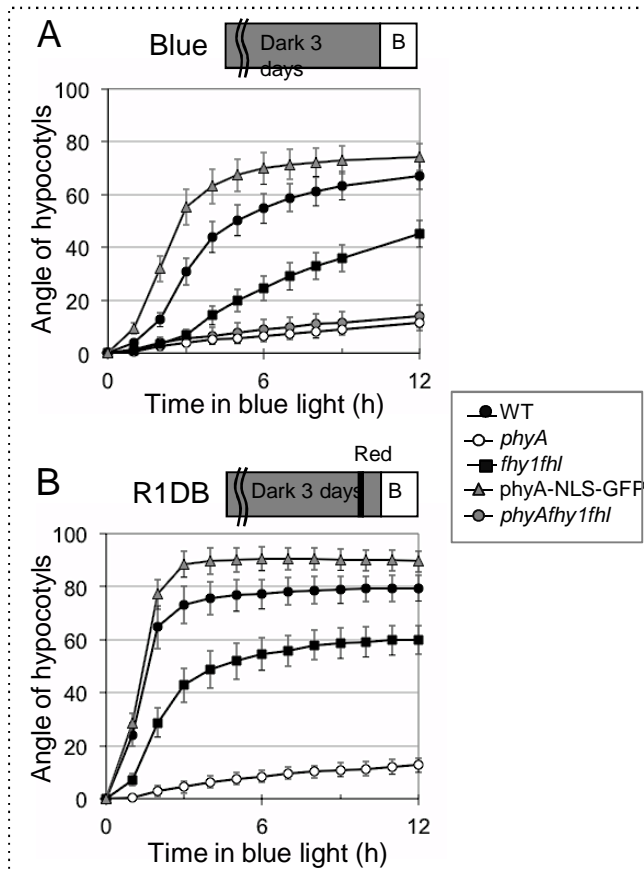


Figure 2. Kinetics of the phototropic response in WT, *phyA*, *fhy1fhl* *phyA fhy1fhl* and *phyA-NLS-GFP*.

(A) Phototropism kinetics from time-laps images of seedlings grown under unidirectional blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$).

(B) Phototropism kinetics from time-laps images under blue light with a red light pre-treatment. For the red light pre-treatment, dark grown seedlings were exposed with R light ($1 \mu \text{mol m}^{-2} \text{s}^{-1}$, 10 seconds) and incubated 1 hour in darkness before exposing them to unilateral blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$).

Data shows average hypocotyl angles ($n=40$, 20 with cotyledons facing blue light and 20 with cotyledons in the opposite direction, see methods) $\pm 2 \text{ X SE}$.

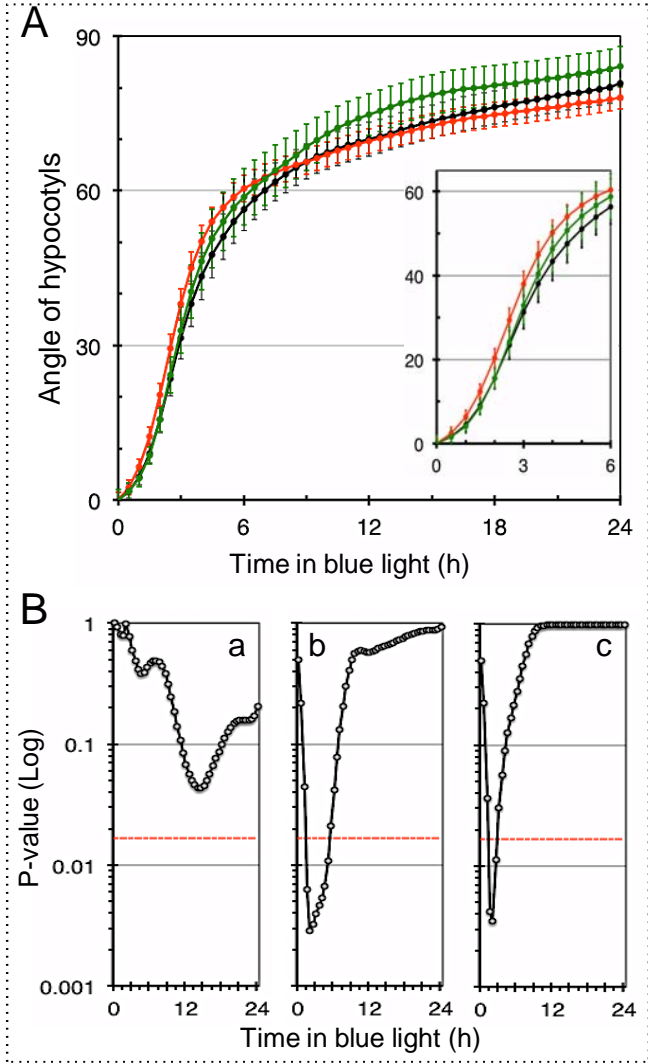


Figure 3. phyA-NLS seedlings have a faster phototropic response than the WT and phyA-GFP seedlings

