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

3

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46

47

48 **SUMMARY**

49

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

54

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

59

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

64

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

70 **INTRODUCTION**

71 Plants are sessile organisms, and to optimize their fitness and competitiveness they  
72 must adapt to changes in their abiotic and biotic environment. From among the  
73 numerous environmental factors light is arguably the most important one, since plants  
74 use light not only as the energy source for photosynthesis but also as a developmental  
75 cue. To harmonize their growth and development with the ambient light environment,  
76 plants have evolved a battery of highly specialized photoreceptors. These  
77 photoreceptors monitor the quality, quantity, duration and direction of the incident  
78 sunlight and include the UVB-sensing UVB-RESISTANCE 8 (Rizzini *et al.*, 2011),  
79 the blue/UVA light absorbing cryptochromes, phototropins and ZTL-like  
80 photoreceptors (Christie, 2007; Yu *et al.*, 2010; Chaves *et al.*, 2011) and the red (R)  
81 and far-red (FR) light absorbing phytochromes (Franklin & Quail, 2010).  
82 Phytochromes (phy) are chromoproteins that exist as dimers, and each monomer  
83 contains a covalently linked open tetra-pyrrol chain chromophore. In the model plant  
84 *Arabidopsis thaliana* the phytochromes are encoded by a small multigene family  
85 (Sharrock & Quail, 1989; Clack *et al.*, 1994). Phytochromes cycle between their  
86 biologically inactive (Pr) and active (Pfr) forms and act as light quality/quantity  
87 dependent molecular switches. phyA is a highly specialized far-red sensor, since a  
88 very low level of phyA Pfr (~0.1 % of total phyA) generated by FR or a low-ratio  
89 R/FR light is already sufficient to launch signaling. It follows that phyA regulates the  
90 so-called very low fluence (VLFR) and high-irradiation responses to far-red light (FR-  
91 HIR), and thereby plays a dominant role in mediating transition from  
92 skotomorphogenesis to photomorphogenesis (Casal *et al.*, 2014).

93         According to the generally accepted concept, the overwhelming majority of  
94 molecular events underlying phyA-controlled photomorphogenesis take place in the  
95 nucleus. Light in a quality- and quantity-dependent fashion induces translocation into  
96 and accumulation of phyA Pfr in the nuclei (Kircher *et al.*, 1999). PhyA does not have  
97 endogenous nuclear localization signal (NLS) motifs, and import of phyA Pfr is  
98 mediated by the NLS-containing FAR-RED ELONGATED HYPOCOTYL1 and  
99 FHY1-like proteins that shuttle between the nucleus and the cytoplasm (Hiltbrunner *et al.*  
100 *et al.*, 2005; Hiltbrunner *et al.*, 2006; Rausenberger *et al.*, 2011). PhyA Pfr localized in  
101 the nucleus interacts with a battery of negative regulatory proteins, including  
102 CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), SUPPRESSOR OF PHYA-  
103 105 1-4 (SPA1-4) and PHYTOCHROME INTERACTING FACTORS (PIFs). The

104 very early steps of phyA signaling result in (i) the inactivation or alteration of the  
105 substrate specificity of the COP1/SPA1-4 complex that targets proteins to  
106 degradation, (ii) disruption of the binding of PIF transcription factors (TFs) to their  
107 cognate promoters and/or initiating their degradation, and (iii) induction of  
108 transcriptional cascades that modulate the expression of 2500–3000 genes of the  
109 Arabidopsis genome in a FR light-dependent fashion (Tepperman *et al.*, 2001). In this  
110 aspect it is worth noting that phyA is ubiquitously expressed (Somers & Quail, 1995;  
111 Hall *et al.*, 2001), and FR light readily penetrates plant tissues. It follows that phyA  
112 signaling, at least theoretically, can be induced simultaneously in each cell. If so, then  
113 it would be essential to know to what extent phyA signaling in different cells/tissues  
114 is identical and/or different, and how these signaling cascades are interconnected with  
115 each other to regulate complex photomorphogenic responses such as hypocotyl  
116 growth inhibition or cotyledon expansion. Clearly, a prerequisite to answer these  
117 questions is to collect detailed information about the spatial/temporal features of  
118 phyA-controlled signaling cascades. The first reports addressing this problem  
119 produced data obtained by focused irradiation (spot, micro-beam etc.) targeted to  
120 specific parts/organs/tissues. For example, it was shown that phytochrome localized in  
121 leaves is essential for regulating hypocotyl elongation under shade conditions (Casal  
122 & Smith, 1988a; Casal & Smith, 1988b). Nick *et al.* (1993) reported that  
123 accumulation of anthocyanin and *CHALCONE SYNTHASE* mRNA induced by  
124 microbeam irradiation with FR light in the cotyledons of mustard seedlings is a cell-  
125 autonomous, stochastic response. However, to explain the gradually developing  
126 expression pattern at the whole organ level these authors hypothesized that the  
127 responses of individual cells are integrated by inhibitory, intercellular communication.  
128 Bischoff *et al.* (1997) showed that microbeam irradiation with R light induced  
129 expression of the *CAB:LUC* reporter at distant parts of the transgenic tobacco leaves,  
130 a finding that indicates existence of inductive cell-to-cell signaling. Jordan *et al.*  
131 (1995) concluded that manipulation of spatial distribution by over-expressing oat  
132 phyA in different organs in transgenic tobacco results in different phenotypes, and  
133 that phyA localized in the vascular tissue plays a significant role in regulating stem  
134 elongation by repressing gibberellic acid (GA) biosynthesis. Neuhaus *et al.* (1993),  
135 Bowler *et al.* (1994) and Kunkel *et al.* (1996) used a radically different approach,  
136 namely microinjection of phyA and various other putative signaling compounds into  
137 the tomato *aurea* mutant, which is deficient in photoactive phytochromes. These

138 authors demonstrated that phyA signals in a cell-autonomous fashion in a subset of  
139 hypocotyl cells, but these studies lacked analysis of complex developmental responses  
140 and were limited in time. More recently, Warnasooriya and Montgomery (2009) and  
141 Costigan *et al.* (2011) chose a different approach and analyzed FR-HIR induced  
142 responses in transgenic Arabidopsis plants in which accumulation of the chromophore  
143 required for the activity of all phytochromes was decreased in an organ/tissue specific  
144 fashion by expressing plastid-targeted mammalian biliverdin IX alpha reductase under  
145 the control of selected promoters. These authors concluded that phyA-controlled  
146 developmental responses, including hypocotyl growth inhibition and root elongation  
147 are mediated by long-distance, inter-organ signaling. The caveat of this approach is  
148 that it lowers rather than fully inhibits accumulation of the chromophore, and the  
149 precise amount of the active photoreceptor present in the various tissues/organs is not  
150 known.

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

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

172 **MATERIALS AND METHODS**

173

174 **Cloning, generation of transgenic plants**

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

181

182 **Seedling and plant growth conditions**

183 Surface sterilized seeds stratified for 72 h in the dark (4 °C), after which germination  
184 was induced by 18 h of white light (20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 22 °C). The plates were  
185 subsequently treated as specified in the text. For analysis of flowering time, seeds  
186 were sown on soil, stratified for 72 h in the dark (4 °C) and subsequently treated as  
187 specified.

188

189 **Microscopy techniques**

190 Epifluorescent and light microscopy was performed as described by Bauer *et al.* (  
191 2004). Confocal laser scanning microscopy was performed using a Leica SP5 AOBS  
192 confocal laser scanning microscope (Leica, Germany) on DMI6000 microscope base.  
193 Microscope configuration was the following: objective lens: HC PL APO 20x  
194 (NA:0.7); sampling speed: 100 Hz; line averaging: 3x; pinhole: 200  $\mu\text{m}$ ; scanning  
195 mode: sequential unidirectional; excitation: 488 nm laser (GREEN FLUORESCENT  
196 PROTEIN, GFP), 514 nm laser (YFP); spectral emission detectors: 496-518 nm  
197 (GFP), 545-582 nm (YFP). Brightness and contrast settings were uniformly done on  
198 the corresponding image pairs. GFP and YFP images were pseudo-colored green and  
199 red, respectively. All microscopic manipulations were performed under safe green  
200 light and documentation of cells was performed during the first 60 s of microscopic  
201 analysis. In each experiment at least 20 seedlings from 4 independent transgenic lines  
202 (representing >100 cells/seedling) were analyzed and statistically evaluated.  
203 Frequencies of images supporting or contrasting the conclusions drawn was >95% or  
204 0.1%. Every experiment was repeated 3 times.

205



206 **Hypocotyl length and cotyledon area measurement**

207 After induction of germination, seeds were placed at 22 °C in darkness or in FR light  
208 ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 730 nm, 128 nm full widths at half-maximum). Measurement of  
209 hypocotyl length and cotyledon area was performed as described by *Ádám et al.*  
210 (2013). At least 25 seedlings were used for each line and each experiment.

