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Characterization of photomorphogenic responses and signaling cascades controlled by phytochrome-A expressed in different tissues

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Supporting Information
SUMMARY

(1) The photoreceptor phytochrome A acts as a light-dependent molecular switch and regulates responses initiated by very low fluences of light (VLFR) and high fluences (HIR) of far-red light. PhyA is expressed ubiquitously, but how phyA signaling is orchestrated to regulate photomorphogenesis is poorly understood.

(2) To address this issue, we generated transgenic *Arabidopsis thaliana* phyA-201 mutant lines expressing the biologically active PHYA-YFP photoreceptor in different tissues, and analyzed the expression of several reporter genes, including HY5-GFP and CFP-PIF1 and various FR-HIR dependent physiological responses.

(3) We show that phyA action in one tissue is (i) critical and sufficient to regulate flowering time, and root growth; (ii) control of cotyledon and hypocotyl growth requires simultaneous phyA activity in different tissues, and (iii) changes detected in the expression of reporters are not restricted to phyA-containing cells.

(4) We conclude that FR-HIR-controlled morphogenesis in Arabidopsis is mediated partly by tissue-specific and partly by intercellular signaling initiated by phyA. Intercellular signaling is critical for many FR-HIR induced responses, yet it appears that phyA modulates the abundance and activity of key regulatory transcription factors in a tissue-autonomous fashion.
INTRODUCTION

Plants are sessile organisms, and to optimize their fitness and competitiveness they must adapt to changes in their abiotic and biotic environment. From among the numerous environmental factors light is arguably the most important one, since plants use light not only as the energy source for photosynthesis but also as a developmental cue. To harmonize their growth and development with the ambient light environment, plants have evolved a battery of highly specialized photoreceptors. These photoreceptors monitor the quality, quantity, duration and direction of the incident sunlight and include the UVB-sensing UVB-RESISTANCE 8 (Rizzini et al., 2011), the blue/UVA light absorbing cryptochromes, phototropins and ZTL-like photoreceptors (Christie, 2007; Yu et al., 2010; Chaves et al., 2011) and the red (R) and far-red (FR) light absorbing phytochromes (Franklin & Quail, 2010).

Phytochromes (phy) are chromoproteins that exist as dimers, and each monomer contains a covalently linked open tetra-pyrrol chain chromophore. In the model plant Arabidopsis thaliana the phytochromes are encoded by a small multigene family (Sharrock & Quail, 1989; Clack et al., 1994). Phytochromes cycle between their biologically inactive (Pr) and active (Pfr) forms and act as light quality/quantity dependent molecular switches. phyA is a highly specialized far-red sensor, since a very low level of phyA Pfr (~0.1 % of total phyA) generated by FR or a low-ratio R/FR light is already sufficient to launch signaling. It follows that phyA regulates the so-called very low fluence (VLFR) and high-irradiation responses to far-red light (FR-HIR), and thereby plays a dominant role in mediating transition from skotomorphogenesis to photomorphogenesis (Casal et al., 2014).

According to the generally accepted concept, the overwhelming majority of molecular events underlying phyA-controlled photomorphogenesis take place in the nucleus. Light in a quality- and quantity-dependent fashion induces translocation into and accumulation of phyA Pfr in the nuclei (Kircher et al., 1999). PhyA does not have endogenous nuclear localization signal (NLS) motifs, and import of phyA Pfr is mediated by the NLS-containing FAR-RED ELONGATED HYOCOTYL1 and FHY1-like proteins that shuttle between the nucleus and the cytoplasm (Hiltbrunner et al., 2005; Hiltbrunner et al., 2006; Rausenberger et al., 2011). PhyA Pfr localized in the nucleus interacts with a battery of negative regulatory proteins, including CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), SUPPRESSOR OF PHYA-105 1-4 (SPA1-4) and PHYTOCHROME INTERACTING FACTORS (PIFs). The
very early steps of phyA signaling result in (i) the inactivation or alteration of the substrate specificity of the COP1/SPA1-4 complex that targets proteins to degradation, (ii) disruption of the binding of PIF transcription factors (TFs) to their cognate promoters and/or initiating their degradation, and (iii) induction of transcriptional cascades that modulate the expression of 2500–3000 genes of the Arabidopsis genome in a FR light-dependent fashion (Tepperman et al., 2001). In this aspect it is worth noting that phyA is ubiquitously expressed (Somers & Quail, 1995; Hall et al., 2001), and FR light readily penetrates plant tissues. It follows that phyA signaling, at least theoretically, can be induced simultaneously in each cell. If so, then it would be essential to know to what extent phyA signaling in different cells/tissues is identical and/or different, and how these signaling cascades are interconnected with each other to regulate complex photomorphogenic responses such as hypocotyl growth inhibition or cotyledon expansion. Clearly, a prerequisite to answer these questions is to collect detailed information about the spatial/temporal features of phyA-controlled signaling cascades. The first reports addressing this problem produced data obtained by focused irradiation (spot, micro-beam etc.) targeted to specific parts/organs/tissues. For example, it was shown that phytochrome localized in leaves is essential for regulating hypocotyl elongation under shade conditions (Casal & Smith, 1988a; Casal & Smith, 1988b). Nick et al. (1993) reported that accumulation of anthocyanin and CHALCONE SYNTHASE mRNA induced by microbeam irradiation with FR light in the cotyledons of mustard seedlings is a cell-autonomous, stochastic response. However, to explain the gradually developing expression pattern at the whole organ level these authors hypothesized that the responses of individual cells are integrated by inhibitory, intercellular communication. Bischoff et al. (1997) showed that microbeam irradiation with R light induced expression of the CAB:LUC reporter at distant parts of the transgenic tobacco leaves, a finding that indicates existence of inductive cell-to-cell signaling. Jordan et al. (1995) concluded that manipulation of spatial distribution by over-expressing oat phyA in different organs in transgenic tobacco results in different phenotypes, and that phyA localized in the vascular tissue plays a significant role in regulating stem elongation by repressing gibberellic acid (GA) biosynthesis. Neuhaus et al. (1993), Bowler et al. (1994) and Kunkel et al. (1996) used a radically different approach, namely microinjection of phyA and various other putative signaling compounds into the tomato aurea mutant, which is deficient in photoactive phytochromes. These
authors demonstrated that phyA signals in a cell-autonomous fashion in a subset of hypocotyl cells, but these studies lacked analysis of complex developmental responses and were limited in time. More recently, Warnasooriya and Montgomery (2009) and Costigan et al. (2011) chose a different approach and analyzed FR-HIR induced responses in transgenic Arabidopsis plants in which accumulation of the chromophore required for the activity of all phytochromes was decreased in an organ/tissue specific fashion by expressing plastid-targeted mammalian biliverdin IX alpha reductase under the control of selected promoters. These authors concluded that phyA-controlled developmental responses, including hypocotyl growth inhibition and root elongation are mediated by long-distance, inter-organ signaling. The caveat of this approach is that it lowers rather than fully inhibits accumulation of the chromophore, and the precise amount of the active photoreceptor present in the various tissues/organs is not known.

