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1 **Chloroplasts modulate elongation responses to canopy shade by**
2 **retrograde pathways involving HY5 and ABA**

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20 **Short title:** Interaction of light and retrograde pathways

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29 **ABSTRACT**

30 Plants use light as energy for photosynthesis but also as a signal of
31 competing vegetation. By using different concentrations of norflurazon and
32 lincomycin, we found that the response to canopy shade in *Arabidopsis thaliana*
33 was repressed even when inhibitors only caused a modest reduction in the level
34 of photosynthetic pigments. High inhibitor concentrations resulted in albino
35 seedlings that were unable to elongate when exposed to shade, in part due to
36 attenuated light perception and signaling via phytochrome B and phytochrome-
37 interacting factors. The response to shade was further repressed by a GUN1-
38 independent retrograde network with two separate nodes represented by the
39 transcription factor HY5 and the carotenoid-derived hormone ABA. The unveiled
40 connection between chloroplast status, light (shade) signaling, and
41 developmental responses should contribute to achieve optimal photosynthetic
42 performance under light-changing conditions.

43 INTRODUCTION

44 Life on our planet heavily relies on photosynthesis, i.e. the use of solar
45 energy (sunlight) to fix carbon into organic matter linked to the production of
46 oxygen from water. In plants, the quantity and quality of the incoming light
47 strongly influence growth and development. For example, oxidative stress and
48 eventual damage can occur if the amount of light exceeds the photosynthetic
49 capacity of the chloroplast. By contrast, light supply and hence photosynthetic
50 activity can be compromised by the shading of nearby plants. Under a canopy,
51 plants might actually be exposed to moments of both excess light (e.g. sunflecks)
52 and low light (i.e. shading) during the same day. Even in open habitats, plants
53 are usually found in communities where competition for light might result in
54 overgrowing and eventual shadowing by neighbors.

55 Light quality is an important signal that informs plants of potential
56 competitors. Vegetation absorbs light from the visible region (called
57 photosynthetically active radiation or PAR, 400–700 nm). In particular, it absorbs
58 red light (R, 600–700 nm) but transmits and reflects far-red light (FR, 700–800
59 nm), therefore causing a reduction in the R to FR ratio (R/FR). Both PAR (light
60 quantity) and R/FR (light quality) are greatly reduced under a plant canopy,
61 whereas the presence of nearby plants (without direct vegetation shading)
62 involves a more moderate reduction of R/FR without changes in PAR (Casal,
63 2012; Martinez-Garcia et al., 2014; Fiorucci and Fankhauser, 2017).
64 Independently of the PAR level, a drop in R/FR acts as a signal that strongly and
65 differentially affects elongation of shade-avoiding plants such as *Arabidopsis*
66 *thaliana* and most crops (Martinez-Garcia et al., 2014). Low R/FR signals also
67 cause a decrease in the levels of photosynthetic pigments (chlorophylls and
68 carotenoids) in seedlings and adult plants (Roig-Villanova et al., 2007; Patel et
69 al., 2013; Bou-Torrent et al., 2015; Llorente et al., 2017). These and other
70 responses triggered by a reduced R/FR are collectively known as the shade
71 avoidance syndrome (SAS) and aim to overgrow neighboring plants, readjust
72 photosynthetic metabolism, and eventually launch reproductive development
73 (Franklin, 2008; Casal, 2012; Gommers et al., 2013; Martinez-Garcia et al.,
74 2014).

75 Low R/FR signals indicative of shade are perceived by the phytochrome
76 (phy) family of photoreceptors. Five genes encode the phy family in *Arabidopsis*:

77 phyA to phyE. While phyB is the major phy controlling the responses to shade,
78 other phy members such as phyD and phyE can also redundantly contribute to
79 the control of shade-modulated elongation growth or flowering time (Franklin,
80 2008; Martinez-Garcia et al., 2014). In the case of photolabile phyA, an
81 antagonistic negative role has been reported for the seedling hypocotyl
82 elongation response to shade. Thus, the SAS is induced by phyB deactivation
83 but gradually antagonized by phyA in response to high FR levels characteristic of
84 plant canopy shade (Casal, 2012; Martinez-Garcia et al., 2014). This intrafamily
85 photosensory attenuation mechanism might act to suppress excessive elongation
86 under prolonged direct vegetation shade. It remains unknown whether other SAS
87 responses, including photosynthetic pigment decrease, are also affected by this
88 antagonistic regulation by phyA and phyB. In any case, the balance between
89 positive and negative regulators of the SAS acting downstream phy was found
90 to be instrumental to regulate not only hypocotyl elongation but also carotenoid
91 biosynthesis (Franklin, 2008; Casal, 2013; Bou-Torrent et al., 2015). Positive
92 regulators of the SAS include transcription factors of the basic-helix-loop-helix
93 (bHLH) (e.g. PIFs, BEEs, BIMs) and homeodomain leucine zipper class II
94 (ATHB2, ATHB4, HAT1, HAT2 and HAT3) families, whereas the basic leucine
95 zipper (bZIP) transcription factor HY5 and bHLH family members PIL1, HFR1
96 and PAR1 have negative roles. Among them, PIFs and HY5 have also been
97 found to participate in retrograde signaling during deetiolation, i.e. in the
98 communication between chloroplasts and nucleus when underground seedlings
99 sense the light and change from skotomorphogenic (i.e. heterotrophic) to
100 photomorphogenic (i.e. photosynthetic) development (Ruckle et al., 2007; Martin
101 et al., 2016; Xu et al., 2016). Alterations in the physiological status of the
102 chloroplast in light-grown plants are also signaled to the nucleus by a variety of
103 retrograde pathways that readjust nuclear gene expression accordingly (Baier
104 and Dietz, 2005; Glasser et al., 2014; Chan et al., 2016). Because exposure to
105 shade causes a decrease in the accumulation of chlorophylls and carotenoids
106 that can eventually compromise photosynthesis and photoprotection (Roig-
107 Villanova et al., 2007; Cagnola et al., 2012; Bou-Torrent et al., 2015), we
108 reasoned that the derived effects on chloroplast homeostasis might not be just a
109 consequence but influence the response to shade itself (e.g. in terms of

110 elongation) through retrograde signaling. The work reported here aimed to test
111 this possibility.

112

113 **RESULTS AND DISCUSSION**

114 **Functional chloroplasts are required for full response to simulated shade.**

115 To initially test whether retrograde signals modulate the elongation
116 response to plant proximity, we used two kinds of inhibitors of chloroplast
117 function associated with retrograde signaling: norflurazon (NF), an inhibitor of
118 carotenoid biosynthesis (Chamovitz et al., 1991) and lincomycin (LIN), an
119 inhibitor of chloroplast protein synthesis (Mulo et al., 2003). Both inhibitors were
120 present in the medium used for seed germination and seedling growth (Figure 1).
121 This medium also contained sucrose to sustain growth even in the absence of
122 photosynthesis. As expected, a concentration-dependent bleaching was
123 observed in wild-type (WT) Arabidopsis plants grown under white light (W) with
124 NF or LIN (Figure 1A). The concentration of inhibitors required to obtain albino
125 seedlings was adjusted to our experimental conditions. For example, an albino
126 phenotype was previously observed in WT seedlings grown without sucrose in
127 the presence of 5 μM NF under 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ or with 50 nM NF under 5 μmol
128 $\text{m}^{-2} \text{s}^{-1}$ (Saini et al., 2011). We used intermediate light intensity conditions (20-24
129 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and, most importantly, added sucrose in the medium, which
130 together made it necessary to adjust NF concentration to 200 nM to obtain
131 completely albino seedlings (Figure 1A).

