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

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Short title: Interaction of light and retrograde pathways

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

RESULTS AND DISCUSSION

Functional chloroplasts are required for full response to simulated shade.

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

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

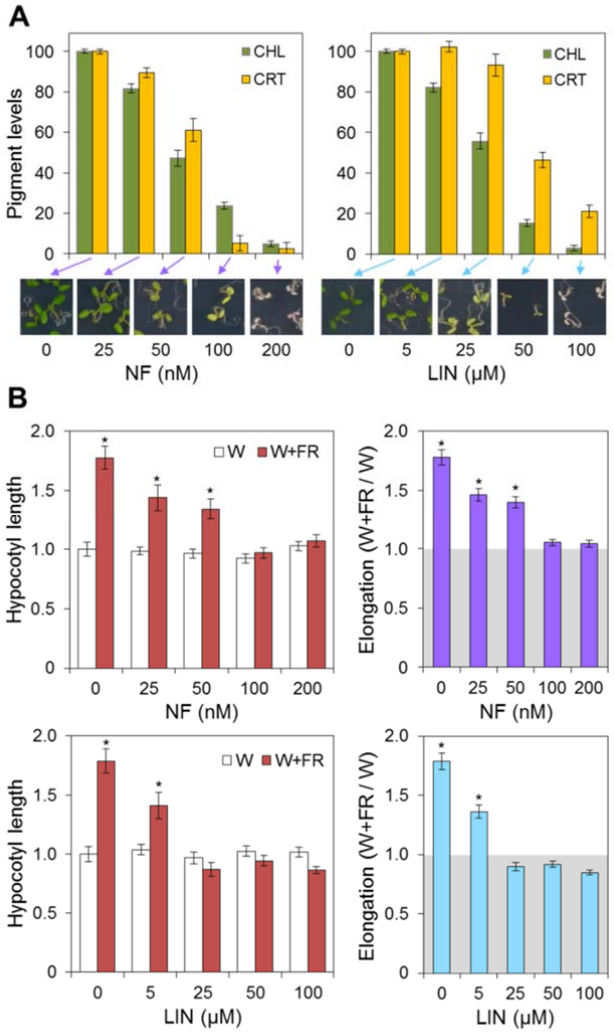

Figure 1. Hypocotyl elongation in response to shade requires functional chloroplasts. (A) WT (Col) plants were germinated and grown under W for 7 days on media with or without the indicated concentrations of NF or LIN. Graphs represent the mean and SEM values of total chlorophyll (CHL) and carotenoid (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 that 1 (T-test, p≤0.05), i.e. responsive to shade by increasing hypocotyl elongation.

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

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

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

Defective chloroplast function impairs phytochrome-mediated shade signaling.

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

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

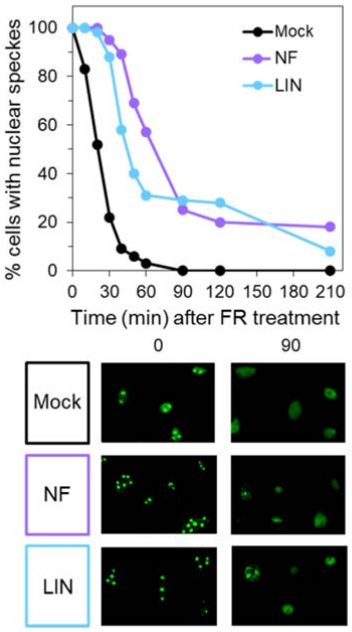

Figure 2. Retrograde signals prevent phyB deactivation in response to shade. Transgenic 35S:PHYB-GFP plants were germinated and grown under W for 7 days on media with or without 5 ≈M NF or 1 mM LIN. Following a 5 min irradiation with FR to simulate shade, the distribution of the phyB-GFP reporter protein 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.

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

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

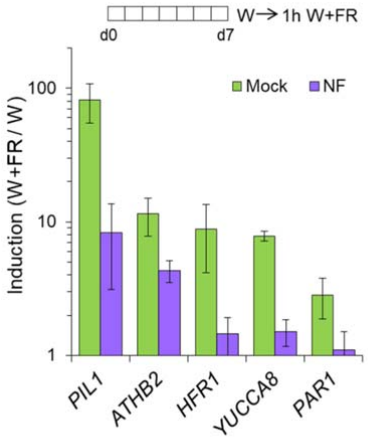

Figure 3. Shade-triggered induction of phytochrome primary target genes is attenuated in bleached seedlings. WT plants were germinated and grown under W for 7 days on media with or without 5 ≈M NF. Before and after 1h of exposure to W+FR, RNA was isolated from seedlings and used to analyze the transcript 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.