(A) Phototropism kinetics of seedlings grown as in Figures 2A (black; WT, green; phyA-GFP, red; phyA-NLS) analyzed by using semi-automatic measurements (HypoPhen). The data are average values of both cotyledon position $\pm 2 \times$ SE, $n > 60$ with the same number of cotyledons for each position. (B) Analysis of a difference in phototropism kinetics for the data shown in (A). Data are represented as pairwise comparisons between WT vs phyA-GFP (a), WT vs phyA-NLS (b) and phyA-GFP vs phyA-NLS (c) for each time point. The p-values resulting from a t-test are represented. The red line represents the 5% threshold corrected for multiple testing.

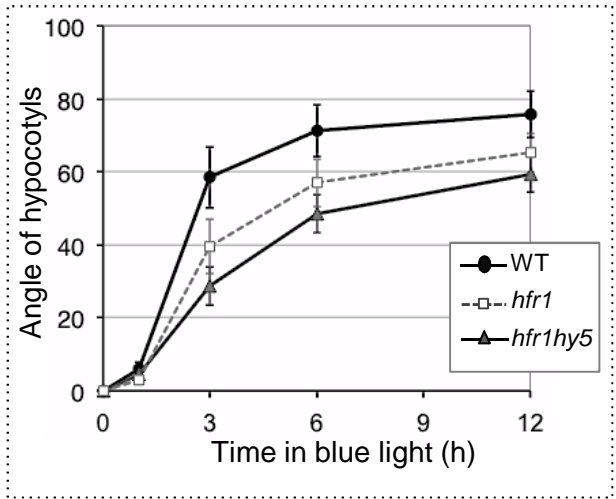


Figure 4. Nuclear components of phyA signaling are required for a fast phototropic response.
Phototropism kinetics of seedlings grown under unilateral blue light ($0.1 \mu \text{ mol m}^{-2} \text{ s}^{-1}$). Data show average hypocotyl angles ($n=40$, 20 with cotyledons facing blue light and 20 with cotyledons in the opposite direction, except for *hfr1hy5* where $n=34$ with 17 seedlings in each orientation see methods) $\pm 2 \times \text{SE}$.

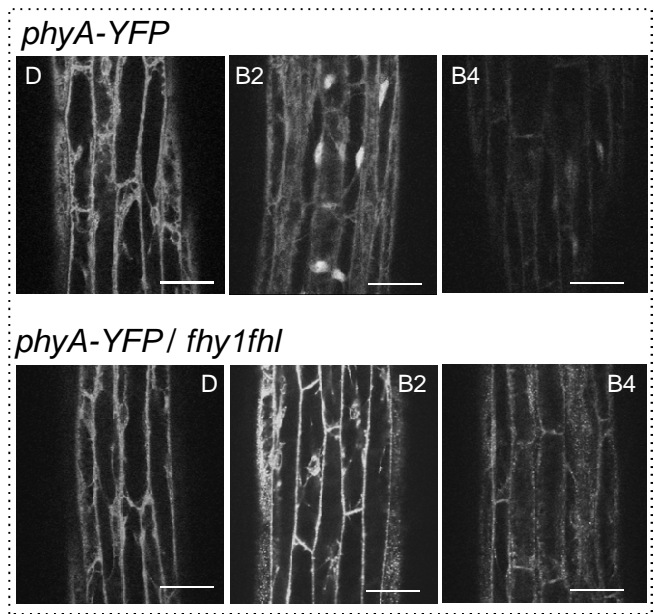


Figure 5. *phyA*-YFP nuclear import depends on FHY1 and FHL in blue light

Three-day-old dark-grown *FHY1FHL* or *fhy1fhl* seedlings transformed with PHYA-YFP were analyzed by confocal laser scanning microscopy. The seedlings were analyzed directly (dark), and after 2 or 4 hours irradiation with blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$). D; Dark, B2 and B4; 2 or 4 hours blue light treatment. Bar, 50 μm .