211

212 **Analysis of flowering time**

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

221

222 **Analysis of phototropism**

223 Seeds were sown on rectangular  $\frac{1}{2}$  MS (Murashige and Skoog medium) agar plates  
224 covered with one sheet of sterilized filter paper. After stratification, the plates were  
225 incubated vertically for 2 days in darkness (23°C). The seedlings were irradiated with  
226 far-red light ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 120 min. Unilateral blue light irradiation ( $1 \mu\text{mol m}^{-2}$   
227  $\text{s}^{-1}$ ) was supplied for 160 min by a projector (Leitz, Wetzlar, Germany) equipped  
228 with a blue light filter (KG45; Optic Balzers, Liechtenstein). For homogeneous  
229 illumination of the etiolated seedlings the plates were placed with an angle of 3° to the  
230 light axis. After scanning of the plates hypocotyl bending was measured with ImageJ  
231 (Schneider *et al.*, 2012).

232

233 **Root growth measurements**

234 Seeds were sown on rectangular  $\frac{1}{2}$  MS agar plates containing 1% of sucrose. The  
235 plates were incubated vertically for 10 days in far-red light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 22 °C.  
236 The plates were scanned and root length was measured with ImageJ.

237

238

239 **RESULTS**

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

242 To ensure tissue/cell type specific localization of the functional phyA-YFP  
243 photoreceptor *in planta*, we expressed the fusion protein under the control of *PHYA*,  
244 *MERISTEM LAYER 1 (ProML1)*, *SUCROSE (SUC)/H<sup>+</sup> SYMPORTER 2 (ProSUC2)*  
245 and *CHLOROPHYLL A/B BINDING PROTEIN 3 (ProCAB3)* promoters in the *phyA-*  
246 *201* mutant. The *ProPHYA* promoter is known to be ubiquitously expressed in  
247 seedlings (Somers & Quail, 1995; Hall *et al.*, 2001), whereas the *ProCAB3*, *ProML1*  
248 and *ProSUC2* promoters had been routinely used in the past to express proteins of  
249 interest exclusively in mesophyll, epidermal or companion cells, respectively  
250 (Sessions *et al.*, 1999; Srivastava *et al.*, 2008; Hategan *et al.*, 2014). For this study we  
251 raised 15–20 independent transgenic lines for each construct, and selected those  
252 which segregated the transgenes as a single Mendelian trait. Transgenic lines  
253 homozygous for the *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and  
254 *ProCAB3:PHYA-YFP* transgenes were then further characterized by western blot,  
255 epifluorescence and confocal microscopy to determine the abundance and tissue-  
256 specificity of the respective fusion protein. We selected 4 transgenic lines for each  
257 construct, and performed all experiments by using progenies of these lines. We also  
258 crossed the selected *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and  
259 *ProCAB3:PHYA-YFP* plants and produced lines expressing the phyA-YFP in two or  
260 three tissue types. For a detailed description of the method applied to identify these  
261 multiple transgenic lines see Supporting Information Methods S1 and Fig. S1. The  
262 transgenic lines were then used to extend and to corroborate results obtained by the  
263 analysis of the parental lines. Fig. 1 shows the typical cellular distribution patterns of  
264 the phyA-YFP protein in the cotyledons and in the hook region of the hypocotyls of  
265 chosen *ProPHYA:PHYA-YFP*, *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP*,  
266 *ProCAB3:PHYA-YFP* transgenic lines, and demonstrates that, depending on the  
267 promoter used, the phyA-YFP fusion protein is detectable either in each cell type  
268 (*ProPHYA*, g-l) or only in the epidermal (*ProML1*, m-r), companion (*ProSUC2*, s-x)  
269 or mesophyll (*ProCAB3*, y-ad) cells. Western blot analysis showed that the total  
270 amount of phyA-YFP in the *ProPHYA:PHYA-YFP* lines is comparable to that of  
271 native phyA in wild type (WT) seedlings (Fig. S2a), but it is approximately 10-12  
272 times lower in the *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and *ProCAB3:PHYA-*  
273 *YFP* transgenic lines (Fig. S2b). To compare the abundance of the phyA-YFP fusion

274 protein in different tissues we determined the amount of phyA-YFP accumulated in  
275 nuclei of epidermal and sub-epidermal cells of hypocotyls after 24 h irradiation with  
276 FR light. We found that abundance of the phyA-YFP fusion protein in the epidermal  
277 cells of *ProML1:PHYA-YFP* and *ProPHYA:PHYA-YFP* does not differ significantly,  
278 but it is much (4-5-fold) lower in the sub-epidermal cells of *ProCAB3:PHYA-YFP* as  
279 compared to *ProPHYA:PHYA-YFP* (Fig. S3). Quantitation of phyA abundance in the  
280 companion cells of the various lines was not feasible by this method; however,  
281 microscopic analysis indicates that the expression level of fusion protein is similar in  
282 the selected *ProSUC2:PHYA-YFP* and *ProPHYA:PHYA-YFP* lines (Fig.1 l, x).  
283 Finally, we compared the expression patterns of the photoreceptor in  
284 *ProPHYA:PHYA-YFP* and the triple transgenic line (obtained by consecutive  
285 crossings of the single *ProML1:PHYA-YFP* with *ProCAB3:PHYA-YFP* and  
286 *ProSUC2:PHYA-YFP*; *ProML1+ProCAB3+ProSUC2:PHYA-YFP*) by confocal  
287 microscopy. Table S2 summarizes the results of these experiments and Fig. S4-S10  
288 illustrate that phyA-YFP is detectable in the epidermis, subepidermal and companion  
289 cells of cotyledons, hypocotyls and various tissues of the root of *ProPHYA:PHYA-*  
290 *YFP* seedlings. Expression of phyA-YFP in the  
291 *ProML1+ProCAB3+ProSUC2:PHYA-YFP* ,triple transgenic line is detectable in the  
292 epidermis, mesophyll and companion cells of cotyledons (Fig. S4,S5), in the  
293 epidermis in the hook and both in the lower and upper part of hypocotyls (Fig. S6-S8)  
294 but its expression in the subepidermal cells of hypocotyls is restricted to the hook  
295 region (Fig.1l,x) whereas in the root we could only detect phyA-GFP in specific cell  
296 files in the epidermis (located in the division/elongation zone) (Fig. S9,S10). Taken  
297 together, we conclude that the expression pattern and the level of phyA-YFP in the  
298 *ProML1+ProCAB3+ProSUC2:PHYA-YFP* transgenic line mimic *ProPHYA:PHYA-*  
299 *YFP* (i) in the epidermis of cotyledon and hypocotyls and partially in root, (ii)  
300 comparable to that in the companion cells but lower in the subepidermal (mesophyll)  
301 cells of cotyledons and hook region and strongly different (iii) in the subepidermal  
302 cells (cortex) of the upper and lower part of hypocotyls and in the roots.

303

304 **Epidermally-expressed phyA-YFP fully restores FR-HIR controlled root growth,**  
305 **but only partially complements the hypocotyl growth inhibition and cotyledon**  
306 **expansion phenotype of the *phyA-201* mutant.**

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

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

331 Fig. 2c shows that inhibition of hypocotyl growth is fully restored in the  
332 *ProPHYA:PHYA-YFP* lines and partially in *ProML1:PHYA-YFP* lines as compared to  
333 WT. In contrast, phyA-YFP expressed in companion and mesophyll cells was not able  
334 to induce any detectable response. Fig. 2c and Fig. S11c illustrate that the  
335 *ProML1:PHYA-YFP* and *ProML1+ProCAB3+ProSUC2:PHYA-YFP* transgenic  
336 seedlings displayed similarly enhanced FR-induced hypocotyl growth inhibition when  
337 compared to *phyA-201*, but were still significantly longer when compared to WT or  
338 *ProPHYA:PHYA-YFP*. Taken together, we conclude that the action of phyA-YFP  
339 localized in the epidermis contributes to FR-dependent inhibition of hypocotyl

340 growth, but signaling by phyA localized in different cell/tissue types is also required  
341 to fully complement the phenotype of the *phyA-201* mutant.  
342 To test if the apparently prominent role of epidermis-localized phyA in regulating FR-  
343 dependent hypocotyl and root elongation as well as cotyledon expansion was due to  
344 its altered stability, we determined the degradation kinetics of phyA-YFP in  
345 *ProML1:PHYA-YFP* and *ProPHYA:PHYA-YFP* transgenic lines by *in vivo*  
346 spectroscopy. Fig. S12 demonstrates that degradation of the phyA-YFP fusion protein  
347 in *ProML1:PHYA-YFP* is identical to that of the total phyA in *ProPHYA:PHYA-YFP*  
348 seedlings. Thus we conclude that degradation of phyA is comparable in different  
349 tissues, and tissue-specific differential degradation does not play a major role in  
350 regulating phyA signaling.

