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Research Article

Light signals generated by vegetation shade facilitate acclimation to low light in shade-avoider plants

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Abstract

When growing in search for light, plants can experience continuous or occasional shading by other plants. Plant proximity causes a decrease in the ratio of R to far-red light (low R:FR) due to the preferential absorbance of R light and reflection of FR light by photosynthetic tissues of neighboring plants. This signal is often perceived before actual shading causes a reduction in photosynthetically active radiation (low PAR). Here, we investigated how several Brassicaceae species from different habitats respond to low R:FR and low PAR in terms of elongation, photosynthesis, and photoacclimation. Shade-tolerant plants such as hairy bittercress (*Cardamine hirsuta*) displayed a good adaptation to low PAR but a poor or null response to low R:FR exposure. In contrast, shade-avoider species, such as Arabidopsis (*Arabidopsis thaliana*), showed a weak photosynthetic performance under low PAR but they strongly elongated when exposed to low R:FR. These responses could be genetically uncoupled. Most interestingly, exposure to low R:FR of shade-avoider (but not shade-tolerant) plants improved their photoacclimation to low PAR by triggering changes in photosynthesis-related gene expression, pigment accumulation, and chloroplast ultrastructure. These results indicate that low R:FR signaling unleashes molecular, metabolic, and developmental responses that allow shade-avoider plants (including most crops) to adjust their photosynthetic capacity in anticipation of eventual shading by nearby plants.

Introduction

Light is essential for plants as a source of energy and environmental information. Shading by nearby individuals can reduce light quantity (i.e. photon supply) and hence compromise photosynthetic activity and growth, a problematic situation in intensive cropping systems. To deal with the outcomes of mutual shading, plants have developed response mechanisms based on the perception of light quality, i.e. spectral information (Casal, 2013; Martinez-Garcia et al., 2010). The preferential absorbance of red (R) light and reflection of far-red (FR) light by photosynthetic tissues results in a decreased ratio of R to FR (R:FR) when light is reflected

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from or filtered through green stems and leaves. The low R:FR is a very reliable light signal that announces the close presence of nearby plants that may compete for resources.

Plants growing in ecosystems where access to light is restricted (e.g. in forest understories) show a shade-tolerant habit by adapting their light capture and utilization systems to low light intensity conditions. In contrast, plants growing in open habitats are shade-avoiders (also referred to as shade-intolerant or sun-loving). In shade-avoider plant species, such as Arabidopsis (*Arabidopsis thaliana*) and most sun-loving crops, perception of the low R:FR signal by the phytochrome photoreceptors activates a signaling pathway that eventually triggers a set of responses known as the shade-avoidance syndrome (SAS). The most prominent phenotype following exposure to low R:FR is elongation (e.g. of seedling hypocotyl, leaf petiole, and stem internode tissues), intended to overgrow neighboring competitors and outcompete them in the access to light. If the neighboring individuals overgrow and eventually shade the plant, the consequent reduction in light quantity (i.e. in the amount of radiation available for photosynthesis) results in additional and stronger SAS responses such as reduced leaf size, attenuated defense mechanisms, and early flowering (Roig-Villanova and Martinez-Garcia, 2016).

The most extensively studied SAS response by far is hypocotyl elongation in *A. thaliana* (At). In this species, low R:FR inactivates phytochrome B (phyB), releasing PHYTOCHROME INTERACTING FACTORs (PIFs) that can then regulate gene expression and promote elongation growth. This response is also repressed by negative SAS regulators such as ELONGATED HYPOCOTYL 5 (HY5), amongst many others (Cifuentes-Esquivel et al., 2013; Ciolfi et al., 2013). Biological activity of these transcription factors can be modulated by additional components of the SAS regulatory network such as LONG HYPOCOTYL IN FAR-RED 1 (HFR1, which binds PIFs to prevent their binding to target genes) and phytochrome A (phyA, which gets stabilized in shade and then promotes HY5 accumulation; Ciolfi et al., 2013; Martinez-Garcia et al., 2014; Yang et al., 2018). Both HFR1 and phyA hence act as additional SAS repressors that were recently found to be instrumental for the adaptation to shade. Indeed, the shade-tolerant hairy bittercress (*Cardamine hirsuta*), a close relative of At, does not elongate when exposed to low R:FR unless the function of phyA or HFR1 is genetically lost in mutant plants (Hay et al., 2014; Molina-Contreras et al., 2019; Paulisic et al., 2021).

Differences between shade-avoider and shade-tolerant species are not restricted to changes in elongation after exposure to low R:FR. Photoacclimation (i.e. the ability of plants to adjust photosynthesis to changes in the incident light with specific phenotypic changes) also diverges. Variation of photoacclimation responses among species on day-to-week time scale has been associated to two main strategies (Murchie and Horton, 1997; Ptushenko and Ptushenko, 2019). The first one consists of an alteration of photosynthetic pigment content, which positively corresponds with photosynthetic capacity. The second one involves changes in the photosynthetic machinery, which appears to be more important in plant species from environments where temporal and spatial variations in light irradiance are common, e.g. margins of woodlands. Combinations of these two main strategies give rise to the observed diversity in photoacclimation. In the case of At and *C. hirsuta* (Ch), a differential response to low R:FR in terms of photosynthetic pigment accumulation has been observed. Chlorophyll and carotenoid levels drop about 20% in At plants grown under low R:FR conditions, whereas the decrease is attenuated in Ch plants (Molina-Contreras et al., 2019). Whether photosynthetic capacity and/or chloroplast ultrastructure is differentially impacted by low R:FR in these species remains unknown. In terms of light quantity, the shade-avoider At showed a lower capacity to acclimate to reduced photosynthetically active radiation (low PAR) but a higher capacity to acclimate to intense light (high PAR) compared to the shade-tolerant Ch (Molina-Contreras et al., 2019). A similar physiological behavior has been described for shade-avoider and shade-tolerant species of the genus *Tradescantia* (Benkov et al., 2019), a model to study the ecology of photosynthesis and the mechanisms of photoacclimation in plants (Ptushenko and Ptushenko, 2019). The possible connections between low R:FR signaling and photoacclimation responses in plants remain, however, virtually unknown. Here, we explored natural and engineered genetic diversity to investigate this connection using different Brassicaceae species.

Results

Different Brassicaceae species present divergent photoacclimation responses

We previously showed that, compared to sun-loving At Col-0, shade-tolerant Ch Ox exhibits a better ability to maintain photosynthesis after transfer to low PAR but a stronger chlorophyll loss when light intensity increases (Molina-Contreras et al., 2019). To better characterize the photoacclimation responses of these two Brassicaceae species, both At and Ch were germinated and grown for 7 d under control conditions of a photosynthetic photon flux density (PPFD) in the PAR region of 20–24 μmol m^{-2} s^{-1} (W_{20}). Then they were transferred to either lower PAR (W_4, PPFD of 4 μmol m^{-2} s^{-1}) or higher PAR (W_{200}, PPFD of 200 μmol m^{-2} s^{-1}) for up to seven more days (Figure 1). Light curve analysis at day 3 after the transfer already showed clearly opposite responses of At and Ch, i.e. a better photosynthetic activity of Ch compared to At when transferred to W_4 and a better activity of At compared to Ch when transferred to W_{200} (Figure 1A). Derived parameters such as maximum electron transport rate (ETRm) and photosynthetic rate in light-limited region of the light curve (alpha) also illustrated that At performed better than Ch after transfer to higher light (W_{200}) but worst after transfer to lower light (W_4; Figure 1B). Other photosynthetic parameters such as maximum quantum efficiency of PSII (Fv/Fm) and light use