132 The presence of inhibitors had no significant effect on hypocotyl length
133 under W (Figure 1B). However, exposure to FR-enriched W (W+FR) to simulate
134 canopy shade progressively impaired elongation as levels of photosynthetic
135 pigments (chlorophylls and carotenoids) decreased. Importantly, inhibition of
136 shade-triggered elongation growth was observed at concentrations of NF or LIN
137 that only slightly reduced the levels of photosynthetic pigments and had no visual
138 impact on seedling pigmentation (e.g. 25 nM NF or 5 μM LIN), suggesting that
139 even moderate alterations in chloroplast function might influence the response to
140 shade. Hypocotyl elongation in response to shade was completely blocked at
141 concentrations of NF causing more than a 80% loss of chlorophylls, whereas an
142 even lower reduction (50%) was required for a lack of response in LIN-treated
143 seedlings (Figure 1B). In both cases, completely bleached seedlings did not

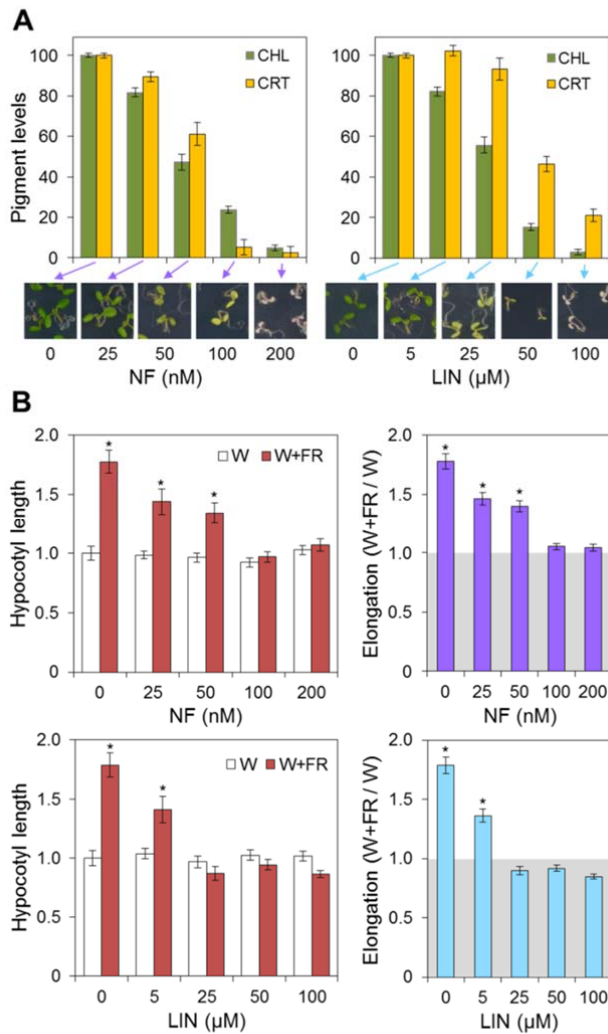


Figure 1. Hypocotyl elongation in response to shade requires functional chloroplasts. (A) WT (Col) plants were germinated and grown under W for 7 days on media with or without the indicated concentrations of NF or LIN. Graphs represent the mean and SEM values of total chlorophyll (CHL) and carotenoid (CRT) contents of at least $n=8$ independent samples (pools of seedlings) from two different experiments. Pigments were quantified by spectrophotometric methods and represented relative to the levels found in the absence of inhibitors. Pictures show the phenotype of representative seedlings. (B) Hypocotyl elongation in 7-day-old seedlings germinated and grown on media supplemented with the indicated concentrations of NF or LIN under W or exposed to W+FR during the last 5 days. Graphs in the left represent the length of the hypocotyls (mean and SEM of $n=100$ seedlings grown in different plates in at least 2 independent experiments) relative to the value in samples grown under W in the absence of inhibitors. Graphs in the right represent the elongation response to shade of the same samples. They show the ratio of hypocotyl length under W+FR relative to that under W. A value of 1 means no growth differences between W and W+FR, values above 1 indicate higher growth under W+FR, and values below 1 indicate lower growth under W+FR. Asterisks mark values statistically higher than 1 (T-test, $p < 0.05$), i.e. responsive to shade by increasing hypocotyl elongation.

144 elongate at all when exposed to W+FR compared to W controls (Figure 1B),
 145 suggesting that functional chloroplasts are required for the elongation response
 146 to canopy shade.

147 We next aimed to confirm that the disrupted elongation response to W+FR
 148 observed in bleached seedlings was not due to energetic constraints. If non-
 149 photosynthetic seedlings lacking chlorophylls maintain an intrinsic capacity to
 150 grow, it would be expected that their hypocotyls would elongate when treated
 151 with growth-promoting hormones such as brassinosteroids, auxins, or
 152 gibberellins. In agreement, seedlings grown in the presence of NF concentrations
 153 that completely blocked photosynthetic development (2μ M) were able to
 154 elongate very similarly to control green seedlings when treated with any of these
 155 hormones (Figure S1). The same hormone treatments caused a similar growth

156 response in the case of mutant *hdr-3* seedlings, which are unable to produce the
157 precursors for chlorophyll and carotenoid biosynthesis in chloroplasts and hence
158 display an albino phenotype (Pokhilko et al., 2015). We therefore conclude that
159 functional chloroplasts are not required for hormone-mediated hypocotyl
160 elongation (at least in sucrose-supplemented media) but are necessary for
161 growth in response to shade signals.

162

163 **Defective chloroplast function impairs phytochrome-mediated shade**
164 **signaling.**

165 Phytochromes are the main photoreceptors involved in shade perception
166 and signal transduction, with phyB having a predominant role in Arabidopsis
167 (Casal, 2012; Martinez-Garcia et al., 2014; Fiorucci and Fankhauser, 2017). To
168 address whether treatment with NF or LIN had an impact on phytochrome
169 signaling, we used transgenic *35S:PHYB-GFP* plants expressing a biologically
170 active GFP-tagged version of the phytochrome (Yamaguchi et al., 1999). Under
171 W, the phyB-GFP fusion protein shows a characteristic distribution in nuclear
172 speckles, presumably the site where active photoreceptor proteins interact with
173 other nuclear factors to mediate light signaling (Yamaguchi et al., 1999). We
174 observed that only minutes after exposing *35S:PHYB-GFP* seedlings to an end-
175 of-day FR treatment to simulate shade, the green fluorescence associated to the
176 phyB-GFP reporter became more disperse in the nuclei of epidermal hypocotyl
177 cells (Figure 2), likely reflecting phyB inactivation. Strikingly, this shade-mediated
178 inactivation process was clearly delayed in albino seedlings germinated and
179 grown in the presence of NF (5 μ M) or LIN (1 mM). While mock (i.e. green)
180 seedlings displayed evenly distributed nuclear phyB-GFP fluorescence in all
181 analyzed cells 90 min after the light treatment, inhibitor-grown (i.e. albino)
182 seedlings still showed nuclear speckles in some cells after 210 min. These
183 results suggest that functional chloroplasts are required for proper phyB
184 inactivation in response to shade signals. Consistent with this conclusion, the
185 shade-triggered and phytochrome-dependent stabilization of photolabile PIF3
186 was attenuated in NF-bleached seedlings (Figure S2).

187 To further confirm whether phytochrome function was altered in albino
188 seedlings, we next analyzed the expression of rapidly shade-induced
189 phytochrome primary target genes in WT plants either treated or not with NF

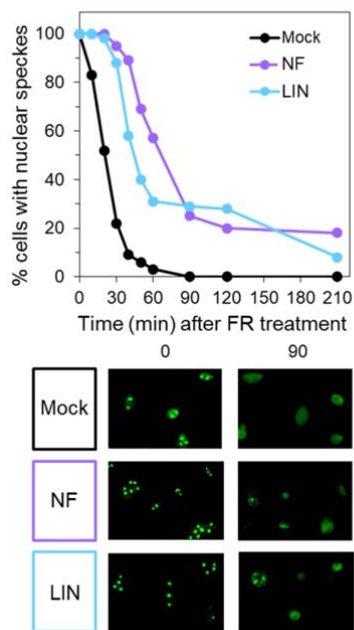


Figure 2. Retrograde signals prevent phyB deactivation in response to shade. Transgenic 35S:PHYB-GFP plants were germinated and grown under W for 7 days on media with or without 5 μ M NF or 1 mM LIN. Following a 5 min irradiation with FR to simulate shade, the distribution of the phyB-GFP reporter protein in nuclei from epidermal hypocotyl cells was analyzed by confocal laser scanning microscopy. A total of ten seedlings from three independent experiments were observed for each treatment and time point. Graph shows mean and SEM values of the percentage of cells showing nuclear speckles in a total of 90 nuclei per treatment and time point. Lower panels show representative images of nuclei from seedling hypocotyl cells 0 and 90 min after the light treatment.