351

### 352 **Blue light induced phototropism is modulated by phyA-YFP localized in** 353 **mesophyll cells**

354 In Arabidopsis, blue light dependent phototropism is primarily mediated by the  
355 *PHOTOTROPIN* photoreceptors, but blue light induced bending of hypocotyls was  
356 shown to be affected by phyA (Janoudi *et al.*, 1997). It was even found that the early  
357 phototropic response in blue light is blocked in *phyA* mutant background (Kami *et al.*,  
358 2012). The mechanism by which the ubiquitously expressed phyA modulates this  
359 early phototropic response is unknown, thus we were interested in determining the  
360 spatial requirements for phyA action. To this end we grew transgenic *phyA-201*  
361 seedlings expressing the phyA-YFP fusion in tissue-specific fashion in darkness, and  
362 illuminated them with unilateral blue light after FR pre-irradiation for 120 min. Fig. 3  
363 demonstrates that *ProPHYA:PHYA-YFP* seedlings exhibit a fully complemented  
364 response, *ProCAB3:PHYA-YFP* a significant response (50% complementation),  
365 whereas phototropic curvatures of *ProML1:PHYA-YFP* and *ProSUC2:PHYA-YFP*  
366 seedlings in blue light did not differ from that of the *phyA-201* mutant. To corroborate  
367 these data we also determined the phototropic response of *ProML1+ProCAB3:PHYA-*  
368 *YFP* and *ProML1+ProCAB3+ProSUC2:PHYA-YFP* transgenic seedlings. We found  
369 that phototropic curvature of the double and triple transgenic seedlings was identical  
370 to that of *ProCAB3:PHYA-YFP* (Fig. 3). Collectively, these data suggest that for  
371 phyA-modulated phototropism (i) signaling by phyA-YFP localized in companion  
372 and epidermal cells is largely dismissible, and (ii) the action of phyA-YFP in sub-

373 epidermal, mainly in the cortical cells of the hook region plays an important role to  
374 regulate blue light induced early phototropic response.

375

### 376 **phyA-YFP localized in companion cells of vascular bundles regulates**

### 377 **FR-accelerated transition to flowering**

378 It has been shown that, similarly to the CRYPTOCHROME2 blue light receptor,  
379 phyA is involved in regulating the time of flowering in Arabidopsis (Mockler *et al.*,  
380 2003). In contrast to phyB, these photoreceptors not only up-regulate the transcription  
381 of CONSTANS (CO) (Endo *et al.*, 2013), but also stabilize CO in the long-day  
382 afternoon. Accordingly, *phyA* mutants compared to WT flowered late in long day  
383 conditions (Neff and Chory 1998) but not in short day conditions when the light  
384 period was extended with FR irradiation.(Johnson *et al.*, 1994). To test if the  
385 localization of phyA is critical for regulating flowering time, we performed the  
386 standard FR day-extension assay on transgenic plants expressing the phyA-YFP  
387 photoreceptor in a tissue-specific fashion. Fig. 4a demonstrates that expression of  
388 phyA-YFP under the control of the *ProPHYA* promoter resulted in full  
389 complementation of the delayed flowering phenotype of the *phyA-201* mutant. phyA-  
390 YFP localized in epidermal and mesophyll cells appears to be inactive concerning the  
391 regulation of flowering time, as *ProML1:PHYA-YFP* and *ProCAB3:PHYA-YFP* lines  
392 flowered as late as the *phyA-201* mutant. In contrast, *ProSUC2:PHYA-YFP* plants  
393 expressing phyA-YFP in vascular bundles exhibited, similarly to *ProPHYA:PHYA-*  
394 *YFP*, a fully complemented response. We also determined the accumulation of *FT*  
395 mRNA in the various transgenic lines. Our data clearly demonstrate that FR day-  
396 extension induces up-regulation of *FT* transcription in the *ProPHYA:PHYA-YFP* and  
397 *ProSUC2:PHYA-YFP* but not in the *ProML1:PHYA-YFP* and *ProCAB3:PHYA-YFP*  
398 lines (Fig. 4b). Taken together, we conclude that phyA-YFP localized in vascular  
399 bundles is necessary and sufficient to regulate FR-induced acceleration of flowering  
400 time.

401

### 402 **phyA-YFP controls FR-HIR dependent accumulation of HY5-GFP and**

### 403 **degradation of CFP-PIF1 fusion proteins in tissue-autonomous manner**

404

405 Two hallmarks of phyA-controlled FR-HIR signaling are FR induced transcription  
406 and accumulation of the bZIP type transcription factor ELONGATED HYPOCOTYL

407 5 (HY5) (Osterlund *et al.*, 2000), and induction of the rapid degradation of the  
408 majority of bHLH-type PIF transcription factors (Leivar *et al.*, 2012). These events  
409 represent very early steps of phyA-controlled signaling, and play an essential role in  
410 establishing the complex signaling network (Ma *et al.*, 2001). Our data show that  
411 phyA (Fig. S4-S10) and PIF1 (see later Fig. 6) are highly expressed in all tissues  
412 tested, whereas expression level of HY5 (Fig. 5) is low (around the threshold of  
413 detection) in etiolated seedlings. To test whether FR light dependent modulation of  
414 the abundance of these TFs is altered by manipulating the distribution/localization of  
415 the photoreceptor we produced WT, *ProML1:PHYA-YFP* and  
416 *ProML1+ProSUC2:PHYA-YFP* and *phyA-201* lines that also expressed  
417 *ProHY5:HY5-GFP*, and monitored FR-induced changes in the abundance of HY5-  
418 GFP by confocal laser scanning microscopy. Fig. 5 clearly demonstrates that (i) the  
419 abundance of HY5-GFP is low in all tissues of etiolated seedlings, and that (ii) FR  
420 light promotes accumulation of HY5-GFP only in the cells of those tissues which also  
421 express the phyA-YFP photoreceptor. Namely, in wild-type seedlings FR treatment  
422 uniformly increased the fluorescence in epidermal, mesophyll and vascular cells,  
423 whereas the same treatment, for example, induced accumulation of the HY5-GFP  
424 fusion protein only in the epidermis of the *ProML1:PHYA-YFP* line and additionally  
425 in the companion cells of *ProML1+ProSUC2:PHYA-YFP* transgenic seedlings. In  
426 contrast, FR illumination did not induce expression of *ProHY5:HY5-GFP* in  
427 transgenic *phyA-201* lines lacking the active photoreceptor (Fig. S13). We used the  
428 same experimental approach to monitor FR-induced degradation of PIF1. PIF1  
429 negatively regulates chlorophyll biosynthesis and seed germination in the dark, and  
430 light-induced degradation of PIF1 relieves this negative regulation to promote  
431 photomorphogenesis (Huq *et al.*, 2004). We expressed CFP-PIF1 in *ProML1:PHYA-*  
432 *YFP*-harboring *phyA-201* seedlings. Fig. 6 shows that the abundance of CFP-PIF1 is  
433 high, and the protein is readily detectable in all cell types of etiolated seedlings. This  
434 figure also demonstrates that a short exposure to FR light induced rapid degradation  
435 of the fusion protein in the epidermal, mesophyll and companion cells of wild-type  
436 seedlings, whereas in the *ProML1:PHYA-YFP* seedlings degradation of the fusion  
437 protein was detectable only in the epidermal cells. These data strongly suggest that for  
438 controlling PIF1 and HY5 abundances phyA acts in a tissue-autonomous fashion, and  
439 intercellular communication between the cells of different tissues does not play a  
440 major role.