Whilst these studies revealed important spatial/temporal features of phyA-controlled photomorphogenic responses, they provided limited molecular information about the events of phyA-controlled signaling cascades at the molecular level. phyA contains no DNA-binding motifs, but Chen et al. (2014) demonstrated by chromatin immunoprecipitation sequencing and RNA sequencing methods that phyA associates with the promoters of hundreds of not only FR light induced but also stress/hormone regulated genes. These authors postulated that by relying on this mechanism phyA has the capacity to directly regulate rapid adaptation of plants to their changing environment by controlling/integrating multiple biological processes. However, these experiments were not designed to address whether phyA binding to the promoters is different in different cell types, thus provided little if any information about the spatial aspects of phyA signaling.

To obtain more precise information about the tissue specificity of molecular events mediating phyA signaling in FR-HIR, we chose a yet different approach. Namely, we (i) generated transgenic lines expressing the phyA-YFP (YELLOW FLUORESCENT PROTEIN) fusion protein in the phyA-201 mutant under the control of its own as well as different tissue-specific promoters, (ii) characterized a broad array of FR-HIR light-induced developmental responses at the physiological level, and (iii) complemented these studies by analyzing the accumulation/degradation of specific reporter constructs in the wild type and/or in transgenic lines expressing the phyA-YFP photoreceptor in different tissues.
MATERIALS AND METHODS

Cloning, generation of transgenic plants
For details of constructing the transgenes used in this study, see Supporting Information Methods S1 and Supporting Information Table S1. Throughout the study we used *Arabidopsis thaliana* L. (Heynh.) *phyA-201* mutant (Reed et al., 1993), (Ler ecotype). The chimeric constructs were transformed into *Arabidopsis* as described by Clough & Bent, (1998). Independent homozygous lines expressing one Mendelian copy of the transgene were selected for further analysis.

Seedling and plant growth conditions
Surface sterilized seeds stratified for 72 h in the dark (4 °C), after which germination was induced by 18 h of white light (20 µmol m⁻² s⁻¹, 22 °C). The plates were subsequently treated as specified in the text. For analysis of flowering time, seeds were sown on soil, stratified for 72 h in the dark (4 °C) and subsequently treated as specified.

Microscopy techniques
Epifluorescent and light microscopy was performed as described by Bauer et al. (2004). Confocal laser scanning microscopy was performed using a Leica SP5 AOBRS confocal laser scanning microscope (Leica, Germany) on DMI6000 microscope base. Microscope configuration was the following: objective lens: HC PL APO 20x (NA:0.7); sampling speed: 100 Hz; line averaging: 3x; pinhole: 200 µm; scanning mode: sequential unidirectional; excitation: 488 nm laser (GREEN FLUORESCENT PROTEIN, GFP), 514 nm laser (YFP); spectral emission detectors: 496-518 nm (GFP), 545-582 nm (YFP). Brightness and contrast settings were uniformly done on the corresponding image pairs. GFP and YFP images were pseudo-colored green and red, respectively. All microscopic manipulations were performed under safe green light and documentation of cells was performed during the first 60 s of microscopic analysis. In each experiment at least 20 seedlings from 4 independent transgenic lines (representing >100 cells/seedling) were analyzed and statistically evaluated. Frequencies of images supporting or contrasting the conclusions drawn was >95% or 0.1%. Every experiment was repeated 3 times.
Hypocotyl length and cotyledon area measurement
After induction of germination, seeds were placed at 22 °C in darkness or in FR light (20 µmol m⁻² s⁻¹, 730 nm, 128 nm full widths at half-maximum). Measurement of hypocotyl length and cotyledon area was performed as described by Ádám et al. (2013). At least 25 seedlings were used for each line and each experiment.

Analysis of flowering time
Following stratification, seedlings were grown in short day (8 h white light; 130 µmol m⁻² s⁻¹ /16 h dark) or in short day extended by 8 h FR light (8 h white light; 130 µmol m⁻² s⁻¹/8 h far red light; 30 µmol m⁻² s⁻¹/ 8 h dark). Irradiation with FR light was performed in a FR light field (730 nm, 128 nm full width at half-maximum). After 15 days, all plants were grown in short day without FR irradiation. Flowering time of each plant was determined by counting the days until flower buds became visible in the centre of the rosette. At least 9 plants were used for each line and light condition. All experiments were repeated two times.

Analysis of phototropism
Seeds were sown on rectangular ½ MS (Murashige and Skoog medium) agar plates covered with one sheet of sterilized filter paper. After stratification, the plates were incubated vertically for 2 days in darkness (23°C). The seedlings were irradiated with far-red light (10 µmol m⁻² s⁻¹) for 120 min. Unilateral blue light irradiation (1 µmol m⁻² s⁻¹) was supplied for 160 min by a projector (Leitz, Wetzlar, Germany) equipped with a blue light filter (KG45; Optic Balzers, Liechtenstein). For homogeneous illumination of the etiolated seedlings the plates were placed with an angle of 3° to the light axis. After scanning of the plates hypocotyl bending was measured with ImageJ (Schneider et al., 2012).

Root growth measurements
Seeds were sown on rectangular ½ MS agar plates containing 1% of sucrose. The plates were incubated vertically for 10 days in far-red light (20 µmol m⁻² s⁻¹) at 22 °C. The plates were scanned and root length was measured with ImageJ.

RESULTS
Generation of transgenic *phyA-201* lines expressing phyA-YFP in tissue-specific fashion