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

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

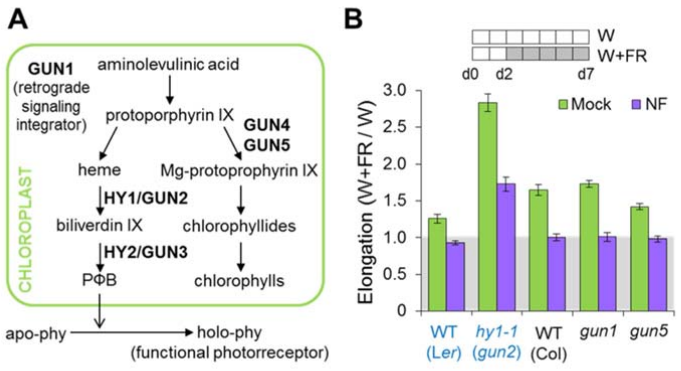

Figure 4. gun mutants show different elongation responses to shade. (A) Roles of GUN proteins in retrograde signaling and production of chlorophylls, heme, and the phytochrome chromophore. (B) Elongation responses to shade in mutants defective in some of the GUN proteins represented in (A). Mutants and their respective WT backgrounds (Ler for hy1/gun2 and Col for the rest) were germinated and grown as indicated with or without 5 ≈M NF. Graph represents the mean and SEM values of at least two independent experiments with nħ25 seedlings each.

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

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

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

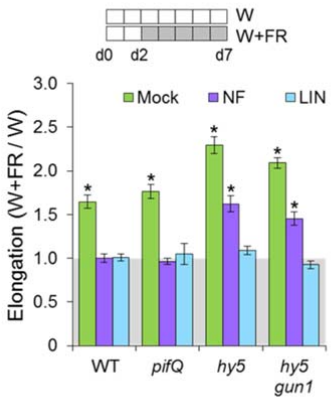

Figure 5. HY5 represses shade-triggered hypocotyl elongation in a GUN1-independent manner. WT (Col) as well as single (hy5), double (hy5 gun1) and quadruple (pifQ) mutant lines were germinated and grown as indicated with or without 5 ≈M NF or 1 mM LIN. Graph represents the mean and SEM elongation values of at least two independent experiments with n≈25 seedlings each. Asterisks mark statistically significant responses to shade (T-test, p≈0.05).

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

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

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

Carotenoid-derived products repress shade-triggered hypocotyl elongation.

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

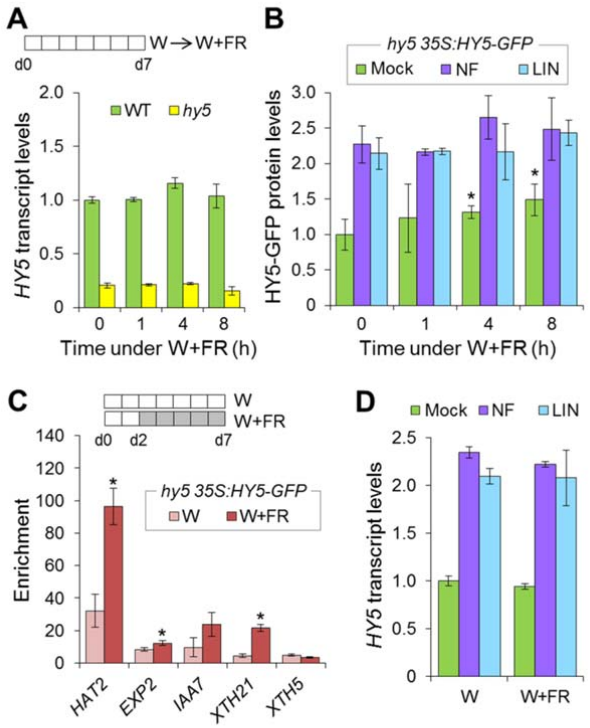

Figure 6. HY5 levels are regulated by shade and retrograde signals. (A) Levels of HY5-encoding transcripts in WT and hy5 plants germinated and grown under W for 7 days and then exposed to W+FR for the indicated times. Transcript levels were quantified by qPCR and represented relative to those in W-grown 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 ≈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 ≈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).