441

442 **phyA-YFP regulates FR-HIR dependent transcription of genes in tissue-**  
443 **autonomous and non-tissue-autonomous fashion**

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

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

461 Expression of *ProGA2ox1* is below detection level in the hypocotyls and  
462 cotyledons of etiolated seedlings and significantly upregulated by FR treatment in the  
463 epidermal and sub-epidermal cells of both organs of WT as well as in triple transgenic  
464 seedlings. However FR-induced upregulation of *ProGA2ox1:CFP-NLS* was also  
465 readily detected not only in the epidermis but also in the sub-epidermal cells of  
466 hypocotyls (Fig. 7) and cotyledons of *ProML1:PHYA-YFP* seedlings (Fig. S14).  
467 These data demonstrate that upregulation of *GA2ox1* in the sub-epidermis is mediated  
468 by mobile signal(s) generated by phyA action in the epidermis cells. The expression  
469 pattern of *ProXTH17* differed from that of *ProGA2ox1*. CFP fluorescence was not  
470 detectable in the cotyledon, but was quite strong both in the epidermis and sub-  
471 epidermis of the hypocotyl of etiolated WT, *ProML1:PHYA-YFP* and triple transgenic  
472 seedlings. Irradiation by FR light radically changed these patterns. FR light  
473 upregulated transcription of *ProXTH17* only in the sub-epidermal cells of cotyledons  
474 of WT, *ProML1:PHYA-YFP* and triple transgenic seedlings (Fig. S14). These data



475 indicate that expression of *ProXTH17* is restricted to the mesophyll cells in this organ,  
476 and that phyA localized only in the epidermal cells is sufficient to enhance expression  
477 of *ProXTH17* in the mesophyll cells. In other words, we conclude that FR light  
478 modulated transcription of *ProXTH17* is (i) at least partly regulated by intercellular  
479 signaling, (ii) mobile signal(s) generated in the epidermis is/are sufficient to induce its  
480 expression in mesophyll cells devoid of phyA. In contrast to cotyledons, FR light  
481 strongly down-regulates expression of *ProXTH17* both in the epidermis and the sub-  
482 epidermis of the hypocotyl of WT, *ProMLI:PHYA-YFP* and triple transgenic  
483 seedlings (Fig. 7).

484 Expression of *ProIAA19:CFP-NLS* displayed a unique pattern. This reporter was not  
485 detectable in the cotyledons of dark-grown seedlings, but was highly expressed in the  
486 epidermis and sub-epidermis of the hypocotyls of WT, *ProMLI:PHYA-YFP* and triple  
487 transgenic seedlings (Fig. 7). FR irradiation dramatically reduced expression of the  
488 reporter in all cell types in WT seedlings, but was completely ineffective to reduce  
489 CFP fluorescence detectable in the epidermis and sub-epidermis of *ProMLI:PHYA-*  
490 *YFP* and triple transgenic seedlings. We interpret these results to indicate that the  
491 repressor of the transcription of *ProIAA19* is not activated/produced either in the  
492 *ProMLI:PHYA-YFP* or triple transgenic seedlings. We have shown that the amounts  
493 of phyA present in the epidermis of *ProMLI:PHYA-YFP*, *ProPHYA:PHYA-YFP* and  
494 triple transgenic seedlings do not differ significantly, thus we conclude that signaling  
495 launched by phyA localized in the epidermis is not sufficient to down-regulate  
496 expression of *ProIAA19* in this tissue. It follows that the signal which is produced  
497 either in the sub-epidermal or vascular cells (or both) in WT seedlings is absent or  
498 below optimal level in the *ProMLI:PHYA-YFP* and triple transgenic lines.  
499 Collectively, analysis of the expression characteristics of these four reporter  
500 constructs at the cellular resolution level convincingly demonstrates that phyA  
501 signaling in FR-HIR is mediated partly by intercellular signaling.

502

503

## 504 **DISCUSSION**

505 We produced transgenic *phyA-201* plants expressing the phyA-YFP photoreceptor  
506 under the control of its own promoter or selectively in epidermal, mesophyll and  
507 companion cells. By crossings we also generated plants that contain phyA in two or  
508 three tissue types. The distribution pattern and abundance of phyA-YFP in the

509 *ProML1+ProCAB3+ProSUC2:PHYA-YFP* line was only partially identical to that of  
510 phyA-YFP in the *ProPHYA:PHYA-YFP* line due to the low expression level of the  
511 *ProCAB3:PHYA-YFP* transgene and the lack of expression of *ProSUC2:PHYA-YFP*  
512 in the hypocotyl and root. We note that the reduced level of phyA in the mesophyll  
513 cells is likely due to the fact that the basal level activity of the *ProCAB3* promoter,  
514 which itself is highly upregulated by phyA signaling, was sufficient only to induce  
515 low level accumulation of phyA in etiolated tissue. Upon FR treatment the activity of  
516 the *ProCAB3* promoter is enhanced, but accumulation of phyA is simultaneously  
517 reduced by the degradation of phyA Pfr, thus we conclude that the steady-state levels  
518 of phyA remained below sub-optimal when compared to *ProPHYA:PHYA-YFP*  
519 seedlings.

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

530 Analysis of FR-HIR induced photomorphogenic responses exhibited by the  
531 selected transgenic lines clearly demonstrated that the output of phyA-YFP drastically  
532 differs in the different tissues. We show that phyA is capable of regulating a subset of  
533 FR-HIR dependent responses in tissue-autonomous fashion (i.e. phyA action in one  
534 tissue is sufficient to complement the *phyA-201* phenotype), whereas other responses  
535 are clearly regulated by simultaneous phyA signaling in different tissues. For example  
536 the *ProSUC2:PHYA-YFP* and *ProPHYA:PHYA-YFP* lines, expressing the  
537 photoreceptor in their vascular bundles, fully restore the flowering phenotype of the  
538 *phyA-201* mutant. These data demonstrate that phyA-dependent stabilization of CO in  
539 the vascular cells can occur without phyA signaling in any other tissues, similarly to  
540 CRYPTOCHROME2 (Endo *et al.*, 2007) but in contrast to phyB (Endo *et al.*, 2005).  
541 However, it is evident that, beyond regulating flowering time, phyA signaling in the  
542 companion cells also contributes to FR-induced expansion of cotyledons (compare the

543 phenotypes of *ProML1:PHYA-YFP*, *ProSUC2:PHYA-YFP* and  
544 *ProML1+ProSUC2:PHYA-YFP*, Fig. 2) but appears not to be critical for FR-regulated  
545 phototropism and root elongation.  
546 phyA-YFP levels in *ProCAB3:PHYA-YFP* as well as in the double and triple  
547 transgenic lines reach only about 20-25% of the levels detected in the  
548 *ProPHYA:PHYA-YFP* line. In these lines expression of *ProCAB3:PHYA-YFP* was  
549 restricted to the mesophyll/subepidermal cells of the cotyledon and the hook region of  
550 the hypocotyl, whereas it was also highly expressed in other parts of the hypocotyl in  
551 the *ProPHYA:PHYA-YFP* lines. Nevertheless, phyA signaling restricted to these cells  
552 restored up to 50% of the FR-sensitized phototropic response in transgenic *phyA-201*  
553 mutants that expressed the *ProCAB3:PHYA-YFP* or  
554 *ProML1+ProCAB3+ProSUC2:PHYA-YFP* but not the *ProML1:PHYA-YFP* or  
555 *ProSUC2:PHYA-YFP* transgenes (Fig. 3). Thus we hypothesize that phyA presence in  
556 the subepidermal cells of hook is critical to regulate this response, and signaling by  
557 the photoreceptor from other tissues/cells might have limited importance. This  
558 hypothesis is in harmony with findings demonstrating that cellular re-distribution of  
559 PHOTOTROPIN1 is mediated by FR and takes place in the upper part of hypocotyls  
560 (Han *et al.*, 2008) and also with a more recent study investigating the spatial features  
561 of PHOTOTROPIN1-mediated blue light dependent phototropism (Preuten *et al.*,  
562 2013). However, phyA signaling in the mesophyll cells was also shown to contribute  
563 to restoring FR-induced expansion of the cotyledons of the *phyA-201* mutant but not  
564 to the regulation of flowering time or root elongation (Fig. 2, Fig. 4).  
565 Expression of the *ProML1:PHYA-YFP* transgene was sufficient to restore FR-HIR  
566 induced root elongation of the *phyA-201* mutant, similarly to *ProPHYA:PHYA-YFP*  
567 (Fig. 2). It was reported that local phyA signaling in the root is dismissible (Costigan  
568 *et al.*, 2011), and shoot-derived, phyA-controlled signal regulates elongation of roots  
569 in FR (Salisbury *et al.*, 2007). Our data show that the action of phyA in the mesophyll  
570 cells or vasculature is not required and phyA in the root of *ProML1:PHYA-YFP* line is  
571 expressed only in a few epidermis cells located at the boundary of dividing/elongation  
572 zone (Fig. S9,S10). Thus we conclude that the signal is likely generated by the action  
573 of phyA of epidermal location in the hypocotyls, cotyledons but not in the root (Fig.  
574 S9,S10) It is assumed that auxin plays a critical role in regulating root elongation.  
575 However, it remains to be determined how signaling by phyA in the epidermis  
576 modulates local synthesis and/or transport of auxin to promote root elongation

577 (Grieneisen *et al.*, 2007). *phyA* localized in the epidermis also contributes to  
578 inhibition of hypocotyl elongation and cotyledon expansion (Fig. 2), but not to the  
579 regulation of flowering time (Fig. 4) or phototropism (Fig. 3).