To ensure tissue/cell type specific localization of the functional phyA-YFP photoreceptor *in planta*, we expressed the fusion protein under the control of *PHYA*, *MERISTEM LAYER 1 (ProML1)*, *SUCROSE (SUC)/H⁺ SYMPORTER 2 (ProSUC2)* and *CHLOROPHYLL A/B BINDING PROTEIN 3 (ProCAB3)* promoters in the *phyA-201* mutant. The *ProPHYA* promoter is known to be ubiquitously expressed in seedlings (Somers & Quail, 1995; Hall *et al.*, 2001), whereas the *ProCAB3*, *ProML1* and *ProSUC2* promoters had been routinely used in the past to express proteins of interest exclusively in mesophyll, epidermal or companion cells, respectively (Sessions *et al.*, 1999; Srivastava *et al.*, 2008; Hategan *et al.*, 2014). For this study we raised 15–20 independent transgenic lines for each construct, and selected those which segregated the transgenes as a single Mendelian trait. Transgenic lines homozygous for the *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and *ProCAB3:PHYA-YFP* transgenes were then further characterized by western blot, epifluorescence and confocal microscopy to determine the abundance and tissue-specificity of the respective fusion protein. We selected 4 transgenic lines for each construct, and performed all experiments by using progenies of these lines. We also crossed the selected *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and *ProCAB3:PHYA-YFP* plants and produced lines expressing the phyA-YFP in two or three tissue types. For a detailed description of the method applied to identify these multiple transgenic lines see Supporting Information Methods S1 and Fig. S1. The transgenic lines were then used to extend and to corroborate results obtained by the analysis of the parental lines. Fig. 1 shows the typical cellular distribution patterns of the phyA-YFP protein in the cotyledons and in the hook region of the hypocotyls of chosen *ProPHYA:PHYA-YFP*, *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP*, *ProCAB3:PHYA-YFP* transgenic lines, and demonstrates that, depending on the promoter used, the phyA-YFP fusion protein is detectable either in each cell type (*ProPHYA*, g-l) or only in the epidermal (*ProML1*, m-r), companion (*ProSUC2*, s-x) or mesophyll (*ProCAB3*, y-ad) cells. Western blot analysis showed that the total amount of phyA-YFP in the *ProPHYA:PHYA-YFP* lines is comparable to that of native phyA in wild type (WT) seedlings (Fig. S2a), but it is approximately 10-12 times lower in the *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and *ProCAB3:PHYA-YFP* transgenic lines (Fig. S2b). To compare the abundance of the phyA-YFP fusion
protein in different tissues we determined the amount of phyA-YFP accumulated in nuclei of epidermal and sub-epidermal cells of hypocotyls after 24 h irradiation with FR light. We found that abundance of the phyA-YFP fusion protein in the epidermal cells of ProML1:PHYA-YFP and ProPHYA:PHYA-YFP does not differ significantly, but it is much (4-5-fold) lower in the sub-epidermal cells of ProCAB3:PHYA-YFP as compared to ProPHYA:PHYA-YFP (Fig. S3). Quantitation of phyA abundance in the companion cells of the various lines was not feasible by this method; however, microscopic analysis indicates that the expression level of fusion protein is similar in the selected ProSUC2:PHYA-YFP and ProPHYA:PHYA-YFP lines (Fig. 1 l, x).

Finally, we compared the expression patterns of the photoreceptor in ProPHYA:PHYA-YFP and the triple transgenic line (obtained by consecutive crossings of the single ProML1:PHYA-YFP with ProCAB3:PHYA-YFP and ProSUC2:PHYA-YFP; ProML1+ProCAB3+ProSUC2:PHYA-YFP) by confocal microscopy. Table S2 summarizes the results of these experiments and Fig. S4-S10 illustrate that phyA-YFP is detectable in the epidermis, subepidermal and companion cells of cotyledons, hypocotyls and various tissues of the root of ProPHYA:PHYA-YFP seedlings. Expression of phyA-YFP in the ProML1+ProCAB3+ProSUC2:PHYA-YFP, triple transgenic line is detectable in the epidermis, mesophyll and companion cells of cotyledons (Fig. S4,S5), in the epidermis in the hook and both in the lower and upper part of hypocotyls (Fig. S6-S8) but its expression in the subepidermal cells of hypocotyls is restricted to the hook region (Fig.1l,x) whereas in the root we could only detect phyA-GFP in specific cell files in the epidermis (located in the division/elongation zone) (Fig. S9,S10). Taken together, we conclude that the expression pattern and the level of phyA-YFP in the ProML1+ProCAB3+ProSUC2:PHYA-YFP transgenic line mimic ProPHYA:PHYA-YFP (i) in the epidermis of cotyledon and hypocotyls and partially in root, (ii) comparable to that in the companion cells but lower in the subepidermal (mesophyll) cells of cotyledons and hook region and strongly different (iii) in the subepidermal cells (cortex) of the upper and lower part of hypocotyls and in the roots.

Epidermally-expressed phyA-YFP fully restores FR-HIR controlled root growth, but only partially complements the hypocotyl growth inhibition and cotyledon expansion phenotype of the phyA-201 mutant.
To assess the action of tissue-specifically expressed phyA-YFP we analyzed basic FR-induced photomorphogenic responses, including promotion of root growth and cotyledon expansion as well as inhibition of hypocotyl elongation in the selected transgenic lines. Fig. 2a and Fig. S11a demonstrate that ProPHYA:PHYA-YFP, ProML1:PHYA-YFP as well as the ProML1+ProCAB3+ProSUC2:PHYA-YFP transgenic lines exhibited an identical, fully complemented root phenotype. These figures also show that, in contrast to ProML1:PHYA-YFP, the root length of the ProCAB3:PHYA-YFP and ProSUC2:PHYA-YFP transgenic seedlings was not restored. These results suggest that signaling by phyA-YFP localized in the epidermis is sufficient to fully complement impaired root growth of the phyA-201 mutant, and phyA-YFP signaling originated in the mesophyll or companion cells has negligible effect on controlling this process.

Fig. 2b and Fig. S11b demonstrate that ProPHYA:PHYA-YFP in phyA-201 seedlings displayed a fully restored, even slightly exaggerated FR-induced cotyledon expansion phenotype. ProML1:PHYA-YFP seedlings exhibited a pronounced whereas ProCAB3:PHYA-YFP seedlings showed a weaker but significant response as compared to WT. In contrast, phyA in the vascular tissue lines was completely ineffective in promoting cotyledon expansion of ProSUC2:PHYA-YFP. Interestingly, ProCAB3+ProSUC2:PHYA-YFP seedlings displayed a partially whereas ProML1+ProCAB3:PHYA-YFP and the ProML1+ProCAB3+ProSUC2:PHYA-YFP transgenic seedlings produced a slightly over-expressing phenotype for FR-induced cotyledon expansion. Collectively, these data demonstrate that the simultaneous action of phyA in epidermal and mesophyll cells is critical and sufficient to promote FR-dependent cotyledon expansion.

Fig. 2c shows that inhibition of hypocotyl growth is fully restored in the ProPHYA:PHYA-YFP lines and partially in ProML1:PHYA-YFP lines as compared to WT. In contrast, phyA-YFP expressed in companion and mesophyll cells was not able to induce any detectable response. Fig. 2c and Fig. S11c illustrate that the ProML1:PHYA-YFP and ProML1+ProCAB3+ProSUC2:PHYA-YFP transgenic seedlings displayed similarly enhanced FR-induced hypocotyl growth inhibition when compared to phyA-201, but were still significantly longer when compared to WT or ProPHYA:PHYA-YFP. Taken together, we conclude that the action of phyA-YFP localized in the epidermis contributes to FR-dependent inhibition of hypocotyl
growth, but signaling by phyA localized in different cell/tissue types is also required to fully complement the phenotype of the phyA-201 mutant. To test if the apparently prominent role of epidermis-localized phyA in regulating FR-dependent hypocotyl and root elongation as well as cotyledon expansion was due to its altered stability, we determined the degradation kinetics of phyA-YFP in ProML1:PHYA-YFP and ProPHYA:PHYA-YFP transgenic lines by in vivo spectroscopy. Fig. S12 demonstrates that degradation of the phyA-YFP fusion protein in ProML1:PHYA-YFP is identical to that of the total phyA in ProPHYA:PHYA-YFP seedlings. Thus we conclude that degradation of phyA is comparable in different tissues, and tissue-specific differential degradation does not play a major role in regulating phyA signaling.