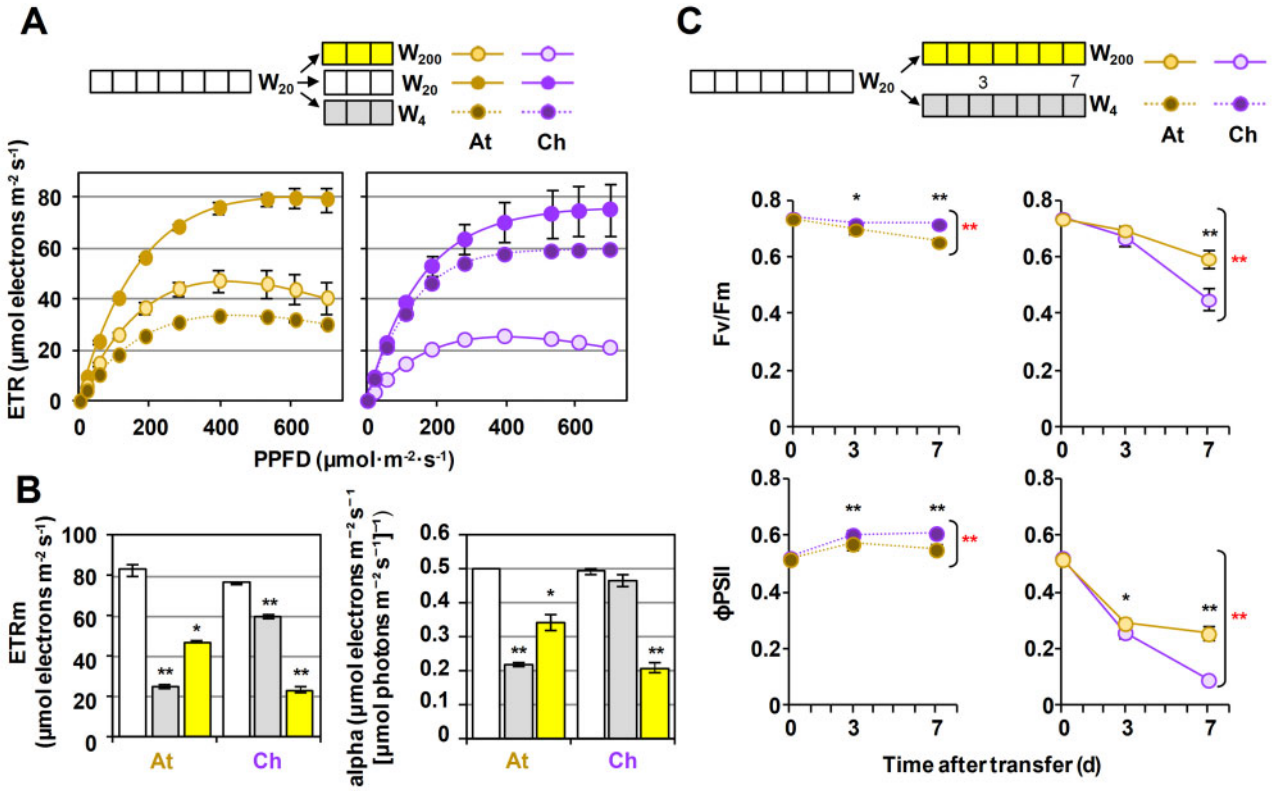

Figure 1 *Arabidopsis thaliana* and *Cardamine hirsuta* show antagonistic photoacclimation responses to higher and lower PAR. A, Light curves of At and Ch seedlings germinated and grown under white light of 20-µmol·m^{-2}·s^{-1} PPFD (W_{20}) for 7 d and then either kept under W_{20} or transferred to either 200 (W_{200}) or 4 (W_4) µmol·m^{-2}·s^{-1} PPFD for 3 more days. Values represent the mean and standard error of $n = 3$ plants for treatment. B, ETRm and photosynthetic rate in the light-limited region of the light curve (alpha) calculated from the curves shown in (A). Asterisks mark statistically significant changes (*t* test $^*P < 0.05$, $^{**}P < 0.01$) in W_4 or W_{200} relative to W_{20}. C, Maximum photochemical efficiency of PSII in the dark-adapted state (Fv/Fm) and effective quantum yield calculated at growth light (ΦPSII) of seedlings germinated and grown for 7 d under W_{20} and then transferred to either W_{200} or W_4 for seven more days. Data were taken at 0, 3, and 7 d after the transfer. Values are mean and standard error of $n = 7$ seedlings per treatment. Black asterisks mark statistically significant differences between At and Ch at each time point (*t* test $^*P < 0.05$, $^{**}P < 0.01$). Red asterisks indicate statistically significant differences between genotypes over time (two-way ANOVA, $^{**}P < 0.01$).

efficiency of PSII (ΦPSII) also showed differences between At and Ch at day 3 after transfer, but these differences became clearer at longer times of exposure to either W_{200} or W_4 (Figure 1C). Specifically, Fv/Fm values were lower in Ch than in At after transfer to higher light, while the opposite was observed when transferred to lower light. A similar trend was observed in the case of ΦPSII (Figure 1C). These results together indicate that Ch tolerates better the transfer to lower PAR (consistent with Ch being more tolerant to shade), while an increase in light irradiance compromises photosynthetic efficiency in Ch more than in shade-avoider At. Based on these results, we used light curve analysis at day 3 or earlier to estimate photoacclimation to lower PAR and Fv/Fm measurements at day 7 to estimate photoacclimation to higher PAR.

Besides At and Ch, the Brassicaceae family (mustards) includes many food crops (e.g. cauliflower, broccoli, radish, cabbage, kale, and similar green leafy vegetables) and a diversity of wild species from forested and open habitats. As a first step to explore the possible connection between low PAR and low R:FR responses, we analyzed photoacclimation and hypocotyl elongation in six different Brassicaceae species or accessions, including At and Ch as controls. The selected wild mustards were alpine rock cress (*Arabis alpina*, Aa), two accessions of shepherd's purse (*Capsella bursa-pastoris*), Freiburg-1 (Cb-F) and Strasbourg-1 (Cb-S), pink shepherd's-purse (*Capsella rubella*, Cr), watercress (*Nasturtium officinale*, No), and London rocket (*Sisymbrium irio*, Si). Initially, we aimed to classify them as shade-avoider or shade-tolerant based on photoacclimation responses. After germination and growth for 7 d under W, seedlings were either kept under control W_{20} or transferred to lower light (W_4). Light curve analyses at day 1 after the transfer already showed differential responses that served to classify the accessions in two groups (Figure 2). Similar to the shade-avoider At, seedlings of Cb-F, Cb-S, and Cr showed a lowering of the curve under W_4 conditions, whereas those of Aa, No, and Si behaved as the shade-tolerant Ch and showed virtually identical light curves under W_{20} and W_4 (Figure 2A). ETRm and alpha values also illustrated that the W_4 treatment led to decreased photosynthetic performance in At, Cb-F, Cb-S, and Cr but not in Ch, Aa, No, and Si (Figure 2B;

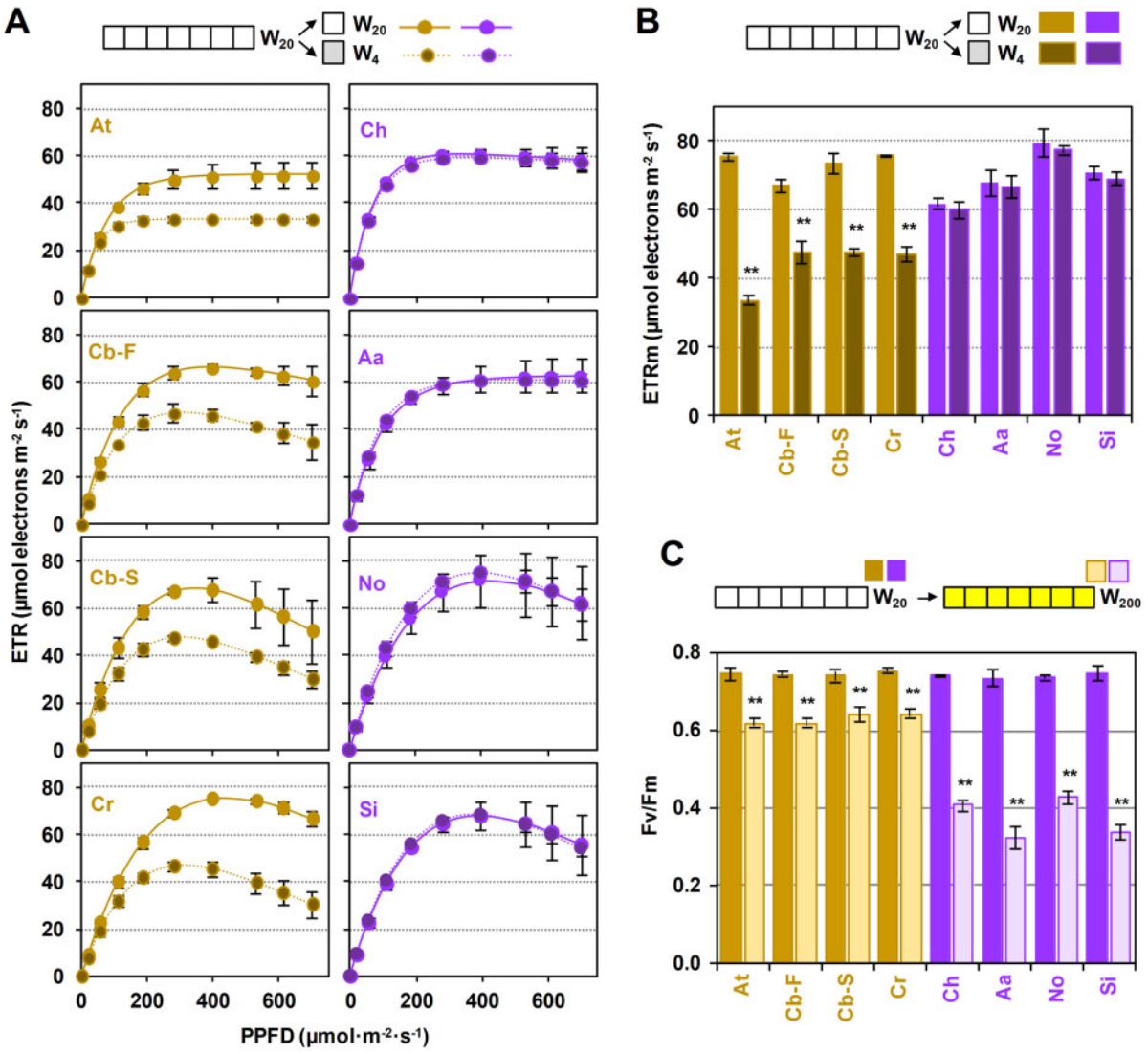

Figure 2 Brassicaceae plants can be grouped with either *A. thaliana* or *C. hirsuta* based on their photoacclimation responses. A, Light curves of *Arabidopsis thaliana* (At), *Capsella bursa-pastoris* (Cb-F and Cb-S), *Capsella rubella* (Cr), *Cardamine hirsuta* (Ch), *Arabis alpina* (Aa), *Nasturtium officinale* (No), and *Sysimbrium irio* (Si) seedlings germinated and grown under white light (W_{20}) for 7 d and then either kept under W_{20} or transferred to lower PAR (W_4) for one more day. Values represent the mean and standard error of $n = 3$ plants for treatment. B, ETRm values calculated from the curves shown in (A). C, Fv/Fm values of seedlings grown for 7 d under W_{20} and then transferred to higher PAR (W_{200}) for seven more days. Mean and standard error of $n = 9$ seedlings per treatment are represented. Asterisks in (B) and (C) mark statistically significant changes (*t* test, $^{**}P < 0.01$) relative to W_{20}.