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

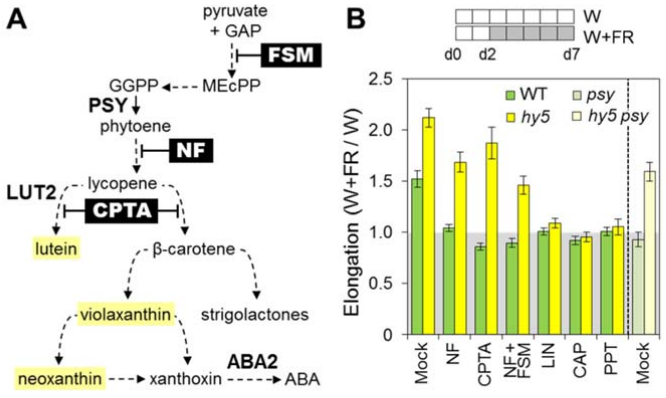

Figure 7. Blockage of the carotenoid pathway derepresses shade-triggered elongation of bleached HY5-defective seedlings. (A) Pathways for the biosynthesis of carotenoids and derived hormones. The steps targeted by NF and other inhibitors and the reactions catalyzed by enzymes that determine metabolic 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 nħ30 seedlings in a representative experiment.

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

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

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

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

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

ABA represses the elongation response to shade.

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

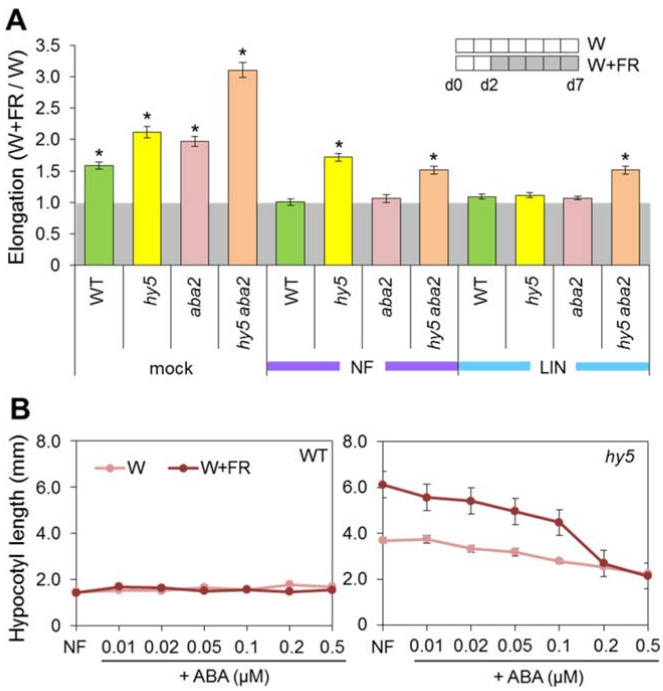

Figure 8. ABA represses shade-triggered hypocotyl elongation independent of HY5. (A) Elongation responses to shade in WT and mutant plants defective in HY5, ABA2, or both. Plants were germinated and grown as indicated with or without 5 ≈M NF or 1 mM LIN. Graph represents the mean and SEM values of a total of nħ25 seedlings from at least two independent experiments. Asterisks mark statistically significant responses to shade (T-test, pĦ0.05). (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 ≈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 nħ25 seedlings in a representative experiment).

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

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

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

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

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

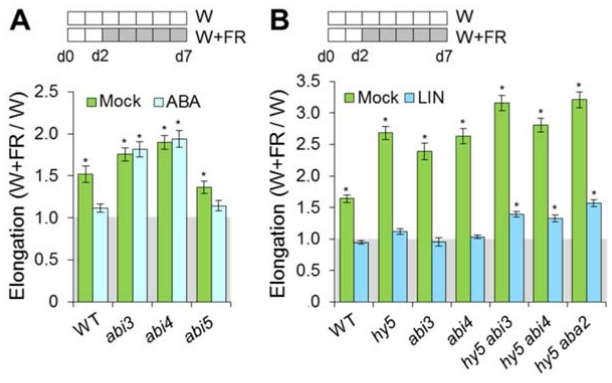

Figure 9. ABI3 and ABI4 but not ABI5 participate in the ABA-mediated repression of shade-induced hypocotyl elongation. (A) Effect of ABA on the elongation responses to shade of WT seedlings and mutants defective in ABI3, ABI4 or ABI5. Plants were germinated and grown as indicated on media with or without 0.2 ≈M ABA. Graph represents the mean and SEM values of a total of nħ25 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 nħ25 seedlings from two independent experiments. Asterisks mark statistically significant responses to shade (T-test, pH0.05).