190 (Figure 3). In particular, we chose genes *PIL1*, *ATHB2*, *HFR1*, *YUCCA8* and
 191 *PAR1* (Roig-Villanova et al., 2006). WT plants grown under W for 7 days were
 192 exposed to W+FR for 1h and then samples were collected and used for RNA
 193 extraction and quantitative RT-PCR (qPCR) analysis. As expected, comparison
 194 of W-grown controls and shade-exposed (1h W+FR) samples showed that all
 195 genes analyzed were induced by shade in green seedlings, ranging from 2-fold
 196 (*PAR1*) to 80-fold (*PIL1*). In NF-grown seedlings, however, the induction was
 197 much reduced (Figure 3). *HFR1*, *YUCCA8* and *PAR1* gene expression hardly
 198 changed after W+FR treatment in albino seedlings, whereas *PIL1* induction was
 199 only 10% compared to that detected in green seedlings and *ATHB2* up-regulation
 200 was less than half. Together, we conclude that the absence of functional
 201 chloroplasts somehow prevents normal light (i.e. shade) perception and signal
 202 transduction by phytochromes.

203 Functional phytochrome holoproteins require the covalent attachment of a
 204 phytychromobilin (P Φ B) chromophore to each phytochrome apoprotein monomer
 205 (Rockwell et al., 2006). The synthesis of P Φ B occurs in the plastid and the early
 206 steps are shared with those required to synthesize heme and chlorophylls (Figure
 207 4). To test whether the observed reduction in shade-triggered phytochrome
 208 inactivation (and hence hypocotyl elongation) in albino seedlings could result for
 209 impaired accumulation of P Φ B, we analyzed the elongation response to shade of

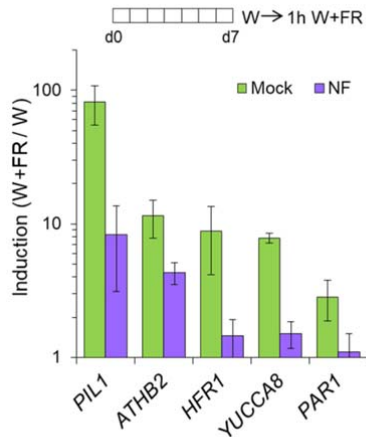


Figure 3. Shade-triggered induction of phytochrome primary target genes is attenuated in bleached seedlings. WT plants were germinated and grown under W for 7 days on media with or without 5 μ M NF. Before and after 1h of exposure to W+FR, RNA was isolated from seedlings and used to analyze the transcript levels of the indicated genes by RT-qPCR. Graph represents the induction response (transcript levels in W vs. those after exposure to W+FR). Mean and SD of n=3 pools of seedlings from independent experiments are shown.

210 Arabidopsis mutants defective in P Φ B synthesis (Parks and Quail, 1991). In
 211 particular, we used the *hy1-1* allele, which was isolated from a fast-neutron
 212 mutagenized population of Landsberg *erecta* (*Ler*) and carries a short deletion
 213 that disrupts its function (Davis et al., 1999). As shown in Figure 4, elongation in
 214 response to W+FR was not repressed but dramatically enhanced in the *hy1-1*
 215 mutant relative to the corresponding WT (*Ler*). Besides showing a much stronger
 216 response to shade under normal growth conditions (i.e. in the absence of
 217 inhibitors), *hy1-1* seedlings were also able to respond to shade and elongate
 218 when treated with NF (Figure 4B). We therefore conclude that treatment with
 219 bleaching inhibitors interferes with phytochrome-dependent signaling by
 220 mechanisms other than defective chromophore availability.

221 Plastid retrograde signaling has been previously shown to interact with
 222 components of light signaling networks to coordinate chloroplast biogenesis with
 223 both the light environment and development (Larkin and Ruckle, 2008; Lepisto
 224 and Rintamaki, 2012; Ruckle et al., 2012; Martin et al., 2016; Xu et al., 2016). In
 225 fact, mutants defective in the P Φ B biosynthetic enzymes HY1/GUN2 and
 226 HY2/GUN3 (Figure 4A) were isolated in a screen for *GENOMES UNCOUPLED*
 227 (*GUN*) mutants that retained partial expression of genes encoding
 228 photosynthesis-related plastidial proteins after NF treatment (Mochizuki et al.,
 229 2001). Other GUN proteins such as GUN5 (Mochizuki et al., 2001) participate in
 230 a different branch of the tetrapyrrole pathway that leads to the production of
 231 chlorophylls (Figure 4A). Unlike other GUN proteins, GUN1 is not an enzyme but
 232 a central integrator of retrograde signaling pathways that was proposed to
 233 coordinate photomorphogenesis with chloroplast function (Koussevitzky et al.,

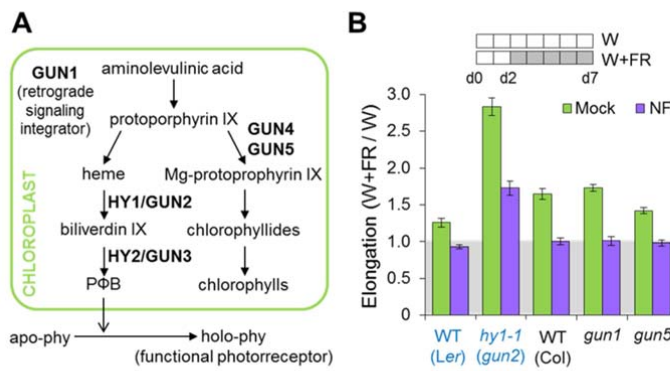


Figure 4. *gun* mutants show different elongation responses to shade. (A) Roles of GUN proteins in retrograde signaling and production of chlorophylls, heme, and the phytochrome chromophore. (B) Elongation responses to shade in mutants defective in some of the GUN proteins represented in (A). Mutants and their respective WT backgrounds (Ler for *hy1/gun2* and Col for the rest) were germinated and grown as indicated with or without 5 μ M NF. Graph represents the mean and SEM values of at least two independent experiments with $n \geq 25$ seedlings each.

234 2007; Ruckle et al., 2007; Ruckle and Larkin, 2009). Similar to WT plants,
 235 mutants *gun1-101* (Ruckle et al., 2007) and *gun5-1* (Mochizuki et al., 2001)
 236 elongated in response to W+FR under normal growth conditions (i.e. when
 237 chloroplasts are functional) but not when chloroplast development was blocked
 238 with NF (Figure 4). Together, the described results suggest that alteration of
 239 chloroplast function impacts a retrograde signaling pathway independent of GUN
 240 proteins that modulates the phytochrome-mediated response to shade.

241

242 **Retrograde pathways repressing shade-triggered hypocotyl elongation** 243 **involve HY5 but not GUN1.**

244 To identify components of the chloroplast-modulated transduction pathway
 245 involved in the response to shade, we next tested the possible role of SAS-
 246 related transcription factors known to be involved in both light and retrograde
 247 signaling: PIFs (Martin et al., 2016) and HY5 (Ruckle et al., 2007; Xu et al.,
 248 2016). A role for PIFs as positive regulators of the response to shade (including
 249 hypocotyl elongation) is well established (Lorrain et al., 2008; Leivar et al., 2012;
 250 Bou-Torrent et al., 2015). However, under our experimental conditions the
 251 quadruple *pifQ* mutant defective in PIF1, PIF3, PIF4 and PIF5 showed a WT
 252 phenotype in terms of shade-triggered hypocotyl elongation in both green and
 253 albino seedlings (Figure 5). HY5 has been proposed to have a function in the
 254 adaptation to prolonged shade and the response to sunflecks, i.e. exposure to
 255 sunlight through gaps in the canopy (Sellaro et al., 2011; Ciolfi et al., 2013). The
 256 role of this elongation-repressing transcription factor in controlling the shade-
 257 promoted growth of seedling hypocotyls, however, remains unclear. Our previous

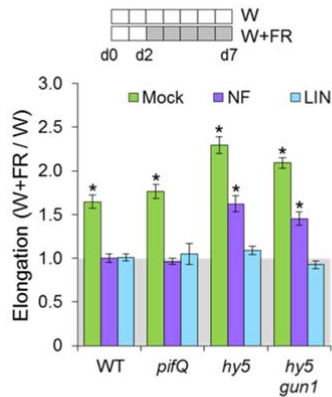


Figure 5. HY5 represses shade-triggered hypocotyl elongation in a GUN1-independent manner. WT (Col) as well as single (*hy5*), double (*hy5 gun1*) and quadruple (*pifQ*) mutant lines were germinated and grown as indicated with or without 5 μ M NF or 1 mM LIN. Graph represents the mean and SEM elongation values of at least two independent experiments with nh25 seedlings each. Asterisks mark statistically significant responses to shade (T-test, $p < 0.05$).