Supplemental Figure S1). We next analyzed photoacclimation to increased irradiation quantifying Fv/Fm before or after transferring 7-d-old W_{20}-grown seedlings to W_{200} for seven additional days. Again, At grouped together with the two accessions of Cb and with Cr as they acclimated much better to high PAR compared to the group formed by Ch, Aa, No, and Si (Figure 2C). Together, these photoacclimation results led to classify the former group as shade-avoiders, and the latter as shade-tolerant species.

Photoacclimation responses can be uncoupled from shade-driven hypocotyl elongation

Next, we investigated whether the classification of the selected mustard species as shade-avoider or shade-tolerant based on their photoacclimation features corresponded with their elongation response to low R:FR. After germination and growth for 3 d under W_{20} (R:FR = 1.5–3.3), seedlings were either kept under W_{20} or transferred to FR-supplemented W_{20} (W_{20}+FR, R:FR = 0.02) for four additional days, and then hypocotyl length was measured (Figure 3). Similar to At, the Cb-F accession showed a strong hypocotyl elongation response, whereas Cb-S, Cr, and No elongated moderately in response to low R:FR. In contrast, Ch, Aa, and Si did not elongate in response to low R:FR (Figure 3A). These results confirm that the elongation response to low R:FR cannot be fully predicted based on the photoacclimation phenotype of a particular accession. Nonetheless, accessions classified as shade-avoider based on their photoacclimation

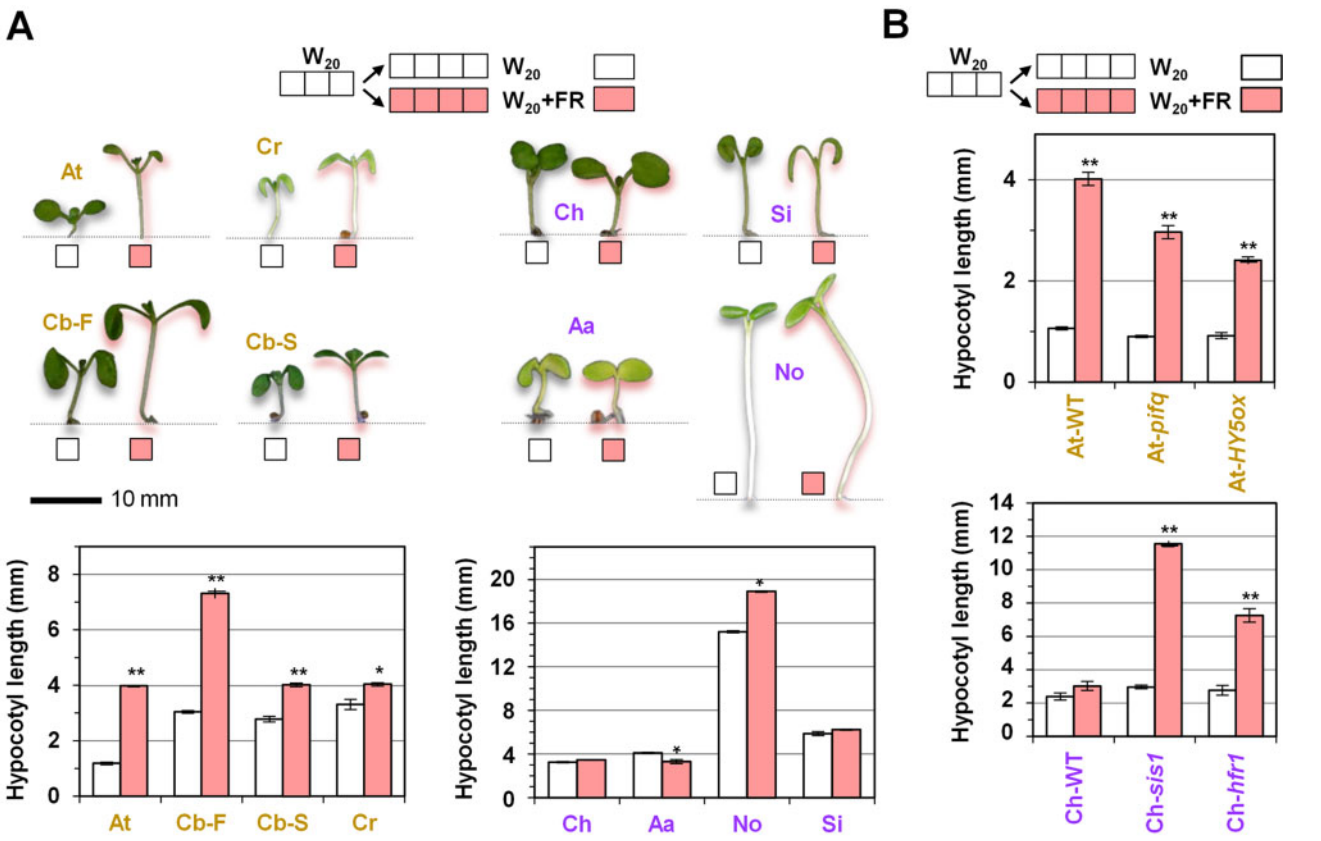

Figure 3 The hypocotyl elongation response to low R:FR is plastic in Brassicaceae plants. A, The indicated genotypes were germinated and grown under W_{20} for 3 d and then either kept under W_{20} or transferred to low R:FR (W_{20}+FR) for four more days. Then, pictures were taken and hypocotyl length was measured. B, Hypocotyl length of the indicated mutants grown as indicated in (A). In both (A) and (B), mean and standard error of measurements from at least 20 seedlings in $n = 3$ independent experiments per treatment are represented. Asterisks mark statistically significant changes in W_{20}+FR relative to W_{20} (*t* test, $^*P < 0.05$, $^{**}P < 0.01$).

behavior (i.e. poor photoacclimation to decreased PAR but good photoacclimation to increased PAR) exhibit a range of elongation responses to low R:FR (i.e. from moderate to strong elongation), whereas plant species with a shade-tolerant photoacclimation responses display either no elongation or a mild shade-avoider phenotype in terms of hypocotyl elongation when exposed to low R:FR (e.g. No).

The shade-avoider or shade-tolerant elongation phenotype in response to low R:FR can be reversed by manipulating the levels of specific SAS regulators. Previous results have shown that At lines overexpressing *HY5* (At-*HY5ox*) display an attenuated hypocotyl response to low R:FR (Ortiz-Alcaide et al., 2019), whereas a similar but weaker response was observed in a quadruple mutant defective in all members of the photolabile PIF quartet (PIFQ), PIF1, PIF3, PIF4 and PIF5 (At-*pifq*; Figure 3B). Despite the different degrees of elongation response to low R:FR, these two lines showed photoacclimation responses to lower PAR very similar to those of wild-type (At-WT) controls (Figure 4). Both light curves (Figure 4A) and ETRm values (Figure 4B) were almost identical in At-WT plants and mutants hyposensitive to low R:FR. In the case of Ch, lines deficient in phyA (Ch-*sis1*) or HFR1 (Ch-*hfr1*) gain the ability to elongate when exposed to low R:FR (Molina-Contreras et al., 2019; Paulisic et al., 2021; Figure 3B). In contrast to the shade-hyposensitive At mutants, the hypersensitive Ch mutant lines appeared to gain a partial shade-avoider phenotype in terms of photoacclimation to low PAR, as lower values of light curves (Figure 4A) and ETRm (Figure 4B) were observed under W_4 compared to W_{20}. However, photoacclimation to increased PAR (W_{200}) estimated from Fv/Fm values and also from chlorophyll levels (Molina-Contreras et al., 2019) was similar for Ch-WT, Ch-*sis1*, and Ch-*hfr1* plants (Figure 4C). We therefore concluded that manipulation of the plant ability to elongate in response to proximity shade hardly impacts their photoacclimation capacity, at least when plants are growing in the absence of the low R:FR signal.