**Blue light induced phototropism is modulated by phyA-YFP localized in mesophyll cells**

In Arabidopsis, blue light dependent phototropism is primarily mediated by the PHOTOTROPIN photoreceptors, but blue light induced bending of hypocotyls was shown to be affected by phyA (Janoudi et al., 1997). It was even found that the early phototropic response in blue light is blocked in phyA mutant background (Kami et al., 2012). The mechanism by which the ubiquitously expressed phyA modulates this early phototropic response is unknown, thus we were interested in determining the spatial requirements for phyA action. To this end we grew transgenic phyA-201 seedlings expressing the phyA-YFP fusion in tissue-specific fashion in darkness, and illuminated them with unilateral blue light after FR pre-irradiation for 120 min. Fig. 3 demonstrates that ProPHYA:PHYA-YFP seedlings exhibit a fully complemented response, ProCAB3:PHYA-YFP a significant response (50% complementation), whereas phototropic curvatures of ProML1:PHYA-YFP and ProSUC2:PHYA-YFP seedlings in blue light did not differ from that of the phyA-201 mutant. To corroborate these data we also determined the phototropic response of ProML1+ProCAB3:PHYA-YFP and ProML1+ProCAB3+ProSUC2:PHYA-YFP transgenic seedlings. We found that phototropic curvature of the double and triple transgenic seedlings was identical to that of ProCAB3:PHYA-YFP (Fig. 3). Collectively, these data suggest that for phyA-modulated phototropism (i) signaling by phyA-YFP localized in companion and epidermal cells is largely dismissible, and (ii) the action of phyA-YFP in sub-
epidermal, mainly in the cortical cells of the hook region plays an important role to
regulate blue light induced early phototropic response.

**phyA-YFP localized in companion cells of vascular bundles regulates**
**FR-accelerated transition to flowering**

It has been shown that, similarly to the CRYPTOCHROME2 blue light receptor,
phyA is involved in regulating the time of flowering in Arabidopsis (Mockler *et al.*, 2003). In contrast to phyB, these photoreceptors not only up-regulate the transcription of CONSTANS (CO) (Endo *et al.*, 2013), but also stabilize CO in the long-day afternoon. Accordingly, *phyA* mutants compared to WT flowered late in long day conditions (Neff and Chory 1998) but not in short day conditions when the light period was extended with FR irradiation (Johnson *et al.*, 1994). To test if the localization of phyA is critical for regulating flowering time, we performed the standard FR day-extension assay on transgenic plants expressing the phyA-YFP photoreceptor in a tissue-specific fashion. Fig. 4a demonstrates that expression of phyA-YFP under the control of the *ProPHYA* promoter resulted in full complementation of the delayed flowering phenotype of the *phyA-201* mutant. phyA-YFP localized in epidermal and mesophyll cells appears to be inactive concerning the regulation of flowering time, as *ProML1:PHYA-YFP* and *ProCAB3:PHYA-YFP* lines flowered as late as the *phyA-201* mutant. In contrast, *ProSUC2:PHYA-YFP* plants expressing phyA-YFP in vascular bundles exhibited, similarly to *ProPHYA:PHYA-YFP*, a fully complemented response. We also determined the accumulation of *FT* mRNA in the various transgenic lines. Our data clearly demonstrate that FR day-extension induces up-regulation of *FT* transcription in the *ProPHYA:PHYA-YFP* and *ProSUC2:PHYA-YFP* but not in the *ProML1:PHYA-YFP* and *ProCAB3:PHYA-YFP* lines (Fig. 4b). Taken together, we conclude that phyA-YFP localized in vascular bundles is necessary and sufficient to regulate FR-induced acceleration of flowering time.

**phyA-YFP controls FR-HIR dependent accumulation of HY5-GFP and degradation of CFP-PIF1 fusion proteins in tissue-autonomous manner**

Two hallmarks of phyA-controlled FR-HIR signaling are FR induced transcription and accumulation of the bZIP type transcription factor ELONGATED HYPOCOTYL
5 (HY5) (Osterlund et al., 2000), and induction of the rapid degradation of the
majority of bHLH-type PIF transcription factors (Leivar et al., 2012). These events
represent very early steps of phyA-controlled signaling, and play an essential role in
establishing the complex signaling network (Ma et al., 2001). Our data show that
phyA (Fig. S4-S10) and PIF1 (see later Fig. 6) are highly expressed in all tissues
tested, whereas expression level of HY5 (Fig. 5) is low (around the threshold of
detection) in etiolated seedlings. To test whether FR light dependent modulation of
the abundance of these TFs is altered by manipulating the distribution/localization of
the photoreceptor we produced WT, ProML1:PHYA-YFP and
ProML1+ProSUC2:PHYA-YFP and phyA-201 lines that also expressed
ProHY5:HY5-GFP, and monitored FR-induced changes in the abundance of HY5-
GFP by confocal laser scanning microscopy. Fig. 5 clearly demonstrates that (i) the
abundance of HY5-GFP is low in all tissues of etiolated seedlings, and that (ii) FR
light promotes accumulation of HY5-GFP only in the cells of those tissues which also
expression the phyA-YFP photoreceptor. Namely, in wild-type seedlings FR treatment
uniformly increased the fluorescence in epidermal, mesophyll and vascular cells,
whereas the same treatment, for example, induced accumulation of the HY5-GFP
fusion protein only in the epidermis of the ProML1:PHYA-YFP line and additionally
in the companion cells of ProML1+ProSUC2:PHYA-YFP transgenic seedlings. In
contrast, FR illumination did not induce expression of ProHY5:HY5-GFP in
transgenic phyA-201 lines lacking the active photoreceptor (Fig. S13). We used the
same experimental approach to monitor FR-induced degradation of PIF1. PIF1
negatively regulates chlorophyll biosynthesis and seed germination in the dark, and
light-induced degradation of PIF1 relieves this negative regulation to promote
photomorphogenesis (Huq et al., 2004). We expressed CFP-PIF1 in ProML1:PHYA-
YFP-harboring phyA-201 seedlings. Fig. 6 shows that the abundance of CFP-PIF1 is
high, and the protein is readily detectable in all cell types of etiolated seedlings. This
figure also demonstrates that a short exposure to FR light induced rapid degradation
of the fusion protein in the epidermal, mesophyll and companion cells of wild-type
seedlings, whereas in the ProML1:PHYA-YFP seedlings degradation of the fusion
protein was detectable only in the epidermal cells. These data strongly suggest that for
controlling PIF1 and HY5 abundances phyA acts in a tissue-autonomous fashion, and
intercellular communication between the cells of different tissues does not play a
major role.
phyA-YFP regulates FR-HIR dependent transcription of genes in tissue-autonomous and non-tissue-autonomous fashion

We also attempted to characterize to what extent regulation of cFR light dependent transcription of genes is affected by expressing phyA in different tissues. To this end first we selected several genes whose transcription was shown to be up- or down-regulated by FR irradiation (Peschke & Kretsch, 2011). Next we constructed reporters containing promoters of the above genes, the CYANO FLUORESCENT PROTEIN (CFP) reporters and SV-40 NLS, and introduced these chimeric constructs into WT, ProML1:PHYA-YFP and ProML1+ProCAB3+ProSUC2:PHYA-YFP lines.