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

602 We have compared at molecular level *phyA* signaling in the different tissues to  
603 understand how *phyA* signaling in different tissues is integrated to control complex  
604 developmental processes such as hypocotyl growth. The data obtained by analyzing  
605 the expression pattern and level of a number of custom-designed molecular reporter  
606 constructs in the transgenic plants convincingly demonstrated that *phyA* (i) regulates  
607 the abundance of key regulatory transcription factors in a tissue-autonomous fashion,  
608 but (ii) also alters the expression of genes in cells lacking the photoreceptor via  
609 intercellular, cell-to-cell signaling under the experimental conditions used. Light-  
610 driven inactivation of COP1 is a key early step in photoreceptor-controlled signaling.

611 It has been shown that FR light activated phyA disrupts the COP1/SPA signaling  
612 complex by interacting with SPA1, which modifies the substrate specificity/activity of  
613 COP1 and thereby promotes accumulation of HY5 (Sheerin *et al.*, 2015).  
614 Interestingly, the SPA1 protein expressed in tissue-specific fashion was shown,  
615 similarly to phyA, to regulate flowering time in tissue-autonomous fashion and to  
616 modulate leaf expansion and hypocotyl growth also via initiating cell-to-cell signaling  
617 (Ranjan *et al.*, 2011). These and our data indicate that (i) cFR light mediated  
618 inactivation of the COP1/SPA1 complex only occurs in cells which do contain phyA,  
619 and (ii) the signal mediating cell-to-cell communication is generated by the action of  
620 phyA/SPA1/COP1 complex via modulating the abundance/activity of HY5 or other  
621 downstream components. This hypothesis is evidently supported by Fig. 5  
622 demonstrating that FR treatment increases the amount of HY5-GFP fusion protein in  
623 tissue-autonomous fashion. Of the bHLH-type PIF1 was shown to interact in a  
624 conformation-dependent fashion with phyA (Khanna *et al.*, 2004) and to be  
625 subsequently phosphorylated and degraded by the 26S proteasome (Al-Sady *et al.*,  
626 2006; Shen *et al.*, 2008). Our data show that (i) FR induced degradation of the  
627 negative regulatory factor PIF1 (Fig. 6) occurs in a tissue-specific fashion, and (ii) this  
628 process does not generate transmittable, non-cell autonomous signal(s) that would  
629 facilitate the degradation of PIF1 in cells of neighboring tissues free of phyA-YFP.  
630 Recent reports provided a conceptual framework for the integration of phytochrome  
631 and phytohormone signaling (de Lucas *et al.*, 2008; Feng *et al.*, 2008; Franklin *et al.*,  
632 2011; Bai *et al.*, 2012; Oh *et al.*, 2012; Zhong *et al.*, 2012); however, these models  
633 need to be adapted to the cellular level to understand synchronization of elongation of  
634 individual cells in different tissues. The tissue/cell-autonomous regulation of key TFs  
635 and phyA association with the promoters of hundreds of genes (Chen *et al.*, 2014)  
636 explain the partially complemented phenotype of tissue-specifically expressed phyA-  
637 YFP and shows that ubiquitous expression of and simultaneous signaling by phyA in  
638 different cells is essential for the control of hypocotyl and cotyledon growth.  
639 However, our data also show altered transcription of *ProGA2ox1* and *ProXTH17* in  
640 cells lacking phyA. We assume that transcription of these genes is not mediated by  
641 HY5 and/or PIFs or phyA associated with the promoters of these genes, since the  
642 abundance of these TFs as well as the substrate specificity of the COP1/SPA complex  
643 do not change upon FR irradiation in those cells which do not contain phyA-YFP. FR  
644 down-regulated transcription of *ProIAA19* represents a yet different mode of phyA

645 action. It appears to require efficient phyA signaling in the mesophyll and epidermis  
646 or only in the mesophyll cells, since FR down-regulation of *ProIAA19* transcription is  
647 detectable only in WT but not in the *ProML1:PHYA-YFP* and triple transgenic line  
648 (Fig. 7). The relatively lower abundance of phyA-YFP in mesophyll cells supports  
649 this conclusion. *ProIAA19* transcription was shown to be regulated by coordinated  
650 action of HY5 and the PICKLE (chromatin remodeller) in the hypocotyl in cFR light  
651 (Jing *et al.*, 2013). Our data indicate that PICKLE-regulated action of HY5 is either  
652 not manifested in epidermis cells or requires a yet unknown factor. It is evident that  
653 transcriptional regulation of *ProGA2ox1*, *ProXTH17* and *ProIAA19* is mediated by  
654 intercellular signaling dependent on phyA action. At present we do not have data at  
655 the whole genome level to estimate the number of genes whose expression is  
656 controlled by intercellular signaling dependent on phyA action, nor about the  
657 chemical nature of these signals. As far the biological function of phyA-controlled  
658 intercellular signaling is concerned, we speculate that it likely provides an additional  
659 regulatory layer to fine-tune integration of signaling cascades induced by light and  
660 other biotic and abiotic factors.

661

662

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672

673

#### 674 **AUTHOR CONTRIBUTION**

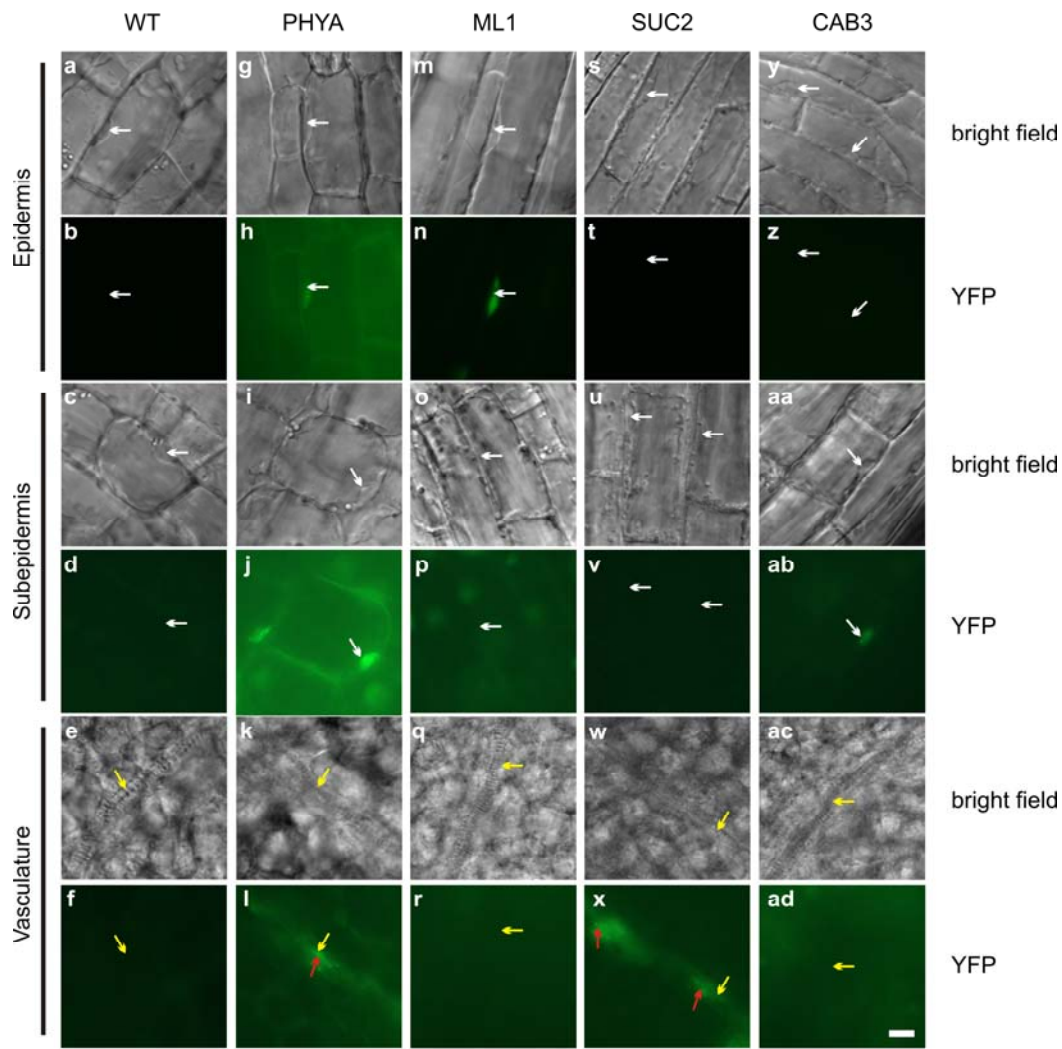
675 D.K., A.V., S.K., E.A., M.L., A.H., C.K., Z.H. performed research;  
676 E.S. and F.N. designed the research and analyzed data; F.N. wrote the paper.