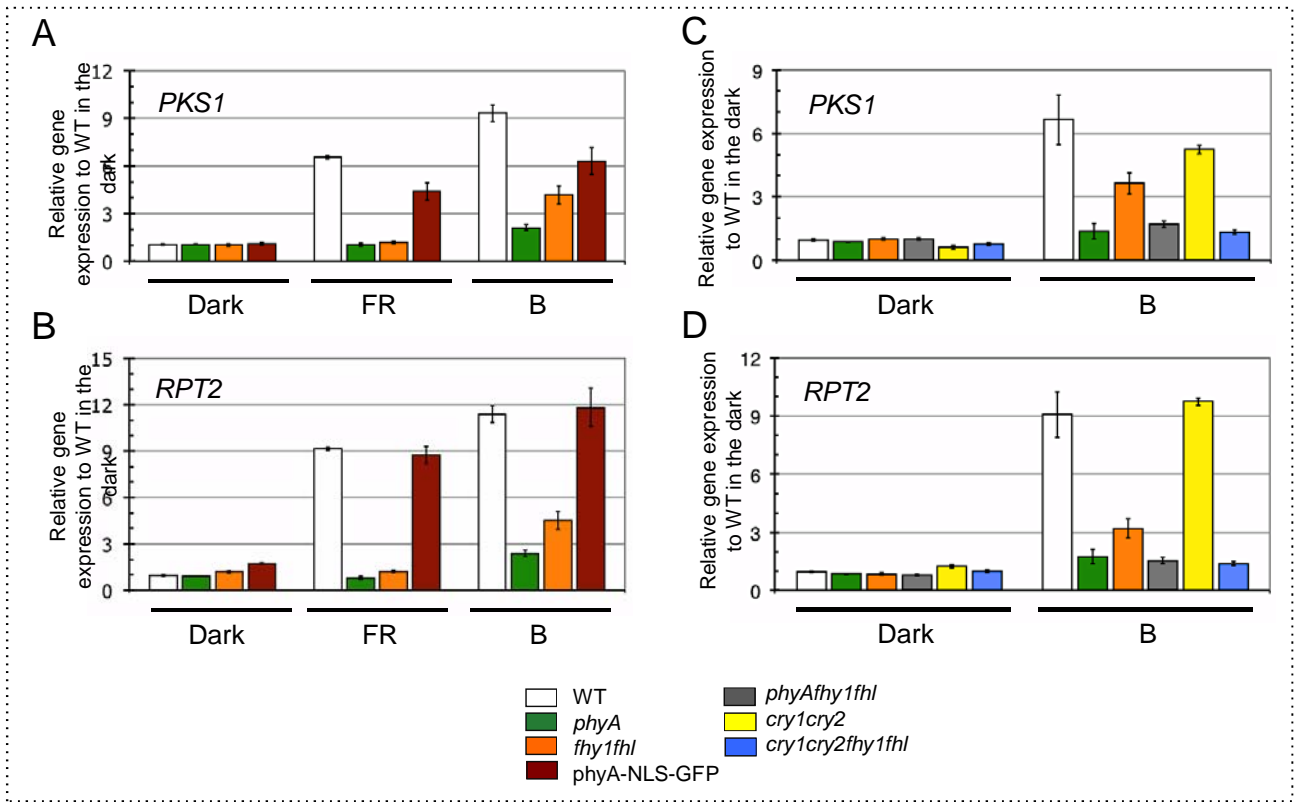


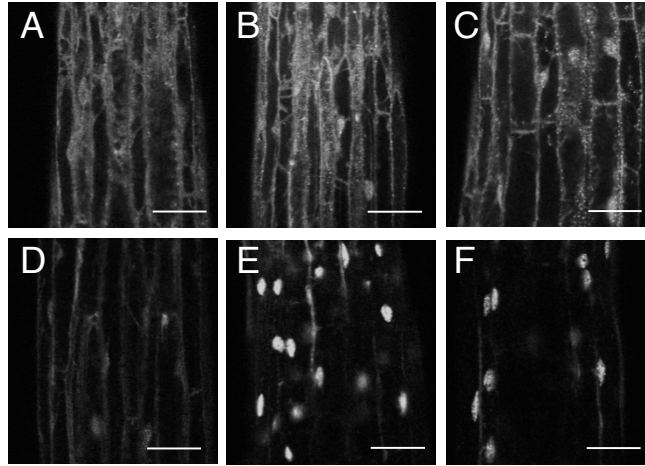
Figure 6. A blue light treatment leads to significant induction of *PKS1* and *RPT2* expression in *fhy1fhl*.