GIBBERELLIN 2-BETA-DIOXYGENASE 1 (GA2ox1) catalyzes the hydroxylation of GA molecules, thus reduces available bioactive GA (Rieu et al., 2008). The enzyme XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 17 (XTH17) is involved in the hydrolysis of xyloglucans, and takes part in the restructuring of xyloglucan cross-links in the cellulose/xyloglucan cell wall framework (Vissenberg et al., 2005). Members of the indole-3-acetic acid inducible (IAA) gene family, including IAA19 are transcription regulators act as repressors of auxin-induced gene expression and were shown to be involved in regulating various hypocotyl and root growth responses (Liscum & Reed, 2002; Tian et al., 2004; Jing et al., 2013).

Expression of ProGA2ox1 is below detection level in the hypocotyls and cotyledons of etiolated seedlings and significantly upregulated by FR treatment in the epidermal and sub-epidermal cells of both organs of WT as well as in triple transgenic seedlings. However FR-induced upregulation of ProGA2ox1:CFP-NLS was also readily detected not only in the epidermis but also in the sub-epidermal cells of hypocotyls (Fig. 7) and cotyledons of ProML1:PHYA-YFP seedlings (Fig. S14). These data demonstrate that upregulation of GA2ox1 in the sub-epidermis is mediated by mobile signal(s) generated by phyA action in the epidermis cells. The expression pattern of ProXTH17 differed from that of ProGA2ox1. CFP fluorescence was not detectable in the cotyledon, but was quite strong both in the epidermis and sub-epidermis of the hypocotyl of etiolated WT, ProML1:PHYA-YFP and triple transgenic seedlings. Irradiation by FR light radically changed these patterns. FR light upregulated transcription of ProXTH17 only in the sub-epidermal cells of cotyledons of WT, ProML1:PHYA-YFP and triple transgenic seedlings (Fig. S14). These data...
indicate that expression of ProXTH17 is restricted to the mesophyll cells in this organ, and that phyA localized only in the epidermal cells is sufficient to enhance expression of ProXTH17 in the mesophyll cells. In other words, we conclude that FR light modulated transcription of ProXTH17 is (i) at least partly regulated by intercellular signaling, (ii) mobile signal(s) generated in the epidermis is/are sufficient to induce its expression in mesophyll cells devoid of phyA. In contrast to cotyledons, FR light strongly down-regulates expression of ProXTH17 both in the epidermis and the sub-epidermis of the hypocotyl of WT, ProML1:PHYA-YFP and triple transgenic seedlings (Fig. 7).

Expression of ProIAA19:CFP-NLS displayed a unique pattern. This reporter was not detectable in the cotyledons of dark-grown seedlings, but was highly expressed in the epidermis and sub-epidermis of the hypocotyls of WT, ProML1:PHYA-YFP and triple transgenic seedlings (Fig. 7). FR irradiation dramatically reduced expression of the reporter in all cell types in WT seedlings, but was completely ineffective to reduce CFP fluorescence detectable in the epidermis and sub-epidermis of ProML1:PHYA-YFP and triple transgenic seedlings. We interpret these results to indicate that the repressor of the transcription of ProIAA19 is not activated/produced either in the ProML1:PHYA-YFP or triple transgenic seedlings. We have shown that the amounts of phyA present in the epidermis of ProML1:PHYA-YFP, ProPHYA:PHYA-YFP and triple transgenic seedlings do not differ significantly, thus we conclude that signaling launched by phyA localized in the epidermis is not sufficient to down-regulate expression of ProIAA19 in this tissue. It follows that the signal which is produced either in the sub-epidermal or vascular cells (or both) in WT seedlings is absent or below optimal level in the ProML1:PHYA-YFP and triple transgenic lines.

Collectively, analysis of the expression characteristics of these four reporter constructs at the cellular resolution level convincingly demonstrates that phyA signaling in FR-HIR is mediated partly by intercellular signaling.

**DISCUSSION**

We produced transgenic phyA-201 plants expressing the phyA-YFP photoreceptor under the control of its own promoter or selectively in epidermal, mesophyll and companion cells. By crossings we also generated plants that contain phyA in two or three tissue types. The distribution pattern and abundance of phyA-YFP in the
ProML1+ProCAB3+ProSUC2:PHYA-YFP line was only partially identical to that of phyA-YFP in the ProPHYA:PHYA-YFP line due to the low expression level of the ProCAB3:PHYA-YFP transgene and the lack of expression of ProSUC2:PHYA-YFP in the hypocotyl and root. We note that the reduced level of phyA in the mesophyll cells is likely due to the fact that the basal level activity of the ProCAB3 promoter, which itself is highly upregulated by phyA signaling, was sufficient only to induce low level accumulation of phyA in etiolated tissue. Upon FR treatment the activity of the ProCAB3 promoter is enhanced, but accumulation of phyA is simultaneously reduced by the degradation of phyA Pfr, thus we conclude that the steady-state levels of phyA remained below sub-optimal when compared to ProPHYA:PHYA-YFP seedlings.

phyA mediates VLFRs, which initiate de-etiolation, and HIRs, which complete de-etiolation under sustained activation with FR. phyA signaling in VLFR and FR-HIR conditions displays characteristic differences and is mediated partly by similar, partly by specific molecular components and events (Casal et al., 2014). The physiological responses brought about by a single or hourly repeated light pulses are generally less robust, and monitoring changes in the expression levels of reporters in VLFR condition requires custom-made, special reporters. To this end we will address tissue autonomous/tissue-to-tissue aspects of phyA signaling in VLFR and the possible inter-dependence of the VLFR and HIR modes of actions of phyA signaling in a separate report.