677

678

## 679 FIGURES AND FIGURE LEGENDS

680



Kirchenbauer et al., Figure 1

681

682 **Figure 1**683 **phyA-YFP is localized exclusively in the epidermal or mesophyll or vascular cells**684 **of the selected transgenic *Arabidopsis phyA-201* seedlings.** Localization of the

685 fusion protein was monitored by epifluorescence microscopy in the hook region [a-d,

686 g-j, m-p, s-v, y-ab] and cotyledons [e, f, k, l, q, r, w, x, ac, ad] of seedlings grown

687 for 2 days in cFR light ( $20 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ). To facilitate comparison of the expression

688 level of phyA-YFP in the tissues of the lines, all images showing the same tissue were

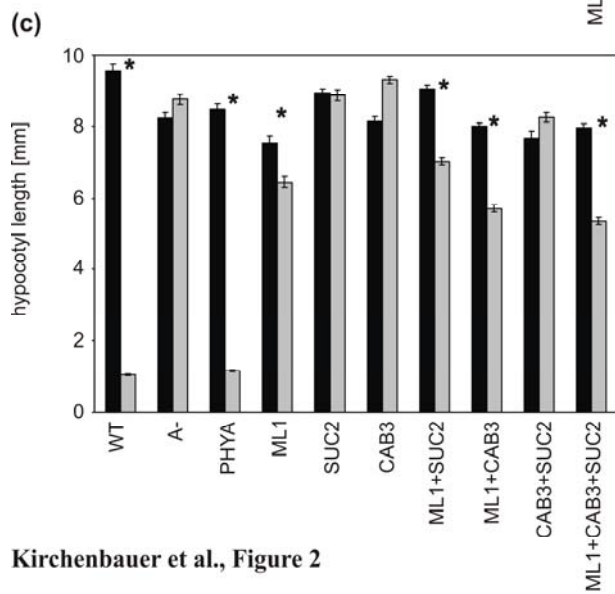
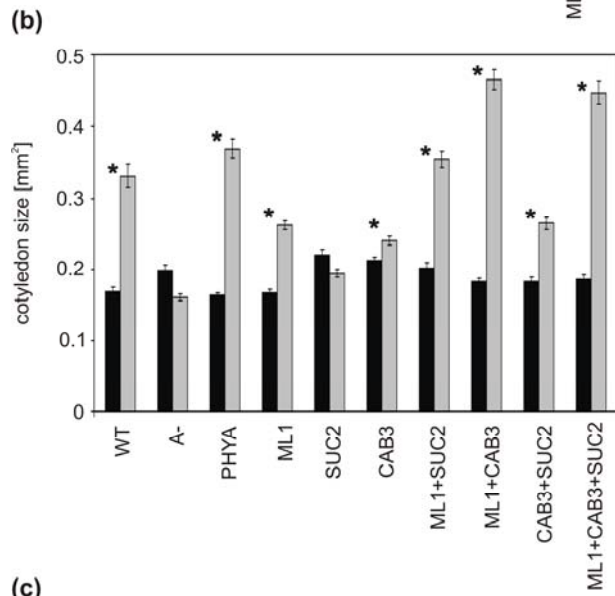
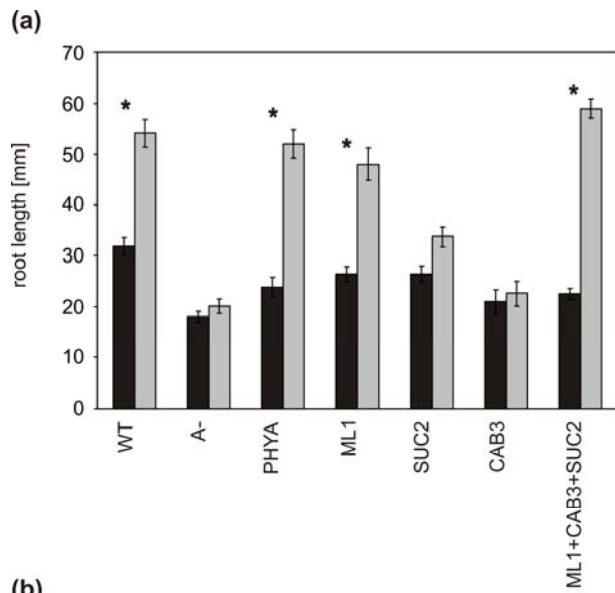
689 obtained after identical exposure times. phyA-YFP is expressed ubiquitously in the

690 *ProPHYA:PHYA-YFP* seedlings [g, i, k bright field microscopy; h, j, l

691 epifluorescence microscopy], it is expressed only in the epidermal cells in the

692 *ProML1:PHYA-YFP* lines [**m, o, q** bright field microscopy; **n, p, r** epifluorescence  
693 microscopy], it shows vascular specific expression in the *ProSUC2:PHYA-YFP* plants  
694 [**s, u, v** bright field microscopy; **t, v, x** epifluorescent microscopy] and is exclusively  
695 localized in the sub-epidermal, mesophyll cells in the *ProCAB3:PHYA-YFP* seedlings  
696 [**y, aa, ac** bright field microscopy; **z, ab, ad** epifluorescence microscopy]. White  
697 arrows mark positions of selected nuclei, yellow arrows point at vascular bundles, red  
698 arrows indicate vascular YFP signal. Scale bar = 10  $\mu$ m. Legend: WT = Ler  
699 (Landsberg *erecta*); PHYA = *ProPHYA:PHYA-YFP*; ML1 = *ProML1:PHYA-YFP*;  
700 SUC2 = *ProSUC2:PHYA-YFP*; CAB3 = *ProCAB3:PHYA-YFP*. Each transgene is  
701 expressed in *phyA-201* background.  
702





704 **Figure 2**

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

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

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

712 **(b) Tissue-specifically expressed phyA-YFP promotes cotyledon expansion of the**  
713 ***phyA-201* mutant in FR light.** After induction of germination transgenic seedlings  
714 were grown for 3 days in constant dark or illuminated with FR light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ).  
715 Absolute surface area of cotyledons ( $\text{mm}^2$ ) is shown [black columns (dark) and gray  
716 columns (far-red)]. For detailed legend see the legend of Figure 2C.

717 **(c) phyA-YFP localized in the epidermis partially restores FR light promoted**  
718 **inhibition of hypocotyl elongation of the *phyA-201* mutant.** After induction of  
719 germination, transgenic seedlings were grown for 3 days in constant dark or  
720 illuminated with FR light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Absolute hypocotyl lengths (mm) are  
721 shown [black columns (dark) and gray columns (far-red)]. Legend: WT = Ler ; A- =  
722 *phyA-201*; PHYA = *ProPHYA:PHYA-YFP*; ML1 = *ProML1:PHYA-YFP*; SUC2 =  
723 *ProSUC2:PHYA-YFP*; CAB3 = *ProCAB3:PHYA-YFP*; ML1+SUC2 =  
724 *ProML1:PHYA-YFP x ProSUC2:PHYA-YFP*; ML1+CAB3= *ProML1:PHYA-YFP x*  
725 *ProCAB3:PHYA-YFP*; CAB3+SUC2 = *ProCAB3:PHYA-YFP x ProSUC2:PHYA-*  
726 *YFP*; ML1+CAB3+SUC2= *ProML1:PHYA-YFP x ProCAB3:PHYA-YFP x*  
727 *ProSUC2:PHYA-YFP*. Each transgene is expressed in *phyA-201* background. Bars  
728 indicate mean of at least 25 seedlings, error bars represent standard error, asterisks  
729 mark lines that display significant differences by the Mann-Whitney U test  
730 (significance  $P < 0.01$ ) after far-red treatment.

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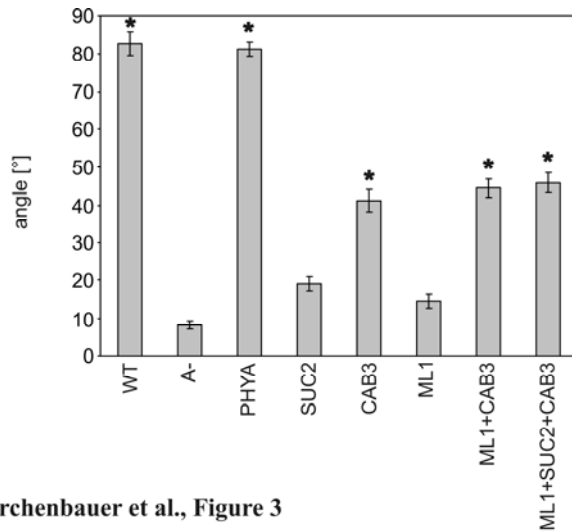
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Kirchenbauer et al., Figure 3