(A) Expression of *PKS1* was measured by RT-qPCR in WT, *phyA*, *fhy1fhl* and *phyA-NLS-GFP*. Three-day-old dark grown seedlings were either kept in the dark or exposed for 1 hour to far-red light (FR; 5 m mol m⁻² s⁻¹) or exposed for 1 hour to blue light (B; 0.5 μ mol m⁻² s⁻¹). Data are average expression of *PKS1* normalized to two control genes and expressed relative to the wild type in the dark +/- 2 x SE. Average from three biological replicas with three technical replicates for each are shown.

(B) Expression of *RPT2* was performed as in panel (A).

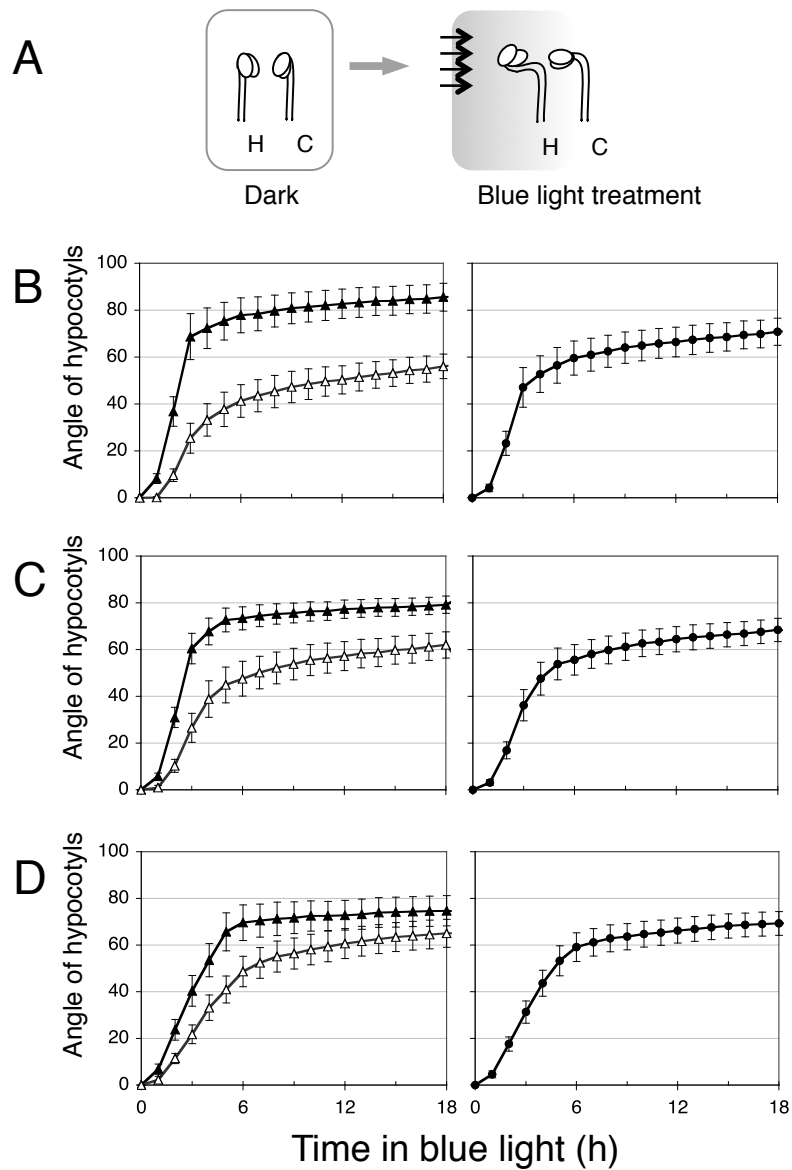
(C) Expression of *PKS1* was performed as in panel (A) but in WT, *phyA*, *fhy1fhl*, *phyAphy1fhl*, *cry1cry2* and *cry1cry2fhy1fhl*.

(D) Expression of *RPT2* was performed as in panel (C).