Activation of low R:FR signaling causes a decrease in pigment levels and photosynthetic activity

Low R:FR signals not only influence hypocotyl elongation but they are also known to reduce the contents of photosynthetic pigments (chlorophylls and carotenoids) in many plant species (Roig-Villanova et al., 2007; Cagnola et al., 2012; Patel et al., 2013; Bou-Torrent et al., 2015; Molina-Contreras et al., 2019). The reduction is observed in both elongating (At-WT) and nonelongating (Ch-WT) seedlings, but it is stronger in the former (Figure 5). *Cardamine hirsuta* mutants that gained the ability to elongate in response to shade, such as Ch-*sis1* and Ch-*hfr1*, also displayed stronger

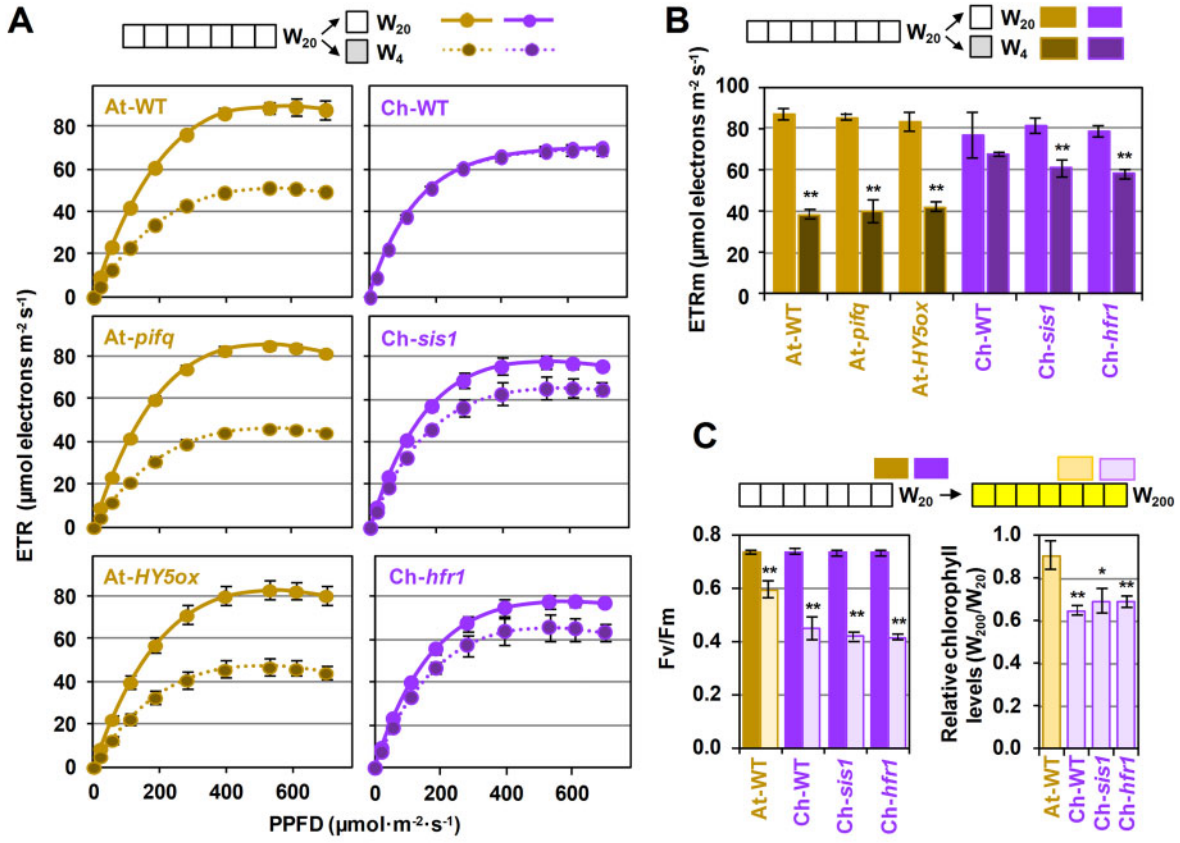

Figure 4 Mutations that alter sensitivity to low R:FR do not impact photoacclimation responses. A, Light curves of At and Ch wild-type and mutant seedlings germinated and grown under W_{20} for 7 d and then either kept under W_{20} or transferred to lower PAR (W_4) for one more day. Values represent the mean and standard error of $n = 3$ plants for treatment. B, ETRm values calculated from the curves shown in (A). C, Fv/Fm values and HPLC-determined relative chlorophyll levels of seedlings grown for 7 d under W_{20} and then transferred to higher PAR (W_{200}) for seven more days. Mean and standard error of $n = 9$ seedlings (Fv/Fm) or $n = 3$ independent pools (HPLC) per treatment are represented. Asterisks in (B) and (C) mark statistically significant changes (*t* test, $^*P < 0.05$, $^{**}P < 0.01$) relative to W_{20}.

reductions in photosynthetic pigment contents relative to Ch-WT after low R:FR exposure (Figure 5A; Molina-Contreras et al., 2019). Conversely, At mutants with a reduced ability to elongate in response to shade, such as At-*pifq* and At-*HY5ox* (Figure 3B), showed attenuated reduction of pigment contents relative to At-WT when exposed to low R:FR (Figure 5A).

To test whether decreases in photosynthetic pigment levels driven by simulated shade exposure might affect photosynthetic activity, we next measured Fv/Fm and ΦPSII in seedlings grown either under W_{20} or under W_{20}+FR (Figure 5B; Supplemental Figure S2A). Indeed, low R:FR was found to result in decreased photosynthetic activity in the lines with strong pigment loss responses independently on the species (At-WT, Ch-*sis1*, and Ch-*hfr1*). ETRm and alpha parameters also tended to be lower in W+FR-exposed At-WT, Ch-*sis1*, and Ch-*hfr1* seedlings compared to W controls (Figure 5C; Supplemental Figure S2B). The effect of low R:FR on photosynthesis was much less dramatic in the rest of the lines (At-*pifq*, At-*HY5ox*, and Ch-WT), which consistently displayed a reduced impact of W_{20}+FR exposure on their photosynthetic pigment levels (Figure 5).

Proximity shade signals have also been found to impact photosynthesis at the level of gene expression. Analyses of low R:FR-triggered transcriptomic changes showed reduced levels of transcripts encoding photosynthesis-related proteins (e.g. enzymes involved in chlorophyll and carotenoid biosynthesis, components of the photosynthetic apparatus, and/or members of the carbon fixation process) in several species, including alfalfa (Lorenzo et al., 2019), maize (Shi et al., 2019), tomato (Cagnola et al., 2012), and At (Leivar et al., 2012). Interestingly, the changes in the expression of photosynthesis-related genes triggered by low R:FR are attenuated in the At-*pifq* mutant compared to At-WT seedlings (Figure 6). This is particularly evident in the case of low R:FR-repressed photosynthetic genes (Figure 6), suggesting that the PIF-mediated regulation of gene expression in response to low R:FR is instrumental for the observed changes in photosynthesis (Figure 5).

Exposure of shade-avoider plants to low R:FR improves their photoacclimation to low PAR

The observation that exposure of low R:FR caused a decreased in photosynthetic activity of At-WT seedlings and