738

739 **Figure 3**

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

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

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

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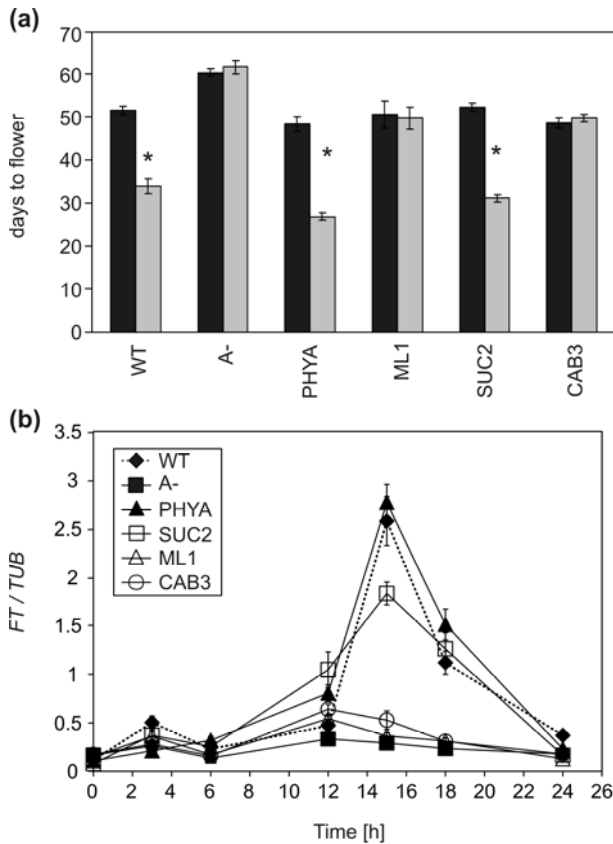
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Kirchenbauer et al., Figure 4

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763 **Figure 4**

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

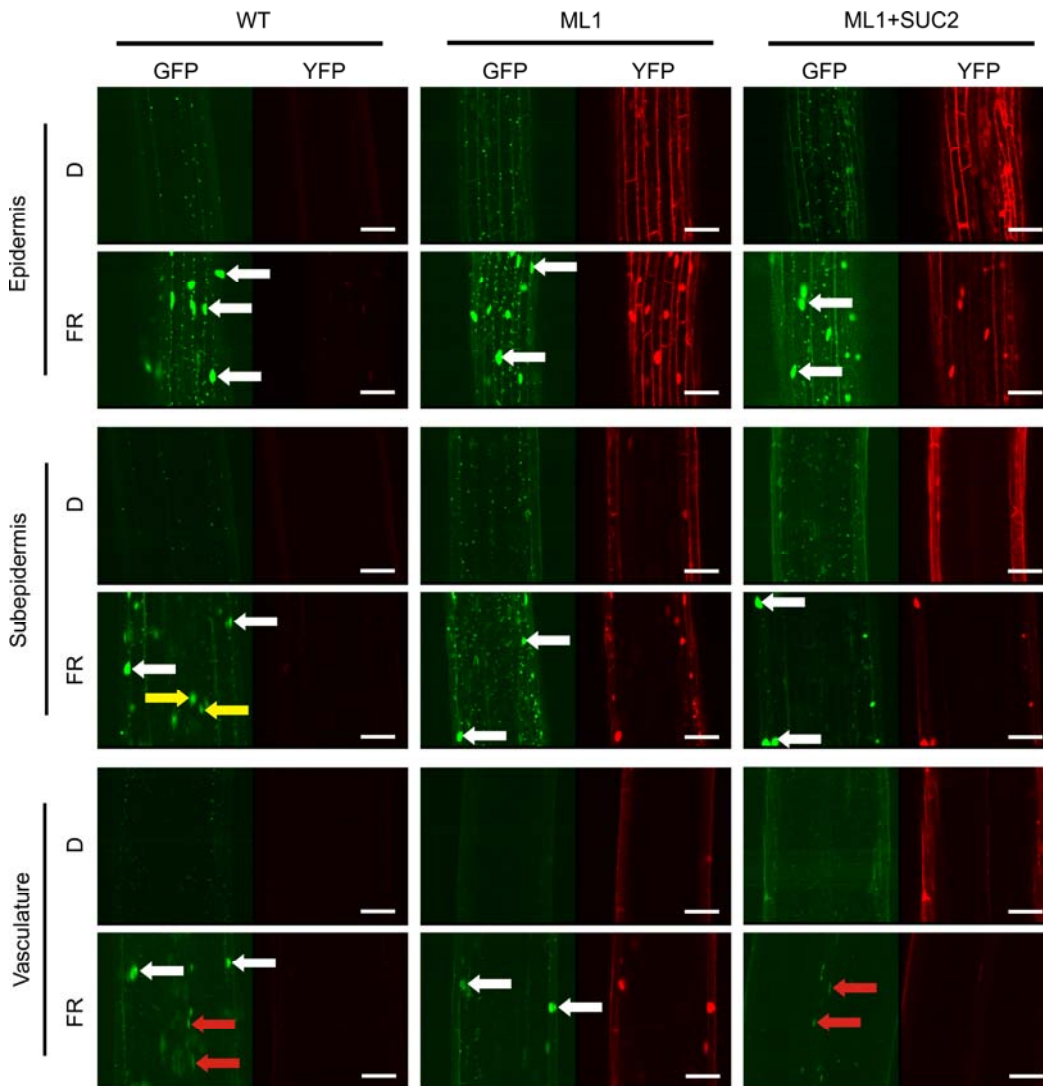
766 **(a) Analysis of the flowering time.**

767 Examined seedlings were grown in short day with (gray bars) or without (black bars)  
 768 8 h FR light ( $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) day extension for 15 days. After day 15 all plants were  
 769 grown in short day without FR irradiation. Bars indicate the number of days to  
 770 bolting. The experiment was repeated 3 times, error bars show standard error of the  
 771 mean; asterisks indicate significant response by the Mann-Whitney U test ( $P < 0.01$ )  
 772 compared to the *phyA-201* mutant. For the detailed name of examined lines see the  
 773 legend of Figure 2C.

774 **(b) Effect of PHYA-YFP on *FT* transcript level**

775 Transgenic seedlings were grown in short day with FR light day extension as  
 776 described above. On day 14 samples were collected at the indicated time points and  
 777 total RNA was isolated. Expression level of *FT* was analyzed by qRT-PCR and the  
 778 obtained values were normalized to the corresponding *TUBULIN (TUB)* mRNA

779 amount. Error bars indicate the standard error of the mean values obtained from three  
780 independent experiments. For the detailed name of examined lines see the legend of  
781 Figure 2C.



Kirchenbauer et al., Figure 5

783

784

**Figure 5**

785

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

786

787

Arabidopsis Ler (WT), and *phyA-201* mutant seedlings harboring *ProML1:PHYA-*

788

*YFP* (ML1) or *ProML1+ProSUC2:PHYA-YFP* (ML1+SUC2) transgene expressing

789

the *ProHY5:HY5-GFP* reporter were grown in darkness (D) for 4 days and irradiated

790

with  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  4 h FR light (FR). Localization and abundance of HY5-GFP

791

(GFP) and PHYA-YFP (YFP) were monitored by confocal laser scanning

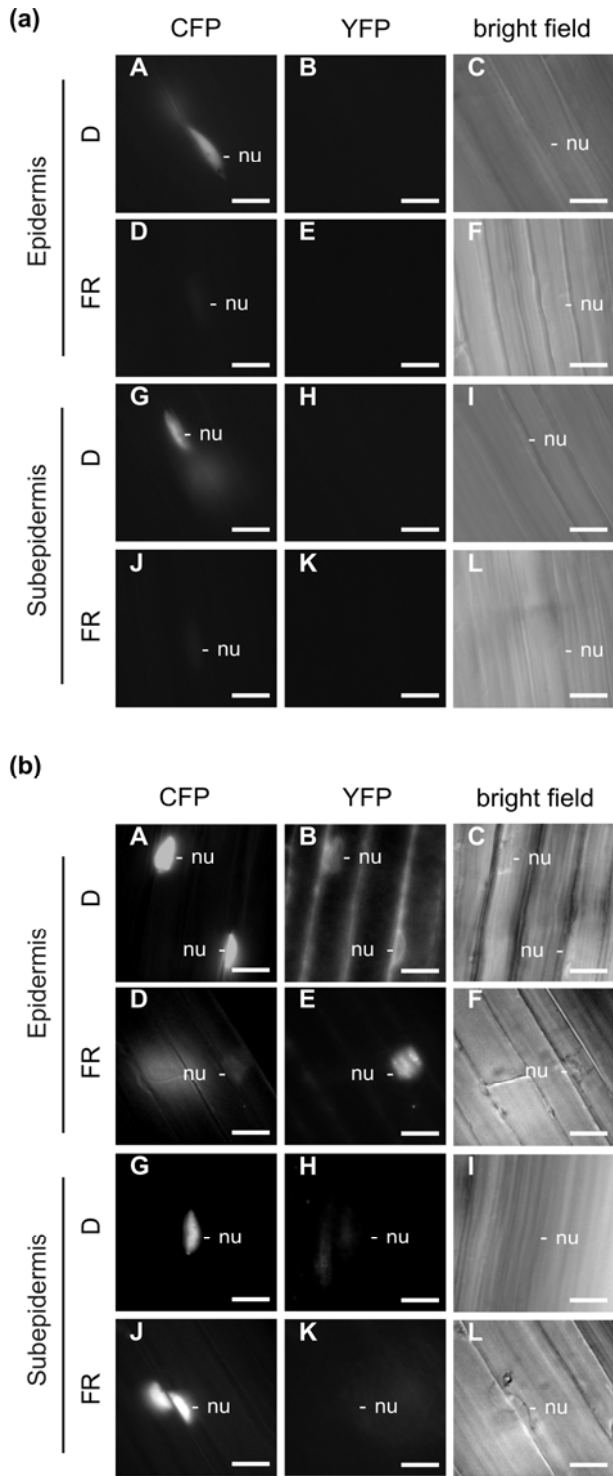
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microscopy. To facilitate comparison of the expression levels of HY5-GFP in

793

different tissues, all images shown were obtained after identical exposure settings.