S 1. Subcellular localization of a constitutively localized phyA

Subcellular localization of phyA-GFP and phyA-NLS-GFP. Dark grown *phyA* seedlings (4 – 5.9 mm length) transformed with PHYA-GFP or PHYA-NLS-GFP were analyzed by confocal laser scanning microscope. The seedlings were analyzed directly (dark) or after 1-4 hours irradiation with blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$). A; phyA-GFP seedling in the darkness, B; phyA-GFP seedling after 1 hour blue light irradiation, C; phyA-GFP seedling after 2 hours blue light irradiation, D; phyA-GFP seedling after 4 hours blue light irradiation, E; phyA-NLS-GFP seedling in the darkness, F; phyA-NLS-GFP seedling after 4 hours blue light irradiation. Bar, 50 μm .



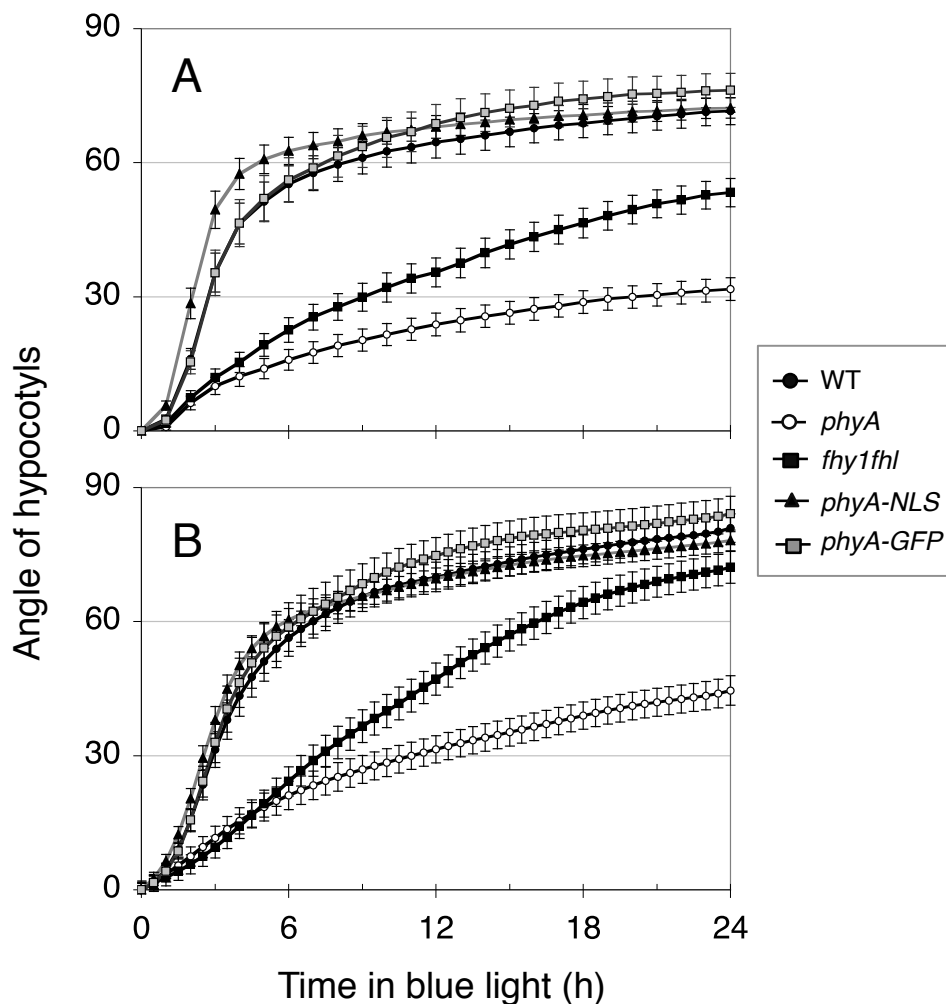
S 2. Phototropism kinetics depends on the length of the hypocotyl and the orientation of the cotyledons

Dark grown seedlings were exposed unilateral blue light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 24 hours.
 A: Schematic representation of the two positions of the cotyledons (C: cotyledon facing blue light, H: cotyledon in the opposite direction) relative to the incoming light.

B : Kinetic analysis of phototropism in seedling with a hypocotyl length of 1 - 3.9 mm. The left panel shows the kinetics for position H (full triangle) and C (open triangle) separately. The right panel shows the average data of seedlings with both positions. Each data are average with $\pm 2 \times \text{SE}$ of hypocotyl angles ($n=50$, 25 hypocotyls of C and 25 of hypocotyls H).