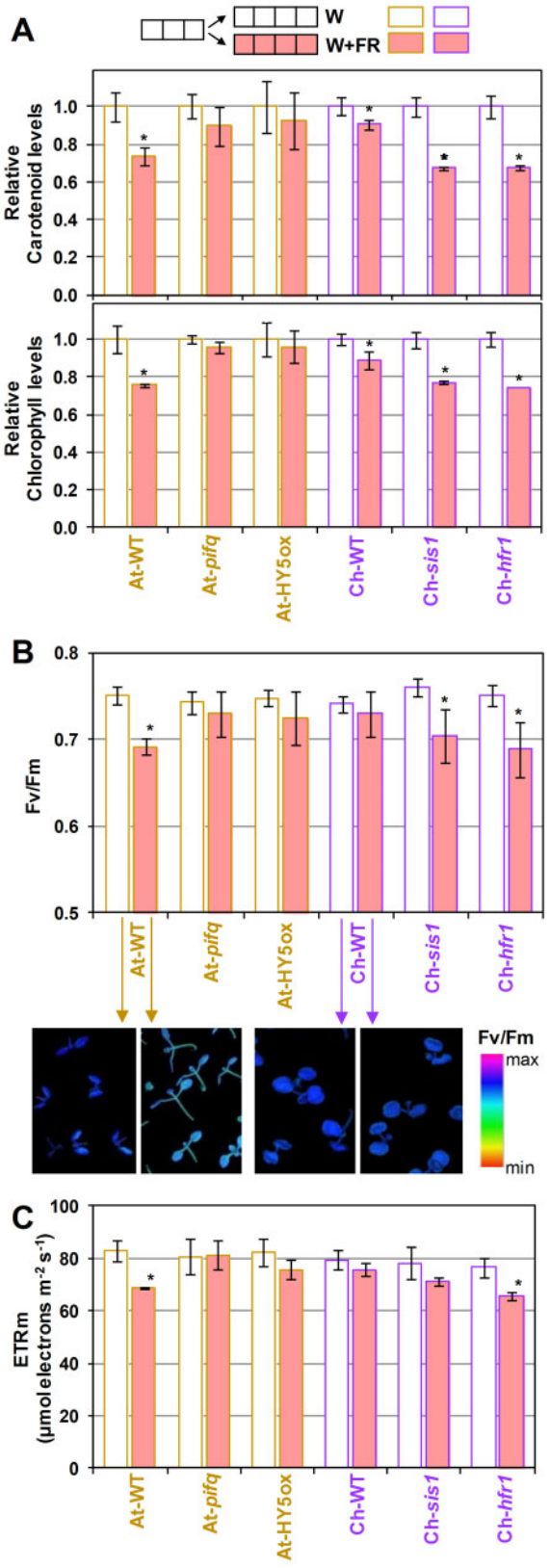

Figure 5 Activation of low R:FR signaling reduces photosynthetic pigment levels and activity. A, The indicated genotypes were germinated and grown under W_{20} for 3 d and then either kept under W_{20} or transferred to low R:FR (W_{20}+FR) for four more days. Then, the levels of photosynthetic pigments (carotenoids and chlorophylls) were quantified spectrophotometrically. B, Fv/Fm values of seedlings germinated and grown as indicated in (A). Lower pictures show false-color images in wild-type seedlings. (C) ETRm values of seedlings germinated and grown as indicated in (A). Mean and standard error of $n = 3$ independent pools of seedlings (A) or $n = 9$ seedlings (B and C) per treatment are represented. Asterisks mark statistically significant changes in W_{20}+FR relative to W_{20} (t test, $^*P < 0.05$).

shade-hypersensitive Ch mutants prompted us to analyze whether this light signal may also cause changes in chloroplast ultrastructure. Cotyledons from At-WT seedlings germinated and grown for 2 d under W_{20} and then either kept in W_{20} or transferred to W_{20}+FR for five additional days were collected and used for transmission electron microscopy (TEM). Chloroplasts from low R:FR-exposed samples were found to exhibit larger grana stacks and contain less and smaller plastoglobules compared to W-grown controls (Figure 7). Interestingly, similar changes are associated to low PAR photoacclimation (Rozak et al., 2002; Lichtenthaler, 2007; Wood et al., 2018). We therefore reasoned that exposure to low R:FR in the absence of any light intensity change might trigger responses to anticipate a foreseeable shading involving a decrease in PAR. To test this hypothesis, we analyzed light curves of WT and mutant seedlings grown in either W_{20} or W_{20}+FR and then transferred to lower PAR (W_4) for 3 d (Figure 8). Pre-exposure of At-WT seedlings to low R:FR (W_{20}+FR) resulted in a strongly attenuated reduction in ETRm after their transfer to lower PAR (Figure 8A). In contrast, At mutants with reduced SAS elongation responses also lost the response to low R:FR in terms of improved photoacclimation to lower PAR (W_4; Figure 8A). Pretreatment with W_{20}+FR had virtually no effect on the photoacclimation of Ch-WT seedlings to lower PAR (W_4) but caused a slight but significant improvement of ETRm in shade-hypersensitive Ch mutants at day 1 after transfer to W_4 (Figure 8A). When analyzing photoacclimation to higher PAR, pre-exposure of At-WT or Ch-WT seedlings to W_{20}+FR resulted in no improvement compared to W_{20}-grown controls (Figure 8B). If anything, Ch-WT seedlings grown under W_{20}+FR photoacclimated worse than W_{20}-grown seedlings when exposed to higher light intensity (Figure 8B).

The battery of mustards that grouped together with At in terms of photoacclimation responses (Cb-F, Cb-S, and Cr; Figure 2; Supplemental Figure S1) also showed improved photoacclimation to reduced PAR when pre-exposed to low R:FR, whereas the simulated shade signal did not have an effect on those clustered with Ch (Aa, No, and Si; Figure 8A). This low R:FR-dependent phenotype was independent of the growing light intensity and photoperiod, as it was also observed in At-WT seedlings growing under W_{200} or W_{200}+FR for 8 h or 16 h a day (i.e. under long day or short day conditions, respectively) and then transferred to W_{15} (Supplemental Figure S3). Because both the response of shade-avoider plants to low R:FR and the acclimation to low light involve a reduced respiration rate to cope with the limited generation of photoassimilates and hence contribute to

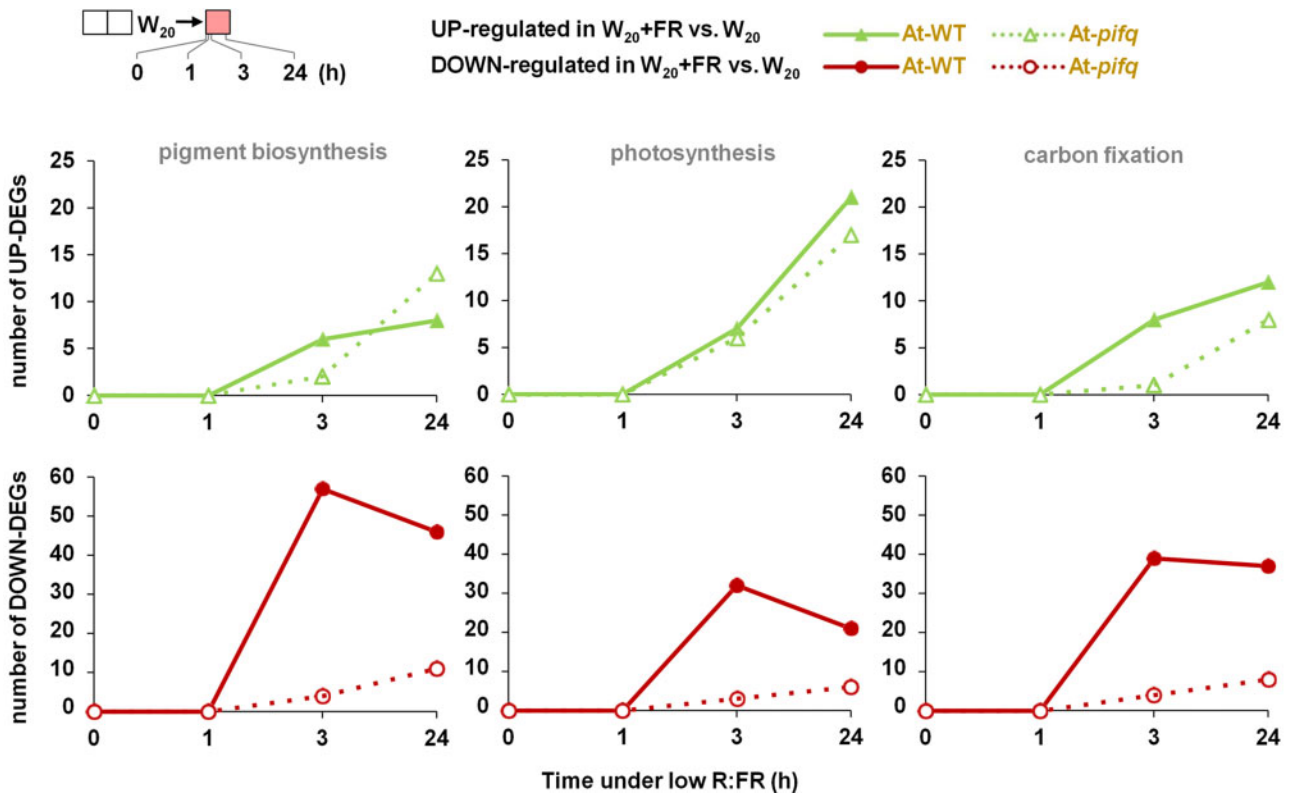

Figure 6 Exposure to low R/FR triggers changes in photosynthetic gene expression that are attenuated in the hyposensitive At-*pifq* mutant. Data were extracted from a publicly available experiment (Leivar et al., 2012). At-WT and At-*pifq* lines were germinated and grown under 19 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR white light (W_{20}, R:FR of 6.48) for 2 d and exposed to low R:FR (W_{20}+FR, R:FR of 0.006) for 0, 1, 3, or 24 h. Plots represent the number of DEGs either up- or downregulated in W_{20}+FR versus W_{20} that are involved in photosynthetic pigment biosynthesis (Kyoto Encyclopedia of Genes and Genomes pathways ath00906 and ath00860), photosynthesis (ath00195 and ath00196), and carbon fixation (ath00710).

carbon balance (Cagnola et al., 2012; Casal, 2013), we next measured changes in respiration in whole wild-type At and Ch seedlings exposed or not to low R:FR and then transferred to reduced PAR (Supplemental Figure S4). In W_{20} controls, respiration (estimated as total oxygen consumption in darkness) was reduced in At seedlings when they were moved to W_4. When exposed to W_{20}+FR, however, respiration was already lower and did not significantly change after transferring to lower PAR. In contrast, Ch seedlings showed similar respiration values in all conditions (Supplemental Figure S4). Based on these data we conclude that detection and transduction of low R:FR signals not only allows shade-avoider plants to overgrow their neighbors but also to pre-adapt their photosynthetic and respiratory machinery to foreseeable conditions of actual shading involving reduced PAR. In contrast, shade-tolerant plants have a better adapted capacity to grow under reduce PAR and do not seem to use the low R:FR signal.