258 work (Bou-Torrent et al., 2015) showed that complete loss of HY5 activity in the
 259 null *hy5-2* mutant (referred to as *hy5* from now on) hardly had an impact in the
 260 elongation of Arabidopsis seedlings exposed to a W+FR treatment mimicking
 261 vegetation proximity ($R/FR = 0.05$). As shown in Figure 5, however, *hy5*
 262 seedlings displayed increased hypocotyl elongation compared to the WT when
 263 illuminated with light of a lower R/FR (0.02), reminiscent of canopy shade. These
 264 results suggest that HY5 is a repressor of hypocotyl elongation in green
 265 seedlings exposed to low or very low R/FR conditions. Consistently, shade-
 266 triggered hypocotyl growth was inhibited in transgenic seedlings
 267 overaccumulating HY5 in a *hy5* background (Figure S3). Similar to WT plants,
 268 the elongation response to canopy shade of *hy5* seedlings was almost
 269 completely blocked with LIN (Figure 5). However, the growth response of HY5-
 270 deficient seedlings was not abolished but just attenuated in NF-supplemented
 271 medium. Similar results were obtained in medium lacking sucrose, but the effects
 272 of HY5 gain or loss of function on the elongation response of green or NF-treated
 273 seedlings, respectively, were much more obvious in the presence of sucrose
 274 (Figure S3). We therefore kept using sucrose-supplemented media for the rest of
 275 the work. Double *hy5 gun1-101* mutants were also found to display a partial
 276 elongation response to shade in NF but not in LIN, similar to that found for the
 277 single *hy5* mutant (Figure 5). Together, the described results show that HY5 is a
 278 repressor of canopy shade-triggered hypocotyl elongation. When this negative
 279 regulator is lost, the elongation response to shade can still be blocked by a
 280 GUN1-independent retrograde pathway that is active in LIN-treated but not in NF-
 281 treated albino seedlings.

282 We next analyzed the levels of HY5 transcripts before and after exposure to
283 our shade conditions (Figure 6). In green WT plants (grown without inhibitors) the
284 levels of *HY5* transcripts were similar under W and up to 8h of our W+FR
285 treatment (Figure 6A). In contrast, immunoblot analysis of a HY5-GFP reporter in
286 complemented *hy5 35S:HY5-GFP* plants showed increased protein levels after
287 the simulated shade treatment (Figure 6B). Chromatin immunoprecipitation
288 experiments also detected increased levels of HY5-GFP bound to target
289 promoters in shade-exposed green seedlings (Figure 6C). Although the
290 endogenous HY5 protein might not behave exactly as the overexpressed GFP-
291 tagged version of the protein, our results are in agreement with previous studies
292 using a different reporter (HY5-myc) that concluded that the low R/FR treatment
293 stabilizes HY5 (Pacin et al., 2016). Post-transcriptional HY5 accumulation when
294 R/FR is low or very low in natural environments (such as in deep or canopy
295 shade) might help to prevent seedlings from exhibiting excessive elongation.

296 Both HY5-encoding transcripts (Figure 6D) and HY5-GFP protein (Figure
297 6B) were higher in albino seedlings grown with LIN or NF independent of the light
298 treatment, suggesting that these inhibitors promote HY5 function by increasing
299 gene expression (or/and transcript stability) and decreasing protein turnover. The
300 observation that hypocotyl length is not reduced in W-grown seedlings in the
301 presence of inhibitors (Figure 1B) despite accumulating higher HY5 levels (Figure
302 6B) suggests that hypocotyl elongation is suppressed to a saturating level by
303 multiple pathways under W and hence it would not be further repressed by
304 increasing HY5 function. In response to W+FR, however, enhanced HY5 activity
305 together with reduced light signaling in bleached WT seedlings would result in no
306 hypocotyl elongation. Only when the repressor activity of HY5 is removed (i.e. in
307 HY5-defective mutants), a second pathway that inhibits the elongation response
308 of albino seedlings becomes apparent in the presence of LIN but not in the
309 presence of NF (Figure 5).

310

311 **Carotenoid-derived products repress shade-triggered hypocotyl**
312 **elongation.**

313 The distinct mode of action of LIN and NF and particularly their differential
314 effect on carotenoid levels is illustrated by their concentration-dependent impact
315 on photosynthetic pigment accumulation (Figure 1A). HPLC analysis of

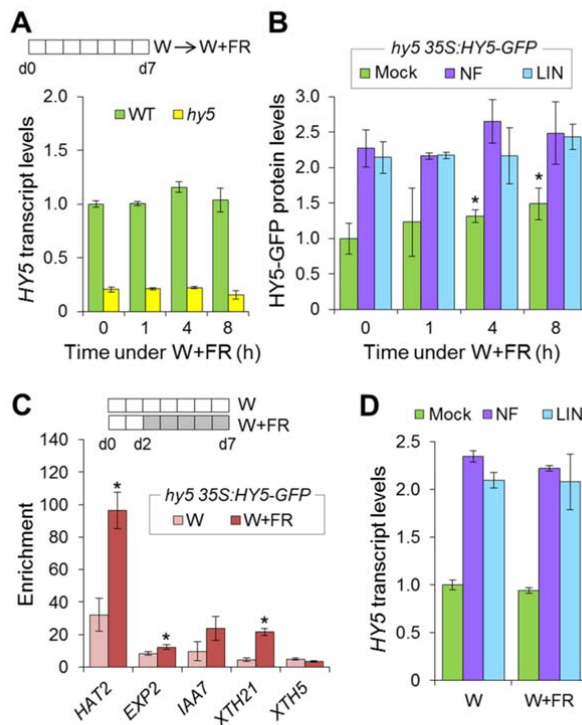


Figure 6. HY5 levels are regulated by shade and retrograde signals. (A) Levels of HY5-encoding transcripts in WT and *hy5* plants germinated and grown under W for 7 days and then exposed to W+FR for the indicated times. Transcript levels were quantified by qPCR and represented relative to those in W-grown WT plants (mean and SEM of n=3 samples corresponding to pools of whole seedlings grown in different experiments). (B) Levels of HY5-GFP protein in *hy5* 35S:HY5-GFP plants germinated and grown under W for 7 days with or without 5 μ M NF or 1 mM LIN and then exposed to W+FR for the indicated times. Protein levels were quantified from immunoblot analysis with a commercial anti-GFP serum. Mean and SEM values (n=3 samples from pools of whole seedlings grown in different experiments) are represented relative to those in plants grown without inhibitors before exposure to shade. Asterisks mark statistically significant differences relative to the 0h timepoint (T-test, p<0.05). (C) Chromatin immunoprecipitation (ChIP) analysis of HY5-GFP binding to the promoters of the indicated genes. After germinating and growing plants of the *hy5* 35S:HY5-GFP line on media without

inhibitors as indicated, ChIP experiments were done using commercial anti-GFP serum. Chromatin from these samples and from no-antibody controls was then used for qPCR amplification of HY5-binding sites in the promoter of the genes. Enrichment was calculated as the ratio of anti-GFP vs. no-antibody values after normalization with input samples (i.e. before ChIP). Graph shows mean and SEM values of n=2 samples from seedlings grown in different experiments. Asterisks mark statistically significant differences in shade-treated samples (T-test, p<0.05). (D) Levels of HY5-encoding transcripts in WT plants germinated on medium with or without 5 μ M NF or 1 mM LIN and grown under W for 2 days followed by 5 additional days under W or under W+FR. Transcript levels were quantified by qPCR and represented relative to those in plants grown under W without inhibitors (mean and SEM of n=3 samples corresponding to pools of whole seedlings grown in different experiments).