Analysis of FR-HIR induced photomorphogenic responses exhibited by the selected transgenic lines clearly demonstrated that the output of phyA-YFP drastically differs in the different tissues. We show that phyA is capable of regulating a subset of FR-HIR dependent responses in tissue-autonomous fashion (i.e. phyA action in one tissue is sufficient to complement the phyA-201 phenotype), whereas other responses are clearly regulated by simultaneous phyA signaling in different tissues. For example the ProSUC2:PHYA-YFP and ProPHYA:PHYA-YFP lines, expressing the photoreceptor in their vascular bundles, fully restore the flowering phenotype of the phyA-201 mutant. These data demonstrate that phyA-dependent stabilization of CO in the vascular cells can occur without phyA signaling in any other tissues, similarly to CRYPTOCHROME2 (Endo et al., 2007) but in contrast to phyB (Endo et al., 2005). However, it is evident that, beyond regulating flowering time, phyA signaling in the companion cells also contributes to FR-induced expansion of cotyledons (compare the
phenotypes of ProML1:PHYA-YFP, ProSUC2:PHYA-YFP and
ProML1+ProSUC2:PHYA-YFP, Fig. 2) but appears not to be critical for FR-regulated
phototropism and root elongation.
phyA-YFP levels in ProCAB3:PHYA-YFP as well as in the double and triple
transgenic lines reach only about 20-25% of the levels detected in the
ProPHYA:PHYA-YFP line. In these lines expression of ProCAB3:PHYA-YFP was
restricted to the mesophyll/subepidermal cells of the cotyledon and the hook region of
the hypocotyl, whereas it was also highly expressed in other parts of the hypocotyl in
the ProPHYA:PHYA-YFP lines. Nevertheless, phyA signaling restricted to these cells
restored up to 50% of the FR-sensitized phototropic response in transgenic phyA-201
mutants that expressed the ProCAB3:PHYA-YFP or
ProML1+ProCAB3+ProSUC2:PHYA-YFP but not the ProML1:PHYA-YFP or
ProSUC2:PHYA-YFP transgenes (Fig. 3). Thus we hypothesize that phyA presence in
the subepidermal cells of hook is critical to regulate this response, and signaling by
the photoreceptor from other tissues/cells might have limited importance. This
hypothesis is in harmony with findings demonstrating that cellular re-distribution of
PHOTOTROPIN1 is mediated by FR and takes place in the upper part of hypocotyls
(Han et al., 2008) and also with a more recent study investigating the spatial features
of PHOTOTROPIN1-mediated blue light dependent phototropism (Preuten et al.,
2013). However, phyA signaling in the mesophyll cells was also shown to contribute
to restoring FR-induced expansion of the cotyledons of the phyA-201 mutant but not
to the regulation of flowering time or root elongation (Fig. 2, Fig. 4).
Expression of the ProML1:PHYA-YFP transgene was sufficient to restore FR-HIR
induced root elongation of the phyA-201 mutant, similarly to ProPHYA:PHYA-YFP
(Fig. 2). It was reported that local phyA signaling in the root is dismissible (Costigan
et al., 2011), and shoot-derived, phyA-controlled signal regulates elongation of roots
in FR (Salisbury et al., 2007). Our data show that the action of phyA in the mesophyll
cells or vasculature is not required and phyA in the root of ProML1:PHYA-YFP line is
expressed only in a few epidermis cells located at the boundary of dividing/elongation
zone (Fig. S9,S10). Thus we conclude that the signal is likely generated by the action
of phyA of epidermal location in the hypocotyls, cotyledons but not in the root (Fig.
S9,S10) It is assumed that auxin plays a critical role in regulating root elongation.
However, it remains to be determined how signaling by phyA in the epidermis
modulates local synthesis and/or transport of auxin to promote root elongation
(Grieneisen et al., 2007). phyA localized in the epidermis also contributes to inhibition of hypocotyl elongation and cotyledon expansion (Fig. 2), but not to the regulation of flowering time (Fig. 4) or phototropism (Fig. 3).

The triple transgenic lines, with the exception of the partially restored inhibition of hypocotyl elongation and phototropism, exhibited fully complemented phyA-201 phenotype. Since phyA-YFP in the epidermis and vascular tissues are expressed approximately at the same level in these plants as in the ProPHYA:PHYA-YFP we conclude that the action of phyA in the mesophyll cells is critical for the regulation of hypocotyl elongation. This is in good agreement with recent findings obtained by analyzing this response in transgenic lines in which the chromophore was depleted in the mesophyll cells (Warnasooriya & Montgomery, 2009) or phyB was expressed in the mesophyll cells of the cotyledon (Endo et al., 2005). These authors also concluded that the long-distance signal produced in the cotyledons is required for the regulation of hypocotyl growth inhibition. The transgenic lines used in this study are not suitable to study organ-specific signaling, yet we note that the triple transgenic lines had fully developed cotyledons and roots. The apparent contradiction between our data and those published by (Warnasooriya & Montgomery, 2009) can be explained by three mutually non-exclusive mechanisms. Namely, we assume that either (i) the signal derived from the mesophyll cells is insufficient to exclusively regulate hypocotyl growth because of the sub-optimally low level accumulation of phyA brought about by the ProCAB3:PHYA-YFP transgene (ii) in addition to the mesophyll cells, local phyA action in other cell types (epidermis) of the hypocotyl is also required, or (iii) despite the fully complemented size the “metabolic state” of cotyledons of the triple transgenic line is still different from that of the ProPHYA:PHYA-YFP plants, thus the amount of the unknown signaling compound is suboptimal.