Discussion

Plants have been traditionally classified as shade avoider and tolerant based mostly on their natural habitat, although virtually all plants are exposed to at least some degree of shade during their lifetime. As an ecological concept, shade tolerance refers to the capacity of a given plant to tolerate low light levels, but it is also associated with a wide range of traits, including phenotypic plasticity to optimize light capture (Valladares and Niinemets, 2008). Analyzing a range of caulescent herbs, it was suggested that the elongation response upon exposure to low R:FR was dependent on the shade habit, the shade-avoiders elongating the most and the shade-tolerant showing a mild or no elongation response (Smith, 1982). Indeed, elongation might not be the best solution for plants that spend all their lives under a canopy or permanently shaded by other plants. Another important parameter to ascertain the degree of shade tolerance of a plant is photoacclimation capacity, which is essential for plant fitness in environments with changing light input conditions (e.g. those where the growth of nearby plants may suddenly compromise access to light). By taking into account both parameters (the hypocotyl elongation response and the capacity to acclimate to low or high PAR), here we analyzed the shade tolerance of several Brassicaceae species, including the closely related mustard model systems At and Ch. As a rule of thumb, we observed that Ch and other species showing a good photoacclimation response to lower PAR (and badly performing after transfer to higher PAR) showed a poor or null elongation response to low R:FR (Figures 2 and

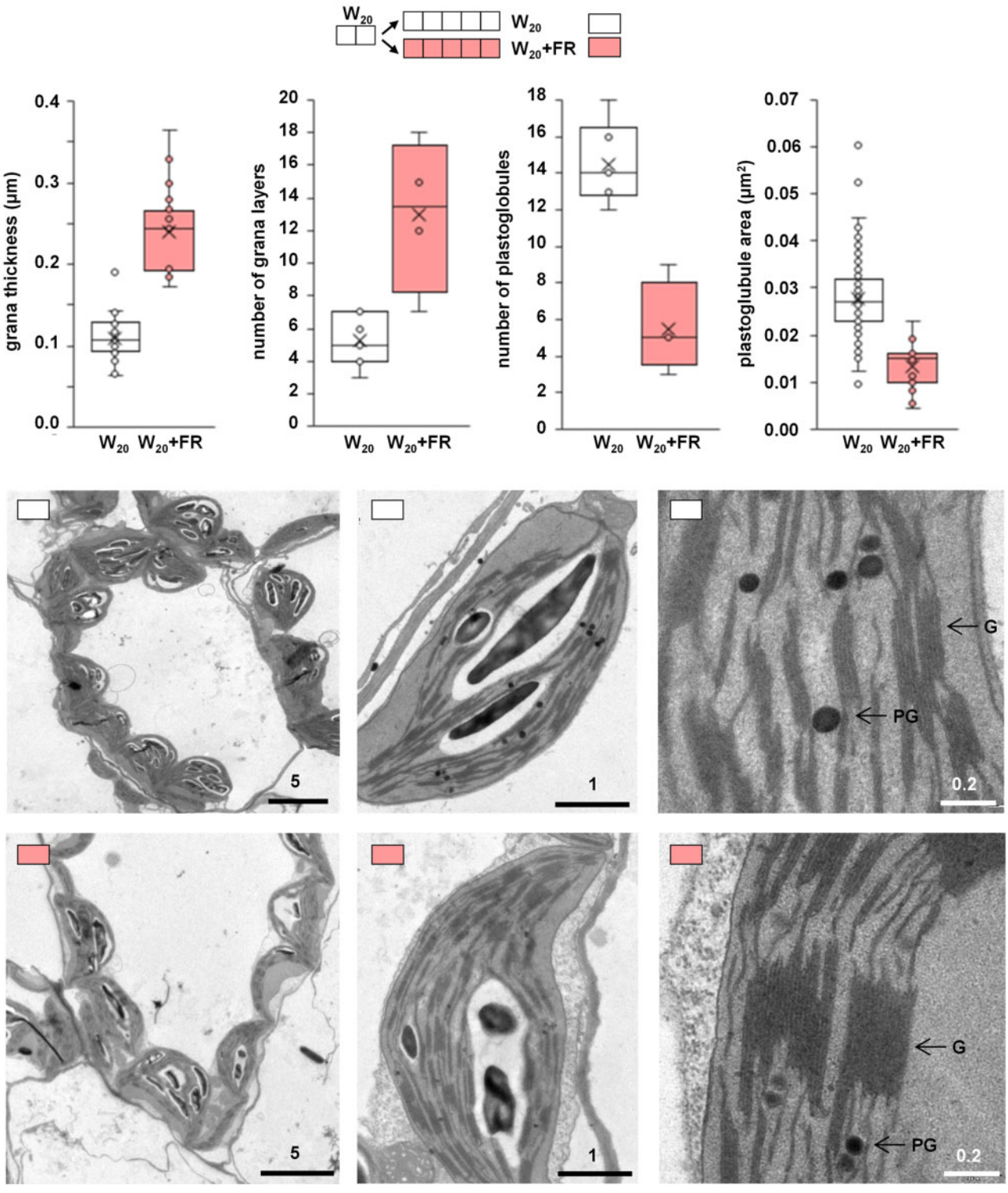

Figure 7 Low R:FR triggers ultrastructural changes in At chloroplasts. At-WT seeds were germinated and grown under W_{20} for 2 d and then either kept under W_{20} or transferred to low R:FR (W_{20}+FR) for 5 more days. Cotyledons were then used for TEM analysis of chloroplast ultrastructure. Representative pictures at different scales (numbers indicate micrometer) are shown. Boxplots show quantification of the indicated parameters from the images. Boxes show the values between the upper and the lower quartile, the cross represents the mean and the horizontal line the median. Whiskers (the upper and lower extremes) and circles represent single data and the ones located outside of the whiskers limit are the outliers (data with the same numerical value are visualized as a single point). For quantifying grana thickness, all the distinguishable structures were used (W_{20} $n = 30$, W_{20}+FR $n = 20$). For quantifying grana layers, four major grana complexes from higher magnifications were measured. For quantifying the number of plastoglobules, at least six individual chloroplasts for each treatment were used. Plastoglobule area was measured for all the plastoglobules (W_{20} $n = 87$, W_{20}+FR $n = 22$). PG, plastoglobules; G, grana.