316 carotenoid contents (Figure S4) confirmed that albino LIN-treated seedlings
 317 accumulated low but detectable levels of lutein and violaxanthin as well as traces
 318 of β -carotene and neoxanthin. By contrast, NF blocks the desaturation of
 319 phytoene, the first committed intermediate of the carotenoid pathway (Figure 7).
 320 As expected, NF-treated seedlings accumulated phytoene (which is colorless and
 321 hence not detected in the spectrophotometric assay used in Figure 1A) but were
 322 virtually devoid of downstream carotenoids (Figure S4). Similar to that observed
 323 with LIN, other bleaching inhibitors that prevent chloroplast development and
 324 cause albinism without specifically blocking the production of carotenoids, such
 325 as the plastid protein synthesis inhibitor chloramphenicol (CAP) or the nitrogen
 326 assimilation inhibitor phosphinotricin (PPT), were found to prevent shade-
 327 triggered elongation growth in WT and HY5-defective mutants (Figure 7B). By
 328 contrast, inhibition of the carotenoid pathway downstream of lycopene by

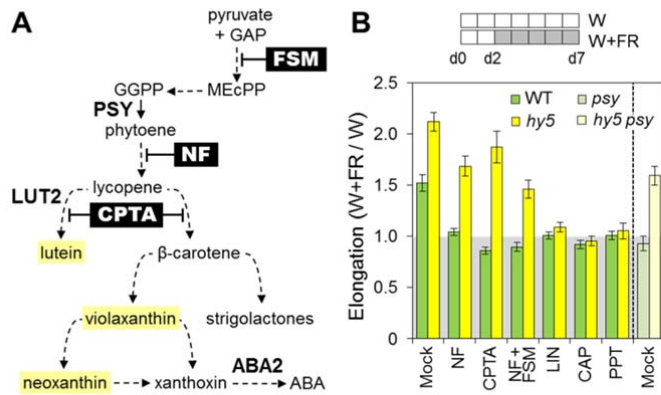


Figure 7. Blockage of the carotenoid pathway derepresses shade-triggered elongation of bleached HY5-defective seedlings. (A) Pathways for the biosynthesis of carotenoids and derived hormones. The steps targeted by NF and other inhibitors and the reactions catalyzed by enzymes that determine metabolic flux to carotenoids (PSY) and ABA (ABA2) are shown. Xanthophylls are boxed in yellow. (B) Elongation responses to shade in WT and mutant plants defective in HY5, PSY, or both. WT and single *hy5* mutant plants were germinated and grown as indicated on media either supplemented or not with concentrations of NF, 2-(4-chlorophenylthio)-triethylamine chloride (CPTA), fosmidomycin FSM), LIN, chloramphenicol (CAP) or phosphinotricin (PPT) producing albino seedlings. Single *psy-1* and double *hy5 psy-1* mutants were only grown without inhibitors. Graph represents the mean and SEM values of nh30 seedlings in a representative experiment.

329 blocking the activity of lycopene cyclases with 2-(4-chlorophenylthio)-
 330 triethylamine chloride (CPTA) resulted in albino seedlings that were able to
 331 respond to shade and elongate when HY5 function was lost (Figure 7).

332 To confirm whether the ability to respond to shade of *hy5* seedlings grown
 333 in the presence of NF or CPTA was specifically due to the blockage of the
 334 carotenoid pathway, we next used Arabidopsis mutants. The enzyme phytoene
 335 synthase (PSY) produces phytoene in the first committed step of the carotenoid
 336 pathway (Figure 7A). Because PSY is encoded by a single gene in Arabidopsis
 337 (Ruiz-Sola and Rodriguez-Concepcion, 2012), the knock-out mutant *psy-1*
 338 (Pokhilko et al., 2015) does not produce phytoene and hence cannot feed the
 339 pathway for the biosynthesis of downstream carotenoids (Figure S4). As a
 340 consequence, the mutant displays an albino phenotype undistinguishable from
 341 that observed in WT seedlings treated with NF or CPTA (Pokhilko et al., 2015).
 342 Similar to that described for WT seedlings grown in the presence of carotenoid
 343 biosynthesis inhibitors, *psy-1* seedlings were unable to elongate when exposed
 344 to W+FR (Figure 7B). However, the elongation response was rescued when both
 345 HY5 and carotenoids were missing in double *hy5 psy-1* mutant seedlings (Figure
 346 7B).

347 Pharmacological or genetic blockage of the carotenoid pathway prevents
 348 the biosynthesis of carotenoids and derived products, but it might also cause an
 349 accumulation of upstream metabolites. Among them, methylerythritol
 350 cyclodiphosphate (MEcPP), an intermediate of the pathway that supplies the

351 metabolic precursors of carotenoids (Figure 7A), has been shown to act as a
352 retrograde signal in response to stress (Xiao et al., 2012). Blockage of MEcPP
353 production with the inhibitor fosmidomycin (Figure 7A), however, did not prevent
354 the elongation response to shade of NF-treated *hy5* seedlings (Figure 7B). We
355 therefore conclude that what allows *hy5* seedlings to respond to shade is not the
356 accumulation of a metabolite upstream PSY but the depletion of a carotenoid-
357 derived product synthesized after the step blocked by CPTA, i.e. downstream of
358 lycopene (Figure 7A).

359 As represented in Figure 7A, lycopene cyclization leads to the production of
360 carotenoids with two β rings (β,β carotenoids such as β -carotene and derived
361 xanthophylls) or with one β and one ϵ ring (β,ϵ carotenoids such as lutein). The
362 production of β,ϵ carotenoids in *Arabidopsis* is completely blocked in the green
363 *lut2* mutant (Figure S4) (Emiliani et al., 2018), which is defective in the only gene
364 encoding lycopene ϵ -cyclase (LCYE/LUT2) in this plant species (Figure 7A)
365 (Ruiz-Sola and Rodriguez-Concepcion, 2012). Loss of β,ϵ carotenoids did not
366 change the elongation response to shade of single *lut2* (vs. WT) or double *hy5*
367 *lut2* (vs. *hy5*) seedlings (Figure S5). We therefore concluded that the effect
368 observed with CPTA (Figure 7B) is not due to the absence of β,ϵ carotenoids but
369 most likely to defects in the β -carotene branch of the carotenoid pathway (Figure
370 7A). Considering all these data together, we speculated that unidentified products
371 derived from β,β carotenoids can repress shade-induced elongation growth in
372 seedlings bleached with LIN and other inhibitors that do not target the carotenoid
373 biosynthesis pathway. The absence of these products in seedlings treated with
374 NF or CPTA, or in the *psy-1* mutant, allows hypocotyl elongation in response to
375 shade but only when the growth-inhibitory effect of HY5 is released.

376

377 **ABA represses the elongation response to shade.**

378 Among the biologically active metabolites derived from β,β carotenoids
379 (Hou et al., 2016), we decided to evaluate the role of ABA as this plant hormone
380 was found to participate in the transduction of chloroplast-derived ROS/redox
381 signals (Baier and Dietz, 2005; Glasser et al., 2014; Chan et al., 2016), to
382 modulate hypocotyl growth (Lau and Deng, 2010; Humplik et al., 2017) and to act
383 together with HY5 in the regulation of several plant cell responses (Chen et al.,
384 2008; Xu et al., 2014). Furthermore, treatment with low R/FR was reported to