C: As in B but with hypocotyl length between 4 - 5.9 mm.

D: As in B but with hypocotyl length between 6 - 8.9mm.



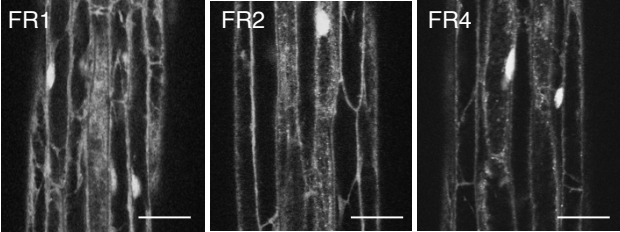
S 3. Comparison of phototropism kinetics by using manual measurement and semi-automatic measurement with same time-laps images

Dark grown seedlings (4 – 5.9 mm length) were exposed unilateral blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$) for 24 hours. The kinetics shows the total average of both cotyledon position with $\pm 2 \times \text{SE}$. All of data were collected more 30 each cotyledon position (WT: 46C +46H, *phyA*: 37C+37H, *fhy1fhl*: 34C+34H, *phyA-NLS*: 61C+ 61H, *phyA-GFP*: 33C +33H).

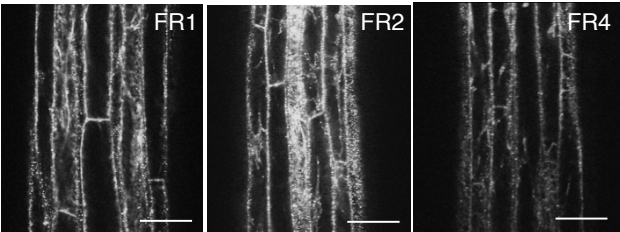
A: Phototropism kinetics by using Manual measurement with ImageJ

B: Phototropism kinetics by using Semi-automatic measurement (HypoPhen). Data for the WT, *phyA-NLS* and *phyA-GFP* are the same as those presented in figure 3.

phyA-YFP

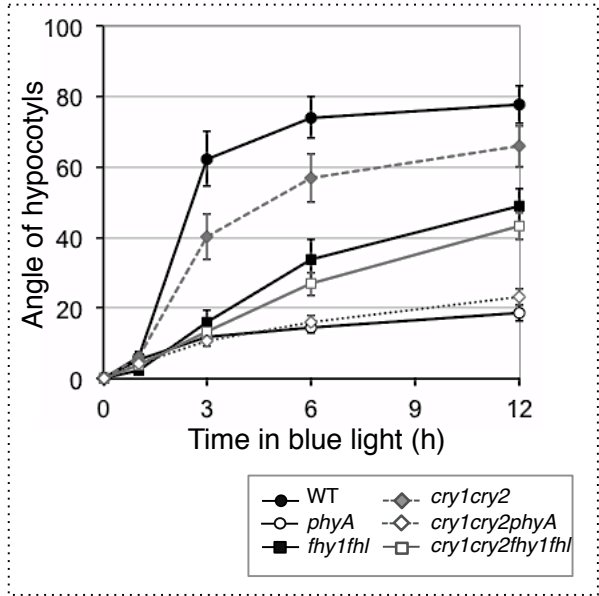


phyA-YFP / fhy1fhl



S 4. phyA localization (phyA-YFP) in WT or *fhy1fhl* under far-red light

Dark grown (4 – 5.9 mm length) PHYA-YFP in *phyA* and *fhy1fhl* seedlings were analyzed by confocal laser scanning microscopy. The seedlings were analyzed directly (dark) after 1, 2 or 4 hours irradiation with far-red light ($5 \mu \text{ mol m}^{-2} \text{ s}^{-1}$). FR1, FR2 and FR4; 1, 2 or 4 hours far-red light treatment. Bar, 50 μm .



S 5. Comparison of phototropism kinetics in WT, *phyA*, *fhy1fhl*, *cry1cry2*, *cry1cry2phyA* and *cry1cry2fhy1fhl* under low blue light

Dark grown seedling (4 – 5.9 mm length) were exposed unilateral blue light ($0.1 \mu \text{mol m}^{-2} \text{s}^{-1}$) for 12 hours. Each data show total average of both cotyledon position ($n=20C+20H$) and $\pm 2 \times \text{SE}$ of hypocotyl angles.