We have compared at molecular level phyA signaling in the different tissues to understand how phyA signaling in different tissues is integrated to control complex developmental processes such as hypocotyl growth. The data obtained by analyzing the expression pattern and level of a number of custom-designed molecular reporter constructs in the transgenic plants convincingly demonstrated that phyA (i) regulates the abundance of key regulatory transcriptions factors in a tissue-autonomous fashion, but (ii) also alters the expression of genes in cells lacking the photoreceptor via intercellular, cell-to-cell signaling under the experimental conditions used. Light-driven inactivation of COP1 is a key early step in photoreceptor-controlled signaling.
It has been shown that FR light activated phyA disrupts the COP1/SPA signaling complex by interacting with SPA1, which modifies the substrate specificity/activity of COP1 and thereby promotes accumulation of HY5 (Sheerin et al., 2015). Interestingly, the SPA1 protein expressed in tissue-specific fashion was shown, similarly to phyA, to regulate flowering time in tissue-autonomous fashion and to modulate leaf expansion and hypocotyl growth also via initiating cell-to-cell signaling (Ranjan et al., 2011). These and our data indicate that (i) cFR light mediated inactivation of the COP1/SPA1 complex only occurs in cells which do contain phyA, and (ii) the signal mediating cell-to-cell communication is generated by the action of phyA/SPA1/COP1 complex via modulating the abundance/activity of HY5 or other downstream components. This hypothesis is evidently supported by Fig. 5 demonstrating that FR treatment increases the amount of HY5-GFP fusion protein in tissue-autonomous fashion. Of the bHLH-type PIF1 was shown to interact in a conformation-dependent fashion with phyA (Khanna et al., 2004) and to be subsequently phosphorylated and degraded by the 26S proteasome (Al-Sady et al., 2006; Shen et al., 2008). Our data show that (i) FR induced degradation of the negative regulatory factor PIF1 (Fig. 6) occurs in a tissue-specific fashion, and (ii) this process does not generate transmittable, non-cell autonomous signal(s) that would facilitate the degradation of PIF1 in cells of neighboring tissues free of phyA-YFP. Recent reports provided a conceptual framework for the integration of phytochrome and phytohormone signaling (de Lucas et al., 2008; Feng et al., 2008; Franklin et al., 2011; Bai et al., 2012; Oh et al., 2012; Zhong et al., 2012); however, these models need to be adapted to the cellular level to understand synchronization of elongation of individual cells in different tissues. The tissue/cell-autonomous regulation of key TFs and phyA association with the promoters of hundreds of genes (Chen et al., 2014) explain the partially complemented phenotype of tissue-specifically expressed phyA-YFP and shows that ubiquitous expression of and simultaneous signaling by phyA in different cells is essential for the control of hypocotyl and cotyledon growth. However, our data also show altered transcription of ProGA2ox1 and ProXTH17 in cells lacking phyA. We assume that transcription of these genes is not mediated by HY5 and/or PIFs or phyA associated with the promoters of these genes, since the abundance of these TFs as well as the substrate specificity of the COP1/SPA complex do not change upon FR irradiation in those cells which do not contain phyA-YFP. FR down-regulated transcription of ProIAA19 represents a yet different mode of phyA
action. It appears to require efficient phyA signaling in the mesophyll and epidermis or only in the mesophyll cells, since FR down-regulation of ProIAA19 transcription is detectable only in WT but not in the ProML1:PHYA-YFP and triple transgenic line (Fig. 7). The relatively lower abundance of phyA-YFP in mesophyll cells supports this conclusion. ProIAA19 transcription was shown to be regulated by coordinated action of HY5 and the PICKLE (chromatin remodeller) in the hypocotyl in cFR light (Jing et al., 2013). Our data indicate that PICKLE-regulated action of HY5 is either not manifested in epidermis cells or requires a yet unknown factor. It is evident that transcriptional regulation of ProGA2ox1, ProXTH17 and ProIAA19 is mediated by intercellular signaling dependent on phyA action. At present we do not have data at the whole genome level to estimate the number of genes whose expression is controlled by intercellular signaling dependent on phyA action, nor about the chemical nature of these signals. As far the biological function of phyA-controlled intercellular signaling is concerned, we speculate that it likely provides an additional regulatory layer to fine-tune integration of signaling cascades induced by light and other biotic and abiotic factors.

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AUTHOR CONTRIBUTION

D.K., A.V., S.K., E.A., M.L., A.H., C.K., Z.H. performed research; E.S. and F.N. designed the research and analyzed data; F.N. wrote the paper.
phyA-YFP is localized exclusively in the epidermal or mesophyll or vascular cells of the selected transgenic Arabidopsis *phyA-201* seedlings. Localization of the fusion protein was monitored by epifluorescence microscopy in the hook region [a-d, g-j, m-p, s-v, y-ab] and cotyledons [e, f, k, l, q, r, w, x, ac, ad] of seedlings grown for 2 days in cFR light (20 µmol m⁻² sec⁻¹). To facilitate comparison of the expression level of phyA-YFP in the tissues of the lines, all images showing the same tissue were obtained after identical exposure times. phyA-YFP is expressed ubiquitously in the *ProPHYA:PHYA-YFP* seedlings [g, i, k bright field microscopy; h, j, l epifluorescence microscopy], it is expressed only in the epidermal cells in the
ProML1:PHYA-YFP lines [m, o, q] bright field microscopy; n, p, r epifluorescence microscopy, it shows vascular specific expression in the ProSUC2:PHYA-YFP plants [s, u, v] bright field microscopy; t, v, x epifluorescent microscopy] and is exclusively localized in the sub-epidermal, mesophyll cells in the ProCAB3:PHYA-YFP seedlings [y, aa, ac] bright field microscopy; z, ab, ad epifluorescence microscopy]. White arrows mark positions of selected nuclei, yellow arrows point at vascular bundles, red arrows indicate vascular YFP signal. Scale bar = 10 µm. Legend: WT = Ler (Landsberg erecta); PHYA = ProPHYA:PHYA-YFP; ML1 = ProML1:PHYA-YFP; SUC2 = ProSUC2:PHYA-YFP; CAB3 = ProCAB3:PHYA-YFP. Each transgene is expressed in phyA-201 background.
Figure 2

Phenotypic analyses of Arabidopsis seedlings expressing phyA-YFP in different tissues.

(a) phyA-YFP expressed in the epidermis can restore FR-promoted root elongation in the phyA-201 mutant

Seedlings were grown on vertically positioned ½ MS plates for 10 days in dark or under continuous FR irradiation and their root length was measured. For detailed legend see the legend of Figure 2C.

(b) Tissue-specifically expressed phyA-YFP promotes cotyledon expansion of the phyA-201 mutant in FR light. After induction of germination transgenic seedlings were grown for 3 days in constant dark or illuminated with FR light (20 µmol m⁻² s⁻¹).

Absolute surface area of cotyledons (mm²) is shown [black columns (dark) and gray columns (far-red)]. For detailed legend see the legend of Figure 2C.

(c) phyA-YFP localized in the epidermis partially restores FR light promoted inhibition of hypocotyl elongation of the phyA-201 mutant. After induction of germination, transgenic seedlings were grown for 3 days in constant dark or illuminated with FR light (20 µmol m⁻² s⁻¹). Absolute hypocotyl lengths (mm) are shown [black columns (dark) and gray columns (far-red)]. Legend: WT = Ler ; A- = phyA-201; PHYA = ProPHYA:PHYA-YFP; ML1 = ProML1:PHYA-YFP; SUC2 = ProSUC2:PHYA-YFP; CAB3 = ProCAB3:PHYA-YFP; ML1+SUC2 = ProML1:PHYA-YFP x ProSUC2:PHYA-YFP; ML1+CAB3= ProML1:PHYA-YFP x ProCAB3:PHYA-YFP; SUC2+CAB3= ProSUC2:PHYA-YFP x ProCAB3:PHYA-YFP; ML1+CAB3+SUC2= ProML1:PHYA-YFP x ProCAB3:PHYA-YFP x ProSUC2:PHYA-YFP. Each transgene is expressed in phyA-201 background. Bars indicate mean of at least 25 seedlings, error bars represent standard error, asterisks mark lines that display significant differences by the Mann-Whitney U test (significance P< 0.01) after far-red treatment.
Kirchenbauer et al., Figure 3

**Figure 3**

**phyA-YFP expressed in mesophyll cells efficiently promotes phototropism in blue light**

Arabidopsis seedlings were grown in darkness for 2 days on vertical ½ MS plates and were irradiated first with far-red light (10 µmol m\(^{-2}\) s\(^{-1}\)) for 120 min and subsequently exposed to unilateral blue light (1 µmol m\(^{-2}\) s\(^{-1}\)) for 160 min. The angle of hypocotyl bending is shown, error bars represent standard error, asterisks indicate significant response by the Mann-Whitney U test (P<0.01) compared to the *phyA-201* mutant.