794 White arrows mark nuclei in the epidermis, yellow arrows point to nuclei in the sub-  
795 epidermal layer, whereas red arrows indicate nuclei in the vasculature. Scale bar = 50  
796  $\mu\text{m}$ .  
797  
798



Kirchenbauer et al., Figure 6

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800

**Figure 6**

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**phyA controls FR induced degradation of CFP-PIF1 fusion protein in tissue-**

802

**autonomous fashion.**



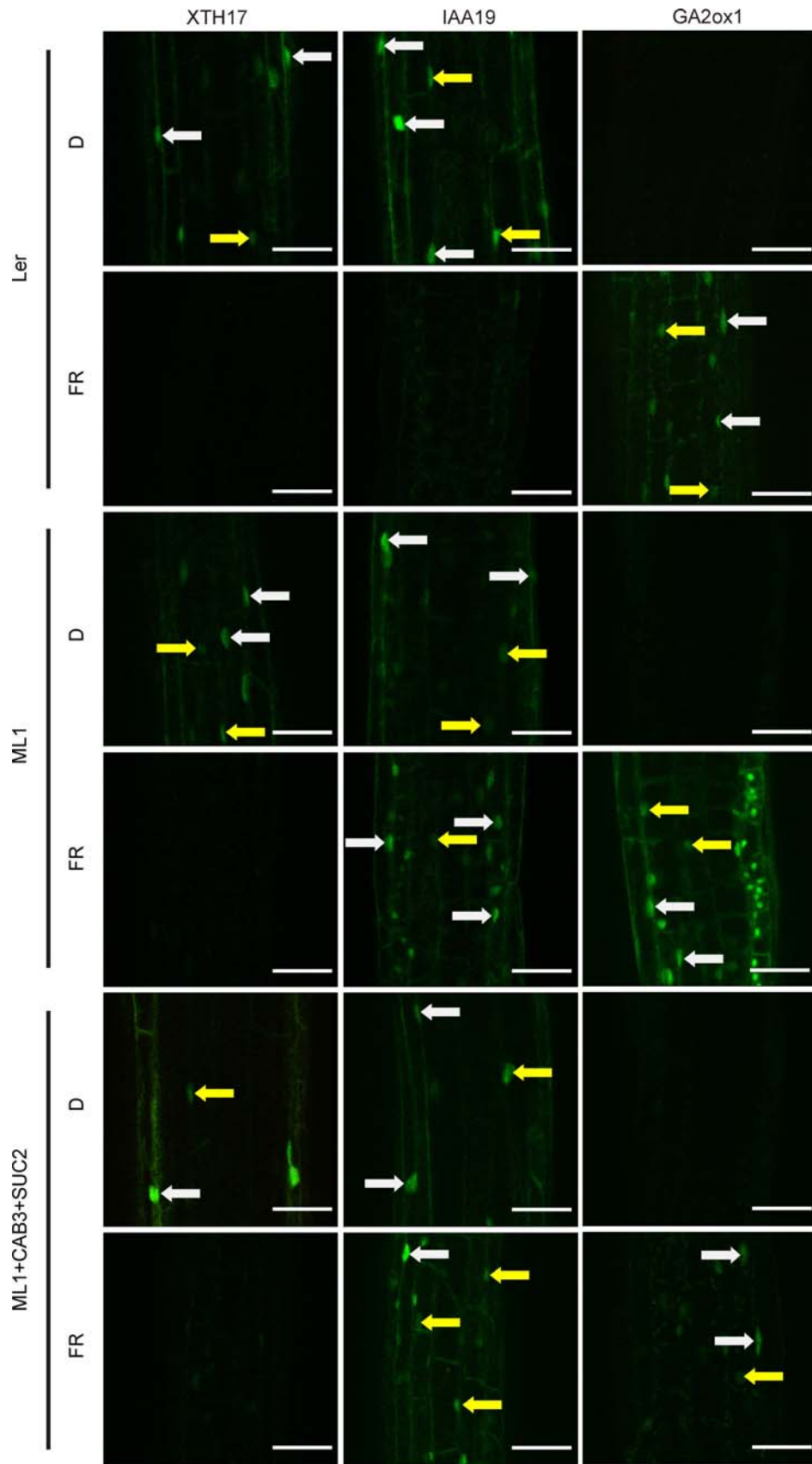
803 **(a) CFP-PIF1 degradation in Arabidopsis Ler wild-type seedlings.** WT seedlings  
804 expressing the *Pro35S:CFP-PIF1* transgene were grown in darkness for 4 days and  
805 either irradiated with FR light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 24 h (**D, E, F, J, K, L**) or further  
806 kept in darkness (**A, B, C, G, H, I**). Localization and abundance of the CFP-PIF1  
807 fusion protein were monitored by epifluorescence microscopy on the 5<sup>th</sup> day with  
808 specific filter sets in the epidermis (**A-F**) or subepidermal cell layer (**G-L**) and  
809 representative cells are shown. Positions of nuclei pair-wise analyzed for CFP  
810 fluorescence (**A, D, G, J**) or YFP (**B, E, H, K**) are marked by nu. **C, F, I, L** show the  
811 respective transmitted light images.

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

817 Note that (**A, B, C**) and (**G, H, I**) as well as (**D, E, F**) and (**J, K, L**) in Figure 6A and  
818 Figure 6B represent the epidermal or subepidermal plane, respectively, at the same  
819 location within the hypocotyl. Scale bar = 10  $\mu\text{m}$ .

820

821



Kirchenbauer et al., Figure 7

823 **Figure 7**  
824 **Different spatial patterns of FR-controlled *ProXTH1*, *ProIAA19* and *ProGA2ox1***  
825 **promoter activity in hypocotyl cells**

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

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#### 1041 **SUPPORTING INFORMATION**

1042 The following materials are available in the online version of this article.

#### 1043 **Supporting Information Figures**

1044 Fig. S1

1045 Application of qRT-PCR method for the selection of homozygous transgenic  
1046 seedlings.

1047 Fig. S2

1048 Expression level of phyA-YFP in the selected transgenic lines.

1049 Fig. S3

1050 Determination of phyA-YFP accumulation at tissue level.

1051 Fig. S4

1052 Detection of PHYA-YFP in the epidermal and mesophyll tissues of the cotyledon.

1053 Fig. S5

1054 Detection of PHYA-YFP in the mesophyll and companion cells of the cotyledon.

1055 Fig. S6  
1056 Detection of PHYA-YFP in the hook region of the hypocotyl.  
1057 Fig. S7  
1058 Detection of PHYA-YFP in the upper part of the hypocotyl.  
1059 Fig. S8  
1060 Detection of PHYA-YFP in the lower part of the hypocotyl.  
1061 Fig. S9  
1062 Detection of PHYA-YFP in the root.  
1063 Fig. S10  
1064 Detection of PHYA-YFP in the root tip.  
1065 Fig. S11  
1066 Root elongation, cotyledon expansion and inhibition of hypocotyl elongation  
1067 regulated by phyA-YFP expressed in different tissues.  
1068 Fig. S12  
1069 phyA degradation in red light.  
1070 Fig. S13  
1071 phyA-YFP controls FR-induced accumulation of HY5-GFP in all tissue types  
1072 examined.  
1073 Fig. S14  
1074 FR-regulated *ProXTH17* and *ProGA2ox1* promoter activity in cotyledon cells.  
1075 **Supporting Information Tables**  
1076 Table S1  
1077 Sequences of oligonucleotides used in the study  
1078 Table S2  
1079 phyA-YFP detectability in different tissues  
1080  
1081 **Methods S1**  
1082