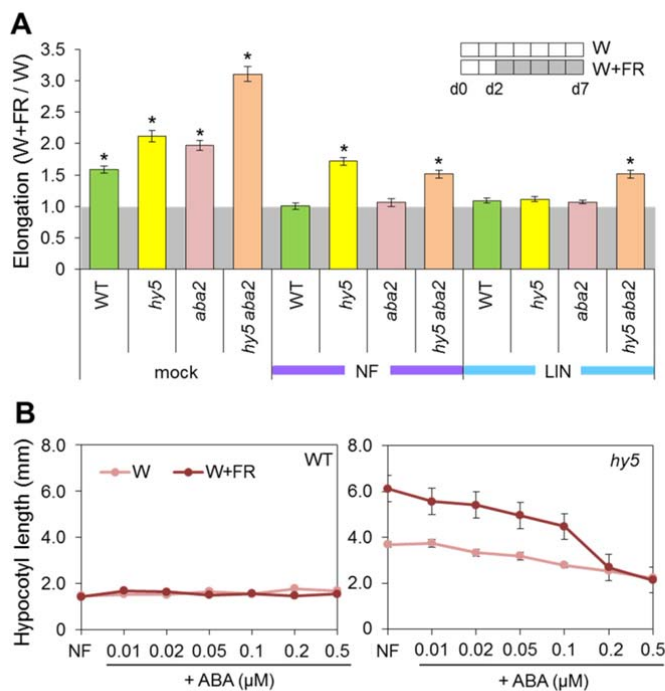


Figure 8. ABA represses shade-triggered hypocotyl elongation independent of HY5. (A) Elongation responses to shade in WT and mutant plants defective in HY5, ABA2, or both. Plants were germinated and grown as indicated with or without 5 μ M NF or 1 mM LIN. Graph represents the mean and SEM values of a total of nh25 seedlings from at least two independent experiments. Asterisks mark statistically significant responses to shade (T-test, $p < 0.05$). (B) Effect of ABA on the elongation of NF-treated seedling hypocotyls in response to shade. WT and *hy5* plants germinated with or without 5 μ M NF plus the indicated concentrations of ABA were grown under W for 2 days followed by 5 additional days under W or under W+FR. Graphs represent hypocotyl length (mean and SEM) of nh25 seedlings in a representative experiment.

385 induce ABA production and signaling in tomato and Arabidopsis (Cagnola et al.,
 386 2012; Gonzalez-Grandio et al., 2013; Holalu and Finlayson, 2017). Indeed, ABA
 387 contents were also found to slightly increase in green seedlings soon (1h) after
 388 exposure to our simulated canopy shade conditions, even though the change
 389 was not statistically significant (Figure S6). As expected, ABA was absent in NF-
 390 treated seedlings but it could be detected in LIN-grown seedlings (Figure S6). If
 391 the presence of ABA in LIN-treated *hy5* seedlings contributed to inhibit their
 392 response to shade, it would be expected that preventing the formation of this
 393 hormone would be sufficient to rescue their response to shade. In agreement, a
 394 genetic blockage of the last step of ABA biosynthesis, catalyzed by the ABA2
 395 protein (Figure 7A), allowed LIN-treated double *hy5 aba2* seedlings to elongate in
 396 response to shade (Figure 8). Single *hy5* and double *hy5 aba2* seedlings had a
 397 very similar response to shade in NF-supplemented medium. By contrast, in
 398 green seedlings grown in the absence of inhibitors (i.e. with functional
 399 chloroplasts) the double mutant elongated more than single *hy5* seedlings when
 400 exposed to shade (Figure 8A). We therefore concluded that HY5 and ABA likely
 401 repress shade-induced hypocotyl elongation by independent pathways. This
 402 conclusion was confirmed by treating NF-grown WT and *hy5* seedlings with
 403 increasing concentrations of ABA (Figure 8B). While no effect was observed in

404 the WT, the ability of *hy5* seedlings to elongate in response to W+FR exposure
405 was progressively repressed as ABA concentration increased. At concentrations
406 of the hormone of 200 nM or higher, which are within the physiological range
407 (Waadt et al., 2014), NF-treated *hy5* seedlings did not respond to shade (Figure
408 8B), similar to that observed with the LIN treatment.

409 Exogenous ABA treatment was also able to repress shade-promoted
410 hypocotyl elongation in green WT seedlings grown without inhibitors (Figure 9).
411 We next used this phenotype to identify ABA-related transcription factors
412 involved in this response. Mutants defective in ABI3 and ABI4 elongated slightly
413 more than WT seedlings when illuminated with W+FR and this response was not
414 repressed by ABA. By contrast, ABI5-defective seedlings showed a WT
415 phenotype in terms of sensitivity of shade-triggered elongation to ABA treatment
416 (Figure 9A). These results suggest that ABI3 and ABI4 but not ABI5 are required
417 for ABA to inhibit hypocotyl elongation. If these transcription factors also
418 transduce the ABA signal in the molecular pathway that blocks elongation in
419 shade-exposed albino seedlings, it would be expected that double mutants
420 lacking both HY5 and ABI3 or ABI4 and grown in the presence of LIN would be
421 able to elongate when exposed to W+FR. Indeed, these double mutants
422 elongated more than their parental lines when both mock (green) and LIN-treated
423 (albino) seedlings were grown under simulated shade (Figure 9B). Shade-
424 triggered elongation of LIN-treated *hy5 abi3* and *hy5 abi4* seedlings, however,
425 was reduced compared to that of ABA-defective *hy5 aba2* seedlings (Figure 9B).
426 These results suggest that ABI3 and ABI4 might not participate in the same ABA
427 signaling pathway eventually repressing hypocotyl elongation but have partially
428 redundant roles in this process (Figure 10).

429

430 **A mechanistic model for the modulation of shade elongation responses by** 431 **plastid-dependent signals.**

432 A model generated based on the described results is shown in Figure 10. In
433 high plant density environments, like those found in forests, prairies or orchard
434 communities, a set of R/FR-dependent adaptive responses are unleashed in
435 shade-avoiding plants. Compared to plant proximity (without direct vegetative
436 shading), canopy shade in nature involves lower R/FR values associated with a
437 reduction in the amount of PAR. Although phyB is the major phytochrome

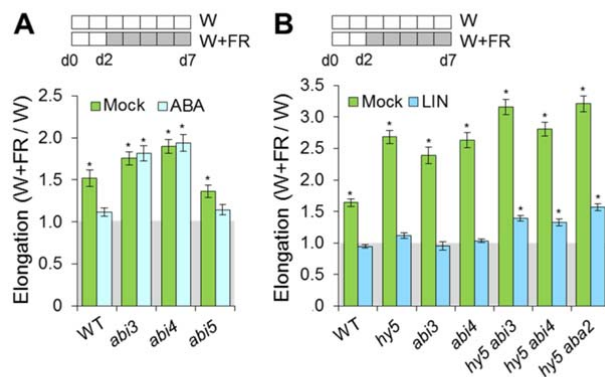


Figure 9. ABI3 and ABI4 but not ABI5 participate in the ABA-mediated repression of shade-induced hypocotyl elongation. (A) Effect of ABA on the elongation responses to shade of WT seedlings and mutants defective in ABI3, ABI4 or ABI5. Plants were germinated and grown as indicated on media with or without 0.2 μ M ABA. Graph represents the mean and SEM values of a total of n \ge 25 seedlings from two independent experiments. (B) Elongation responses to shade of double mutants defective in HY5 and either ABI3 or ABI4. Plants of the indicated genotypes were germinated and grown as illustrated with or without 1 mM LIN. Graph represents the mean and SEM

values of a total of n \ge 25 seedlings from two independent experiments. Asterisks mark statistically significant responses to shade (T-test, p \le 0.05).

438 controlling these responses, the photolabile phyA has an antagonistic negative
 439 role in the shade-mediated regulation of hypocotyl elongation (Ciolfi et al., 2013;
 440 Martinez-Garcia et al., 2014; Wang et al., 2018; Zhang et al., 2018).
 441 Independently of the PAR level, phyB is deactivated by shade of intermediate,
 442 low and very low R/FR, whereas phyA signaling is activated by shade of low and
 443 very low R/FR. As a result, hypocotyl elongation is derepressed under conditions
 444 mimicking vegetation proximity (a response aimed at overgrowing neighbors for
 445 optimal light exposure). Under R/FR values typical of canopy shade, however,
 446 phyA activation prevents seedlings from exhibiting excessive elongation (Figure
 447 10). Our results reported here and elsewhere (Bou-Torrent et al., 2015) suggest
 448 that HY5 represses the hypocotyl elongation response more strongly under
 449 canopy shade. As previously proposed, HY5 might be principally involved in the
 450 phyA-dependent pathway (Ciolfi et al., 2013; Wang et al., 2018; Zhang et al.,
 451 2018) whereas other transcription factors, including growth-promoting PIFs,
 452 would be mostly associated to the phyB-dependent pathway (Figure 10). These
 453 antagonistic phyB/PIFs and phyA/HY5 pathways likely provide young seedlings
 454 with the capacity to rapidly elongate when impending competition is nearby but
 455 also to attenuate excessive growth when growing under a canopy.