For the detailed name of examined lines see the legend of Figure 2C.
Figure 4

**phyA-YFP localized in vascular tissue complements flowering phenotype of the Arabidopsis phyA-201 mutant and elevates FT mRNA levels**

**(a) Analysis of the flowering time.**
Examined seedlings were grown in short day with (gray bars) or without (black bars) 8 h FR light (30 µmol m$^{-2}$ s$^{-1}$) day extension for 15 days. After day 15 all plants were grown in short day without FR irradiation. Bars indicate the number of days to bolting. The experiment was repeated 3 times, error bars show standard error of the mean; asterisks indicate significant response by the Mann-Whitney U test (P<0.01) compared to the phyA-201 mutant. For the detailed name of examined lines see the legend of Figure 2C.

**(b) Effect of PHYA-YFP on FT transcript level**
Transgenic seedlings were grown in short day with FR light day extension as described above. On day 14 samples were collected at the indicated time points and total RNA was isolated. Expression level of FT was analyzed by qRT-PCR and the obtained values were normalized to the corresponding TUBULIN (TUB) mRNA
amount. Error bars indicate the standard error of the mean values obtained from three independent experiments. For the detailed name of examined lines see the legend of Figure 2C.
Figure 5

phyA-YFP controls FR-induced accumulation of HY5-GFP in tissue-autonomous fashion.

Arabidopsis Ler (WT), and phyA-201 mutant seedlings harboring ProML1:PHYA-YFP (ML1) or ProML1+ProSUC2:PHYA-YFP (ML1+SUC2) transgene expressing the ProHY5:HY5-GFP reporter were grown in darkness (D) for 4 days and irradiated with 10 µmol m\(^{-2}\) s\(^{-1}\) 4 h FR light (FR). Localization and abundance of HY5-GFP (GFP) and PHYA-YFP (YFP) were monitored by confocal laser scanning microscopy. To facilitate comparison of the expression levels of HY5-GFP in different tissues, all images shown were obtained after identical exposure settings.
White arrows mark nuclei in the epidermis, yellow arrows point to nuclei in the sub-epidermal layer, whereas red arrows indicate nuclei in the vasculature. Scale bar = 50 µm.
Figure 6

phyA controls FR induced degradation of CFP-PIF1 fusion protein in tissue-autonomous fashion.
(a) CFP-PIF1 degradation in Arabidopsis Ler wild-type seedlings. WT seedlings expressing the Pro35S:CFP-PIF1 transgene were grown in darkness for 4 days and either irradiated with FR light (20 µmol m$^{-2}$ s$^{-1}$) for 24 h (D, E, F, J, K, L) or further kept in darkness (A, B, C, G, H, I). Localization and abundance of the CFP-PIF1 fusion protein were monitored by epifluorescence microscopy on the 5th day with specific filter sets in the epidermis (A-F) or subepidermal cell layer (G-L) and representative cells are shown. Positions of nuclei pair-wise analyzed for CFP fluorescence (A, D, G, J) or YFP (B, E, H, K) are marked by nu. C, F, I, L show the respective transmitted light images.

(b) CFP-PIF1 degradation in transgenic Arabidopsis phyA-201 seedlings expressing ProML1:PHYA-YFP. Localization and abundance of the phyA-YFP and CFP-PIF1 fusion proteins were monitored by epifluorescence microscopy in transgenic ProML1:PHYA-YFP seedlings expressing the Pro35S:CFP-PIF1 treated as described above.

Note that (A, B, C) and (G, H, I) as well as (D, E, F) and (J, K, L) in Figure 6A and Figure 6B represent the epidermal or subepidermal plane, respectively, at the same location within the hypocotyl. Scale bar = 10 µm.
Kirchenbauer et al., Figure 7
Figure 7

Different spatial patterns of FR-controlled ProXTH1, ProIAA19 and ProGA2ox1 promoter activity in hypocotyl cells

Arabidopsis Ler (WT), and phyA-201 mutant seedlings harboring ProML1:PHYA-YFP (ML1) or ProML1+ProCAB3+ProSUC2:PHYA-YFP (ML1+CBA3+SUC2) transgenes expressing ProXTH17:CFP-NLS or ProIAA19:CFP-NLS or ProGA2ox1:CFP-NLS reporters were grown in darkness for 4 days (D) and subsequently irradiated with 16 h FR light (10 µmol m⁻² s⁻¹) (FR). Localization and abundance of the CFP-NLS fluorophore was monitored in the hypocotyl tissues by confocal laser scanning microscopy. White arrows mark nuclei in the epidermis, yellow arrows point to nuclei in the sub-epidermal layer. Scale bar = 50 µm.

REFERENCES


**SUPPORTING INFORMATION**

The following materials are available in the online version of this article.

**Supporting Information Figures**

- Fig. S1: Application of qRT-PCR method for the selection of homozygous transgenic seedlings.
- Fig. S2: Expression level of phyA-YFP in the selected transgenic lines.
- Fig. S3: Determination of phyA-YFP accumulation at tissue level.
- Fig. S4: Detection of PHYA-YFP in the epidermal and mesophyll tissues of the cotyledon.
- Fig. S5: Detection of PHYA-YFP in the mesophyll and companion cells of the cotyledon.
Fig. S6 Detection of PHYA-YFP in the hook region of the hypocotyl.

Fig. S7 Detection of PHYA-YFP in the upper part of the hypocotyl.

Fig. S8 Detection of PHYA-YFP in the lower part of the hypocotyl.

Fig. S9 Detection of PHYA-YFP in the root.

Fig. S10 Detection of PHYA-YFP in the root tip.

Fig. S11 Root elongation, cotyledon expansion and inhibition of hypocotyl elongation regulated by phyA-YFP expressed in different tissues.

Fig. S12 phyA degradation in red light.

Fig. S13 phyA-YFP controls FR-induced accumulation of HY5-GFP in all tissue types examined.

Fig. S14 FR-regulated ProXTH17 and ProGA2ox1 promoter activity in cotyledon cells.

Supporting Information Tables

Table S1 Sequences of oligonucleotides used in the study

Table S2 phyA-YFP detectability in different tissues

Methods S1