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

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

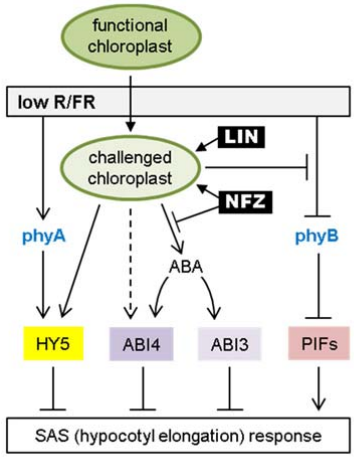

Figure 10. Model for the modulation of shade elongation responses by retrograde signals. In green plants with functional chloroplasts, low R/FR (i.e. canopy shade) signals promote accumulation of growth-promoting PIFs (via phyB deactivation) but also of growth-repressing HY5 (via phyA), likely to prevent 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.

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

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

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

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

Arabidopsis mutants defective in phyB were found to accumulate greater amounts of ABA under well-watered conditions and to be less sensitive to exogenous ABA treatments (Gonzalez et al., 2012). Further supporting a negative role of light for ABA synthesis, dark treatment of previously light-grown plants resulted in increased ABA contents (Weatherwax et al., 1996). A shade-triggered increase in ABA production was reported here (Figure S6) and elsewhere (Cagnola et al., 2012; Gonzalez-Grandio et al., 2013; Holalu and Finlayson, 2017). It is possible that W+FR treatment might promote ABA production to repress the elongation response to shade as part of the mechanism that prevents a too intense commitment (Figure 10). These results together support ABA as a central signal connecting the functional status of the chloroplast with light responses. Interestingly, the plastid-synthesized metabolite 3'-phosphoadenosine 5'-phosphate (PAP), which functions as a retrograde signal during oxidative stress caused by high light exposure and drought, was recently shown to act in concert with ABA signaling in guard cells to mediate stomatal closure and in seeds to mediate dormancy and germination (Pornsiriwong et al., 2017). PAP accumulates when the SAL1 phosphatase that normally degrades this metabolite is inactivated during oxidative stress (Estavillo et al., 2011). SAL1-defective mutants show a short hypocotyl phenotype in the light, indicating that accumulation of PAP can repress hypocotyl elongation (Kim and von Arnim, 2009; Chen and Xiong, 2011). This phenotype is rescued (at least partially) in 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. Futher
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* (L*er*) 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 L*er*
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 or 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 (25μM), 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 μmol m^{-2} 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 μmol m^{-2} 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 μmol/m2/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

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

Chromatin immunoprecipitation

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

Gene expression and immunoblot analyses

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

Quantification of metabolite levels

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

ACKNOWLEDGEMENTS

We thank Elena Monte (CRAG) for comments on the manuscript. Technical support from M. Rosa Rodríguez-Goberna and members of the CRAG core facilities is greatly appreciated. This work was funded by grants BIO2014-59895-P, BIO2014-59092-P, BIO2015-71703-REDT, BIO2017-90877-REDT, BIO2017-85316-R and BIO2017-84041-P from the Spanish Ministry of Science, Innovation, and Universities (MICINN) and grants 2017SGR-1211 and 2017SGR-710 from Generalitat de Catalunya to JFMG and MRC. Funding from the Japan Society for the Promotion of Science (JSPS) KAKENHI grant JP-15H04389 to AN is also acknowledged. We thank the financial support of the MINECO Severo Ochoa Programme for Centres of Excellence in R&D 2016-2019 (SEV-2015-0533) and the Generalitat de Catalunya CERCA Programme to CRAG. MOA and EL were supported by PhD fellowships from Spanish MINECO (BES-2012-052597) and Mexican CONACYT (421688 and SEP “beca complemento”), respectively.

AUTHOR CONTRIBUTIONS

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

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