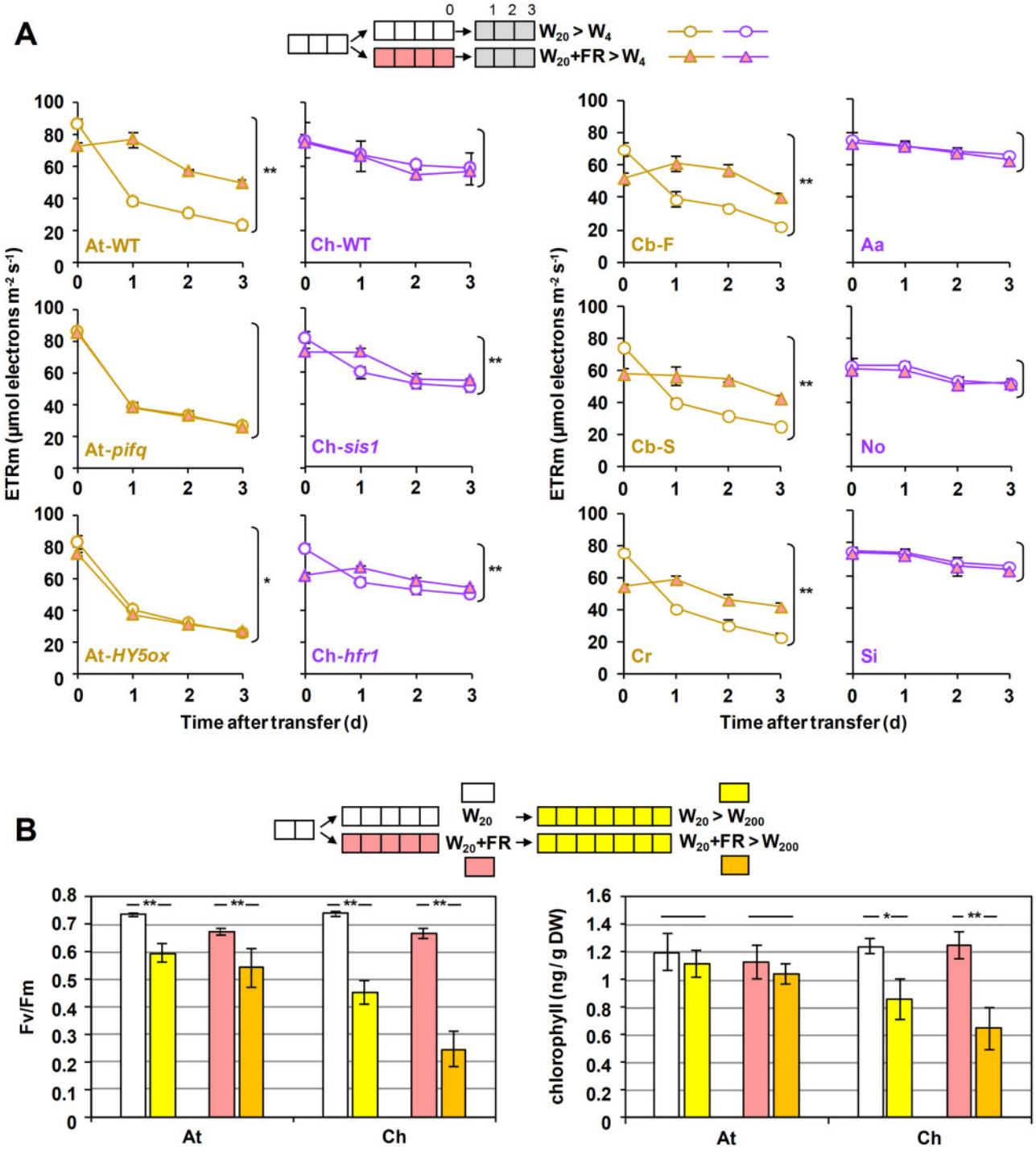

Figure 8 Pre-exposure to low R:FR improves the photoacclimation to low PAR in shade-avoider plants. A, The indicated genotypes were germinated and grown under W_{20} for 3 d, transferred to either W_{20} or W_{20}+FR for 4 d, and then exposed to W_4. Mean and standard error of ETRm values at 0, 1, 2, and 3 d after transfer to W_4 are shown ($n = 3$ seedlings per treatment). Asterisks indicate statistically significant differences between treatments (W_{20} or W_{20}+FR) over time (two-way ANOVA, $^*P < 0.05$, $^{**}P < 0.01$). B, Wild-type At and Ch lines were germinated and grown under W_{20} for 2 d, transferred to either W_{20} or W_{20}+FR for 5 d, and then exposed to W_{200} for 7 more days. Fv/Fm values and HPLC-quantified chlorophyll levels were determined. Mean and standard error of $n = 7$ seedlings (Fv/Fm) or $n = 3$ independent pools (HPLC) per treatment are represented. Asterisks mark statistically significant differences between values before and after exposure to W_{200} (t test, $^*P < 0.05$; $^{**}P < 0.01$).

3). Mustards such as At that photoacclimated poorly to lower PAR but better to higher PAR tended to more conspicuously elongate their hypocotyls in response to low R:FR, but there were exceptions of poorly elongating species such as No (Figures 2 and 3). Furthermore, mutation of genes encoding SAS regulators can dramatically change the

elongation response to low R:FR without improving the photoacclimation phenotype (Figure 4). Together, these results confirm that the capacity for photosynthetic acclimation to changing irradiance is a species-specific trend (Bailey et al., 2001) and a reliable indicator of shade tolerance. The shade-induced hypocotyl elongation response should only be used as a complementary phenotype to classify a plant as shade-tolerant (badly adapted to higher PAR exposure, well adapted to live under lower PAR and poorly responsive to low R:FR) or shade-avoider (well adapted to higher PAR, poor performers under lower PAR that elongate when exposed to low R:FR).

Our results also unveiled that an activation of low R:FR signaling in shade-avoider species such as AtAt-WT and shade-tolerant Ch plants with mutations causing low R:FR hypersensitivity (Ch-*sis1* and Ch-*hfr1*) regulated photosynthesis at multiple levels. We confirmed that exposure to W+FR caused a substantial decrease in the levels of photosynthetic pigments (chlorophylls and carotenoids) in these lines (Roig-Villanova et al., 2007; Bou-Torrent et al., 2015; Molina-Contreras et al., 2019; Paulisic et al., 2021) and proved that the changes had a direct impact on decreasing phytosynthetic activity (Figure 5). Low R:FR treatments are known to trigger changes in gene expression within minutes (Kohnen et al., 2016). These changes, which are often instrumental for altering rapid growth responses, such as hypocotyl or petiole elongation, are usually mediated by PIFs (Hornitschek et al., 2009; Galstyan et al., 2011; Cifuentes-Esquivel et al., 2013; de Wit et al., 2015; Gallemi *et al*, 2017). PIFs were also found to regulate longer-term changes in gene expression such as those affecting photosynthetic genes (Figure 6). Because loss of PIFQ function in the At-*pifq* mutant resulted in a much attenuated response to W+FR compared to At-WT in terms of photosynthetic gene expression (Figure 6) but it also prevented photosynthetic pigment and activity loss (Figure 5), we propose that stabilization of PIFQ proteins following low R:FR exposure triggers a reprogramming of photosynthesis-related gene expression that eventually results in lower pigment levels and reduced photosynthetic activity. Based on the results obtained with other mutants (Figure 5), we speculate that this signaling network is further influenced by factors such as HFR1 and HY5, which prevent PIF binding to target genes by heterodimerization (Hornitschek et al., 2009) or competition for promoter binding sites (Toledo-Ortiz et al., 2014), respectively.

Concomitant with the described molecular and physiological changes, we discovered that low R:FR treatment of At-WT seedlings triggered ultrastructural changes in the chloroplast endomembrane systems resembling those occurring after transfer to low PAR (Figure 7). Grana with more thylakoid layers and increased thickness were observed in the chloroplasts of At seedlings exposed to simulated shade. In contrast, chloroplasts from tobacco (*Nicotiana tabacum*) leaves that received end-of-day-FR treatments (considered to induce similar shade responses as low R:FR) showed fewer thylakoid layers per granum but more small grana spread throughout the chloroplast compared to end-of-day R controls (Kasperbauer and Hamilton, 1984). While these differences in chloroplast ultrastructure might derive from distinct treatments being applied to diverse species, both solutions likely contribute to optimize photosynthesis in the shade, when relatively less photons would strike a leaf. Indeed, leaves that develop under low PAR have chloroplasts with less plastoglobules (which are derived from thylakoid membranes) and more thylakoids per granum (Rozak et al., 2002; Lichtenthaler, 2007; Wood et al., 2018). Based on these results, we suggest that the chloroplast ultrastructural changes observed in At-WT plants grown under low R:FR are most likely aimed to acclimate their photosynthetic machinery to perform better under low PAR by, for instance, allowing a more efficient energy transfer. In agreement, pretreatment with low R:FR improved photoacclimation to low PAR of At-WT seedlings but had no effect in At mutants defective in low R:FR signaling (Figure 8). Further experiments showed that the observed positive effect of low R:FR exposure for acclimation to low PAR can be observed in At-WT plants growing under different light conditions (Supplemental Figure S3) and in other shade-avoider Brassicaceae (Cb-F, Cb-S, and Cr), but not in shade-tolerant species such as Ch, Aa, No, and Si (Figure 8A).

At low irradiances, a proper balance between carbon allocation to growth and to respiration is important to meet the challenges associated with a shade environment. Wild-type At (shade-avoider) but not Ch (shade-tolerant) seedlings showed a drop in dark respiration when irradiation was reduced (Supplemental Figure S4), likely to reduce carbon loss for a better carbon balance. This adaptive mechanism might contribute to explain why shade-avoider and shade-tolerant species appear to show little or no differences in carbon balance under low light conditions (Sterck et al., 2013; Pons and Poorter, 2014). Similar to that observed for photosynthetic activity (Figure 8), the respiration drop observed in At-WT seedlings was attenuated by pre-exposure to low R:FR (Supplemental Figure S4). Interestingly, there is evidence for the specific activation/deactivation of respiratory pathways by the phytochrome system at different levels (Ribas-Carbo et al., 2008; Igamberdiev et al., 2014). Regardless of the signaling pathway connecting low R:FR perception to reduced photosynthesis and respiration, this is likely part of an anticipation mechanism for shade-avoider plants to prepare for the foreseeable reduction in PAR associated with shading. Indeed, low R:FR signals are perceived before actual shading takes place and light becomes limiting, and hence they are considered to act as a warning signal that shading might occur (Martinez-Garcia et al., 2010; Casal, 2013). When shade-avoider plants such as At and most crops (including tomato, cereals, or legumes) grow among taller plants or in a forest understory, they will use the low R:FR signals coming from a closing canopy to elongate (to overgrow its neighbors) but also to readapt its photosynthetic and respiratory machinery to low PAR before

actual shading takes place. In contrast, shade-tolerant plants are adapted to grow under dim light and hence photoacclimation to low PAR is hardly improved even when hypersensitive mutants that show shade-avoider responses in terms of elongation (Figure 3) and photosynthesis (Figure 6) are pre-exposed to low R:FR (Figure 8).