456 During seedling deetiolation, the phyB/PIFs pathway converges with a
 457 GUN1-dependent retrograde pathway to antagonistically regulate the
 458 transcriptional photomorphogenic network (Martin et al., 2016). The GUN1-
 459 mediated retrograde signal involved in this particular process was proposed to
 460 attenuate photomorphogenesis when chloroplast function is challenged and to be

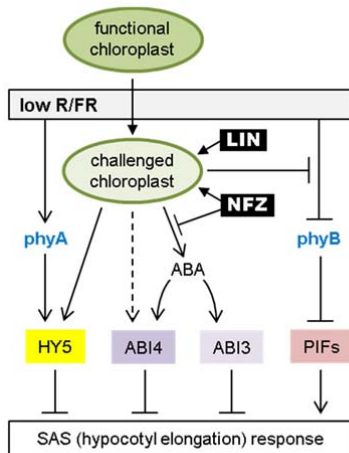


Figure 10. Model for the modulation of shade elongation responses by retrograde signals. In green plants with functional chloroplasts, low R/FR (i.e. canopy shade) signals promote accumulation of growth-promoting PIFs (via phyB deactivation) but also of growth-repressing HY5 (via phyA), likely to prevent an excessive elongation response. Persistent shading or other environmental factors challenging chloroplast function (including exogenous treatment with LIN or NF) can repress phyB inactivation, enhance HY5 expression, and likely promote HY5 stability, eventually resulting in decreased elongation growth. An independent pathway involves ABA, a carotenoid-derived hormone that represses shade-triggered hypocotyl elongation via ABI3 and ABI4. NF (but not LIN) prevents the production of ABA. As a result, loss of both HY5 and ABA in NF-treated *hy5* seedlings allows them to elongate when exposed to low R/FR, whereas this hypocotyl response is blocked by low but detectable levels of ABA in LIN-treated mutants.

461 independent of ABI4 and HY5 (Martin et al., 2016). Our results reported here
 462 suggest that in shade-exposed seedlings, a completely different retrograde
 463 network that is independent of GUN1 but does depend on HY5, ABI3 and ABI4
 464 modulates the antagonistic action of phyA/HY5 and phyB/PIFs signaling
 465 pathways (Figure 10).

466 Prolonged exposure to shade causes a decrease in the accumulation of
 467 chlorophylls and carotenoids that can eventually compromise photosynthesis and
 468 photoprotection (Roig-Villanova et al., 2007; Cagnola et al., 2012; Bou-Torrent et
 469 al., 2015). Our results suggest that such a challenge to the chloroplast functional
 470 status might in turn feedback-regulate the response to shade (Figure 10).
 471 Treatment with low concentrations of NF or LIN (i.e. those causing weak to
 472 moderate reduction in the level of photosynthetic pigments) was sufficient to
 473 repress the hypocotyl elongation response to low R/FR (Figure 1), likely due to
 474 delayed phyB deactivation after a reduction in R/FR (Figure 2). Decreased phyB
 475 deactivation correlated with impaired PIF accumulation (Figure S2) and
 476 attenuated gene expression changes (Figure 3). NF or LIN treatments also
 477 caused an enhanced accumulation of *HY5* transcripts and increased the stability
 478 of the HY5-GFP reporter protein (Figure 6). Together, our findings suggest that
 479 retrograde signals inhibit the SAS by repressing the (positive) phyB/PIFs pathway
 480 and by promoting the (negative) phyA/HY5 pathway (Figure 10).

481 Our work further unveiled ABA as another component of the feedback
 482 mechanism. This carotenoid-derived hormone was found to repress shade-
 483 triggered hypocotyl elongation (Figure 8), likely through the action of the
 484 transcription factors ABI3 and ABI4 (Figure 9). ABI4 has been proposed to

485 participate in GUN1-dependent retrograde signaling (Koussevitzky et al., 2007;
486 Sun et al., 2011; Guo et al., 2016; Xu et al., 2016). However, the results
487 supporting this claim have been repeatedly challenged (Kacprzak et al., 2018).
488 Our data suggest that ABI4 (and ABI3) may act redundantly to transduce the
489 ABA-dependent signal that represses shade-triggered hypocotyl elongation in
490 response to chloroplast dysfunction (Figure 9). While HY5 was previously shown
491 to directly bind and activate the promoter of *ABI5* to promote light-induced
492 hypocotyl inhibition during deetiolation (Chen et al., 2008; Xu et al., 2014), our
493 results suggest that this mechanism does not participate in the control of shade-
494 dependent hypocotyl growth. First, HY5 and ABA appear to repress hypocotyl
495 growth by independent pathways (Figure 8). And second, *ABI5* is not required to
496 transduce the ABA signal eventually repressing the response to shade (Figure 9).

497 Arabidopsis mutants defective in *phyB* were found to accumulate greater
498 amounts of ABA under well-watered conditions and to be less sensitive to
499 exogenous ABA treatments (Gonzalez et al., 2012). Further supporting a
500 negative role of light for ABA synthesis, dark treatment of previously light-grown
501 plants resulted in increased ABA contents (Weatherwax et al., 1996). A shade-
502 triggered increase in ABA production was reported here (Figure S6) and
503 elsewhere (Cagnola et al., 2012; Gonzalez-Grandio et al., 2013; Holalu and
504 Finlayson, 2017). It is possible that W+FR treatment might promote ABA
505 production to repress the elongation response to shade as part of the mechanism
506 that prevents a too intense commitment (Figure 10). These results together
507 support ABA as a central signal connecting the functional status of the
508 chloroplast with light responses. Interestingly, the plastid-synthesized metabolite
509 3'-phosphoadenosine 5'-phosphate (PAP), which functions as a retrograde signal
510 during oxidative stress caused by high light exposure and drought, was recently
511 shown to act in concert with ABA signaling in guard cells to mediate stomatal
512 closure and in seeds to mediate dormancy and germination (Pornsiriwong et al.,
513 2017). PAP accumulates when the *SAL1* phosphatase that normally degrades
514 this metabolite is inactivated during oxidative stress (Estavillo et al., 2011). *SAL1*-
515 defective mutants show a short hypocotyl phenotype in the light, indicating that
516 accumulation of PAP can repress hypocotyl elongation (Kim and von Arnim,
517 2009; Chen and Xiong, 2011). This phenotype is rescued (at least partially) in
518 double *sal1 phyB* and *sal1 hy5* mutants (Kim and von Arnim, 2009; Chen and

519 Xiong, 2011), suggesting that functional phyB and HY5 are required for the PAP-
520 promoted and light-dependent repression of hypocotyl growth. Further
521 experiments should explore whether PAP is the retrograde signal deduced from
522 our data to attenuate the response to shade in terms of hypocotyl elongation by
523 independently inhibiting phyB deactivation, increasing HY5 accumulation, and
524 promoting ABA signaling (Figure 10).

525 Besides ABA, it is possible that other carotenoid-derived products might
526 also contribute to the repression of shade-triggered hypocotyl elongation
527 detected in *hy5* seedlings bleached with LIN, CAP or PPT but not with NF or
528 CPTA (Figure 7). In particular, strigolactones are hormones derived from β -
529 carotene (Figure 7A) that inhibit hypocotyl elongation in the light by a mechanism
530 requiring phytochromes and involving upregulation of *HY5* expression and
531 protein (Tsuchiya et al., 2010; Jia et al., 2014). Other metabolites produced after
532 cleavage of carotenoids include β -cyclocitral, and unknown compounds that
533 modulate developmental and stress responses (Hou et al., 2016). While β -
534 cyclocitral is a relatively well-established retrograde signal associated to oxidative
535 stress (Ramel et al., 2012), its contribution to hypocotyl elongation is unknown.
536 Similarly, no hypocotyl growth alterations have been reported in mutants lacking
537 carotenoid-derived signals that do have an impact on leaf development (van
538 Norman et al., 2007; Avendaño-Vazquez et al., 2014). Whether any of these
539 carotenoid-related metabolites participate in the elongation response to shade
540 remains to be investigated.

541 Collectively, our data support the notion that chloroplasts are plant cell
542 compartments with fundamental roles not only for photosynthesis and
543 metabolism but also for environmental (light) sensing and signaling. Here we
544 show that HY5 and ABA (via ABI3 and ABI4) are nodes of a plastid-modulated
545 network that attenuates the response to shade in terms of hypocotyl elongation.
546 In green plants with functional chloroplasts, light signals associated with canopy
547 shade rapidly promote hypocotyl elongation via the phyB/PIFs pathway.
548 Exposure to low R/FR also triggers negative (growth-repressing) circuits involving
549 the phyA/HY5 pathway and the carotenoid-derived hormone ABA, likely to
550 prevent an excessive response and facilitate the return to non-shade conditions if
551 the low R:FR signal disappears (e.g. if a commitment to the shade-avoidance
552 lifestyle is unnecessary). When maintained, shade further causes a decrease of

553 chlorophyll and carotenoid contents which might eventually disrupt chloroplast
554 homeostasis. Such situation would be then signaled to feedback-regulate the
555 response to the light signal by independently inhibiting phyB deactivation,
556 increasing HY5 accumulation, and promoting ABA signaling. This mechanism
557 connecting the metabolic status of the chloroplast with light (shade) signaling and
558 developmental responses likely contributes to achieve optimal photosynthetic
559 performance.

560

561 **MATERIALS AND METHODS**

562 **Plant material**

563 All mutants used in this work are listed in Table S1. *Arabidopsis thaliana* lines
564 used here were in the Columbia (Col) background with the only exception of *hy1-*
565 *1*, a Landsberg *erecta* (Ler) mutant (Rodriguez-Concepcion et al., 2004). Some
566 of those lines were already available in our lab and previously used in published
567 works, including *hdr-3* (Pokhilko et al., 2015), *gun1-101* (Llamas et al., 2017),
568 *gun5-1* (Llamas et al., 2017), *pifQ* (Toledo-Ortiz et al., 2010), *hy5-2* (Bou-Torrent
569 et al., 2015), *psy-1* (Pokhilko et al., 2015), *lut2* (Emiliani et al., 2018), *aba2* (Ruiz-
570 Sola et al., 2014), and *hy5 35S:HA-HY5* (Toledo-Ortiz et al., 2014). Lines *abi3-8*
571 (*Nambara et al., 2002*), *abi4-1* (*Finkelstein et al., 1998*), *abi5-7* (*Tamura et al.,*
572 *2006*), and *35S:GUS-PIF3* (*Monte et al., 2004*) were requested. For generation of
573 double mutants, single homozygous plants were crossed and the F2 progeny
574 was first screened for the characteristic long hypocotyl phenotype associated to
575 the *hy5* mutation in homozygosis. Long individuals were then PCR-genotyped to
576 identify homozygous mutants for the second gene and confirm that they were
577 also homozygous for *hy5*. For the generation of the *35S:HY5-GFP* construct, the
578 full coding region of the *Arabidopsis HY5* cDNA was PCR-amplified using primers
579 HY5-attB1-F and HY5-attB2-R (Table S2) and cloned into Gateway pDONR-207.
580 Cloning into Gateway pGWB405 eventually generated the construct for the 35S
581 promoter-driven expression of a C-terminal fusion of the sGFP reporter protein to
582 HY5. This construct was used to transform the *hy5-2* mutant by floral dipping.
583 The *hy5 35S:HY5-GFP* line used for the experiments reported here was selected
584 based on complete complementation of the long hypocotyl phenotype associated
585 with the *hy5* mutation and high levels of nuclear GFP fluorescence. Line

586 *35S:PHYB-GFP* was generated by transforming Col-0 plants with the same
587 construct previously found to work in an *Arabidopsis phyB* mutant in the Ler
588 background (Yamaguchi et al., 1999). From the resulting transformants, we
589 selected for further experiments one of the lines showing a clearer accumulation
590 of the phyB-GFP protein in nuclear bodies under W.

591

592 **Growth conditions and treatments**

593 Seeds were surface-sterilized and germinated on solid Murashige and Skoog
594 (MS) medium supplemented with 10 mg/ml of sucrose to provide carbon and
595 energy for albino seedlings to grow. When indicated, the medium was further
596 supplemented with different concentrations of norflurazon (NF, Zorial), lincomycin
597 (LIN, Sigma) or abscisic acid (ABA, Sigma). Other chemicals added to the
598 medium included epibrassinolide (1 μM), gibberellic acid (10 μM), picloram (5
599 μM), 2-(4-chlorophenylthio)-triethylamine chloride (25 μM), fosmidomycin (500
600 μM), chloramphenicol (50 μM), or phosphinotricin (100 μM). When comparing
601 different lines (e.g. WT vs. mutant), they were grown together on the same plate
602 instead of growing each line on a different plate. After stratification for at least 3
603 days at 4°C in the dark, plates were incubated in growth chambers at 22°C under
604 W of 20-24 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (R/FR = 1.6). When indicated, W was
605 supplemented with FR provided by GreenPower LED module HF far-red (Philips)
606 QB1310CS-670-735 light-emitting diode hybrid lamps (Quantum Devices) to
607 simulate canopy shade (20-24 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, R/FR = 0.02). Fluence rates
608 were measured using a Spectrosense 2 meter associated with a 4-channel
609 sensor (Skye Instruments Ltd.) as described (Martinez-Garcia et al., 2014).
610 Grown seedlings were laid out flat on the growth media and digital images were
611 taken to quantify hypocotyl length using the NIH ImageJ software.

612

613 **Microscopy**

614 Whole *35S:PHYB-GFP* seedlings germinated and grown under W for 7 days on
615 media with or without 5 μM NF or 1 mM LIN were exposed to a 5 min pulse of FR
616 (735 nm, 60 $\mu\text{mol/m}^2/\text{s}$) and then kept in the dark. At different timepoints, treated
617 seedlings were placed on glass slides under a safety green light and kept in the
618 dark until observation with an Olympus BX60 FLUOVIEW FV300 microscope.
619 Confocal laser scan images of the hypocotyl area closer to the cotyledons were

620 obtained at different timepoints in the dark with a combination of 488 nm laser
621 excitation and 515 nm longpass filter (LP515; Carl Zeiss Jena). For each
622 timepoint, three sequential images from different focus planes were recorded
623 automatically.

624

625 **Chromatin immunoprecipitation**

626 About 800 µl of seeds from *hy5 35S:HY5-GFP* plants were plated on 8 square
627 (10 cm x 10 cm) plates of sucrose-supplemented medium. After growth for 2
628 days under W, 4 plates were left under W and 4 were transferred to W+FR for 5
629 additional days. For chromatin immunoprecipitation (Moon et al., 2008), each
630 sample was divided in 3 aliquots after crosslinking and sonication: one input, one
631 to be incubated with a 1:1000 dilution of anti-GFP antibody (Life Technologies),
632 and the last one to be processed similarly but without antibody. After DNA
633 isolation, the three samples were used for qPCR analysis of promoter sequence
634 abundance with the primers shown in Table S2. After normalization with the
635 input, enrichment was calculated as the ratio of the signal with vs. without
636 antibody.

637

638 **Gene expression and immunoblot analyses**

639 Total RNA was extracted from whole seedlings and used for qPCR analysis as
640 described (Llamas et al., 2017) with the gene-specific primers listed in Table S2.
641 Protein extraction, immunoblot analysis, and quantification of protein abundance
642 were performed as described (Llamas et al., 2017) using a 1:1000 dilution of anti-
643 GFP serum (Life Technologies).

644

645 **Quantification of metabolite levels**

646 Whole seedlings were frozen in liquid nitrogen, lyophilized, and ground in a
647 mortar for extraction and quantification of photosynthetic pigments and ABA.
648 Chlorophyll and carotenoid levels were measured either by spectrometric
649 methods or by HPLC (Bou-Torrent et al., 2015). ABA content was quantified by
650 LC/ESI-MS/MS as described (Ruiz-Sola et al., 2014).

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666

667 AUTHOR CONTRIBUTIONS

668 MOA, JFMG and MRC designed the research; MOA, EL, and AGC performed
669 research; AN contributed analytic tools; MOA, EL, AN, JFMG and MRC analyzed
670 data; JFMG and MRC wrote the paper.

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