While the observed decrease in respiration and photosynthetic pigment and activity levels in shade-avoider plants appears to be part of the anticipation mechanism to an eventual reduction in PAR, a too committed response might be detrimental if light conditions change (e.g. if shading does not occur or shade plants become exposed again to direct sunlight). We have previously shown that a compensation mechanism exist that represses the response to low R:FR when the photosynthetic capacity of chloroplasts is compromised (Ortiz-Alcaide et al., 2019). The retrograde (i.e. chloroplast-to-nucleus) pathway that adapts low R:FR perception and signaling to the photosynthetic status of the plant involves the antagonistic factors PIFs and HY5, which also participate in retrograde signaling when underground seedlings are illuminated and start their photomorphogenic (i.e. photosynthetic) development (Ruckle et al., 2007; Martin et al., 2016; Xu et al., 2016; Ortiz-Alcaide et al., 2019). The balance of positive and negative regulators together with the chloroplast-mediated control of SAS likely contribute to prevent an excessive response to shade, hence preventing photooxidative damage (resulting from light intensity exceeding the photosynthetic capacity of the plant) and facilitating the return to high R:FR conditions if the low R:FR signal disappears (e.g. if a commitment to the shade-avoidance lifestyle is unnecessary). Together, our work demonstrates that regulation of photosynthetic (chloroplast) performance is both an output and an input of the response of plants to shade. Our results therefore contribute to a better understanding of how plants respond to shade, a knowledge that will contribute to optimally grow crop plants closer together or/and under canopies (e.g. in intercropping settings).

Materials and methods

Plant material and growth conditions

Alpine rock cress (*Arabis alpina*, *pep1-1* mutant; Wang et al., 2009), Arabidopsis (*Arabidopsis thaliana*, Col-0 accession), hairy bittercress (*Cardamine hirsuta*, Oxford, Ox accession; Molina-Contreras et al., 2019), shepherd's purse (*Capsella bursa-pastoris*, accessions Strasbourg-1, Str-1 and Freiburg-1, Fre-1), pink shepherd's-purse (*Capsella rubella*), and London rocket (*Sysimbrium irio*) plants were grown in the greenhouse under long-day photoperiods (16-h light and 8-h dark) to produce seeds, as described (Gallemi et al., 2017). Seeds of *C. bursa-pastoris* were collected by Ruben Alcazar (University of Barcelona, Spain) from wild populations in Strasbourg (France, coordinates: 48.612436, 7.767881; Str-1) and Freiburg (Germany, coordinates: 47.994945, 7.861979; Fre-1). Seeds of *C. rubella*, collected from wild populations in Crete (Greece, coordinates 35.29, 24.42; accession 879) were previously described (Koenig et al., 2019). Seeds of *S. irio* were collected from wild populations in Bellaterra (Barcelona, Spain, coordinates: 41.497731, 2.109558). Seeds of watercress (*Nasturtium officinale*) were provided by a seed company (www.semillasfito.es). *Arabidopsis thaliana* and Ch mutant and transgenic lines were previously available in our laboratories (Molina-Contreras et al., 2019; Ortiz-Alcaide et al., 2019; Paulisic et al., 2021).

For the light acclimation experiments seedlings were germinated and grown in Petri dishes containing solid medium without sucrose (0.5× MS): 2.2 g·L^{-1} MS basal salt mixture (Duchefa), 1% (w/v) agar, 0.25 g·L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma Aldrich), pH 5.7). Normal light conditions refer to white light (W) produced by cool-white vertical fluorescent tubes of a photosynthetic photon flux density in the PAR region (PPFD) of 20-24 µmol m^{-2} s^{-1} (W_{20}) with a R:FR of 1.5–3.3. Low light and high light conditions corresponded to W of PPFD of 4 (W_4) and 200 (W_{200}) µmol m^{-2} s^{-1}, respectively, produced by horizontal fluorescent tubes. Low R:FR treatment was produced by supplementing W_{20} with FR (W_{20}+FR). FR was emitted from a GreenPower LED module HF FR (Philips), providing a R:FR of 0.02 (Martinez-Garcia et al., 2014). For the light acclimation experiments shown in Supplemental Figure S3, seedlings were germinated and grown in Petri dishes, as previously described, but exposed to long-day (16-h light/8-h darkness) or short-day (8-h light/16-h darkness) photoperiods. The light part of the photoperiod was produced by cool-white horizontal fluorescent tubes of 200–210 µmol m^{-2} s^{-1} of PPFD (W_{200}) with R:FR of 2–3.5). In that case, low light conditions corresponded to values of 15 µmol m^{-2} s^{-1} PPFD (W_{15}). In this set-up, low R:FR treatment was produced by supplementing W_{200} with the same FR lamps described above (W_{200}+FR), obtaining a R:FR of 0.2–0.25. Light fluence rates were measured with a Spectrosense2 meter (Skye Instruments Ltd), which provides PPFD (400–700 nm), and photon flux density in 10 nm windows of R (664–674 nm) and FR (725–735 nm) regions to calculate the R:FR (Martinez-Garcia et al., 2014). Full spectra photon distribution of W and W+FR treatments have been described elsewhere (Molina-Contreras et al., 2019).

Measurement of hypocotyl length

For hypocotyl measurement, about 30 seeds of each genotype were germinated and grown on plates containing 0.5× MS solid media. For quantification of hypocotyl length, at least 20 seedlings were analyzed with the FIJI-ImageJ software (Schindelin et al., 2012), as described (Roig-Villanova et al., 2019). All experiments were repeated at least three times with consistent results. Hypocotyl measurements from all the different experiments were averaged.

Photosynthetic measurements and pigment quantification

Whole seedlings were harvested, ground in liquid nitrogen, and the resulting powder was used for quantification of chlorophylls and carotenoids either spectrophotometrically

or by high performance liquid chromatography (HPLC) as described (Bou-Torrent et al., 2015). Chlorophyll fluorescence measurements were carried out on seedlings using a MAXI-PAM fluorometer (Heinz Walz GmbH) as described (Molina-Contreras et al., 2019). Briefly, for every measurement the whole cotyledons of seven seedlings were considered. Effective quantum yield of photosystem II (PSII) under growth light, ΦPSII, was measured as $\Delta F/Fm'$, where ΔF corresponds to $Fm'-F$ (the maximum minus the minimum fluorescence of light-exposed plants). Maximum quantum yield of PSII, Fv/Fm, was calculated as (Fm−Fo)/Fm, where Fm and Fo are, respectively, the maximum and the minimum fluorescence of dark-adapted samples. For dark acclimation, plates were incubated for at least 30 min in darkness to allow the full relaxation of photosystems. Light curves were constructed with 10 incremental steps of actinic irradiance (E; 0, 20, 55, 110, 185, 280, 395, 530, 610, 700 μmol photons·m^{-2}·$s^{-1}$of PPFD). For each step, ΦPSII was monitored every minute and electron transport rate (ETR) was calculated as $E\times\Phi PSII\times 0.84\times 0.5$ (where 0.84 is the light absorptance by an average green leaf and 0.5 is the fraction of absorbed quanta available for PSII). The light response and associated parameters ETRm (maximum electron transport rate) and alpha (photosynthetic rate in light-limited region of the light curve) were characterized by fitting iteratively the model of the rETR versus E curves using MS Excel Solver (Platt et al., 1980). The fit was very good in all the cases ($r > 0.98$).

Respiration measurements

Seedlings were germinated and grown on 0.5× MS plates, as described (Supplemental Figure S4). Before the measurements, seedlings were placed in the dark for about 30 min to avoid light-enhanced dark respiration. Five to 10 seedlings were then collected, immediately weighed, and placed into the respiration cuvette containing the respiration buffer (30-mM MES, pH 6.2, 0.2-mM $CaCl_2$). Oxygen uptake rates were measured in darkness using a liquid-phase Clark-type oxygen electrode (Rank Brothers Ltd.) as previously described (Florez-Sarasa et al., 2009) at a constant temperature of 23°C.

Microarray data analyses

Microarray data corresponding to Col-0 At-WT and At-*pifq* seedlings exposed to low-R:FR for 0, 1, 3, and 24 h (Leivar et al., 2012) were analyzed to select for differentially expressed genes (DEGs) specifically related to photosynthesis. The reported list of DEGs was further filtered using cut-offs of FDR <0.05 and log2-transformed fold change higher than 0.585 for upregulated genes and lower than −0.599 for downregulated genes. Then, photosynthesis-related genes were identified by using the Kyoto Encyclopedia of Genes and Genomes Mapper tool (Kanehisa and Sato, 2020).

Transmission electron microscopy

TEM was carried out as described (Flores-Perez et al., 2008). Chloroplast features in the pictures were quantified by using the FIJI-ImageJ software (Schindelin et al., 2012).

Accession numbers

Sequence data from this article can be found in the EMBL/Genbank and *C. hirsuta* genetic and genomic resource (http://chi.mpipz.mpg.de) data libraries under the following accession numbers: *AT1G02340* (*AtHFR1*), *AT5G11260* (*AtHY5*), *AT2G20180* (*AtPIF1*), *AT1G09530* (*AtPIF3*), *AT2G43010* (*AtPIF4*), *AT3G59060* (*AtPIF5*), *CARHR001660* (*ChHFR1*), and *CARHR009540* (*SIS1*/*ChPHYA*).

Supplemental data

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

Supplemental Figure S1. Alpha values calculated from the light curves shown in Figure 2A.

Supplemental Figure S2. Activation of low R:FR signaling reduces photosynthetic activity.

Supplemental Figure S3. Pre-exposure to low R:FR improves photoacclimation to lower PAR in *A. thaliana* plants grown under photoperiods.

Supplemental Figure S4. Exposure to low R:FR differentially impacts respiration rate of shade-avoider and shade-tolerant plants.

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Conflict of interest statement. The authors declare no competing interests.

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