Light quality signals generated by vegetation shade facilitate acclimation to reduced light quantity in shade-avoider plants

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SUMMARY

- 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 red to far red light (low R:FR) due to the preferential absorbance of red light and reflection of far red 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 elongation, photosynthesis and photoacclimation responses in several Brassicaceae species to explore the possible connections between low R:FR and low PAR.

- A negative correlation was found in shade-tolerant *Cardamine hirsuta* and shade-avoider *Arabidopsis thaliana* seedlings (e.g., shade-tolerance was associated with a good adaptation to low PAR but a poor or null response to low R:FR exposure). However, they could be genetically uncoupled. Most interestingly, exposure to low R:FR of shade-avoider 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.

Key words: chloroplasts, elongation, light, photoacclimation, photosynthesis, shade-avoider, shade-tolerance.

52 **INTRODUCTION**

53   Light is essential for plants as a source of energy and environmental information. Shading by

54 nearby individuals can reduce light quantity (i.e. photon supply) and hence compromise

55 photosynthetic activity and growth. To deal with this challenge, plants have developed response

56 mechanisms based on the perception of light quality, i.e. spectral information (Martinez-Garcia *et*

57 *al.*, 2010; Casal, 2013). The preferential absorbance of red light (R) and reflection of far-red light

58 (FR) by photosynthetic tissues results in a decreased ratio of R to FR (R:FR) when light is reflected

59 from or filtered through green stems and leaves. The low R:FR is a very reliable light signal that

60 announces the close presence of nearby plants that may compete for resources.

61   Plants growing in ecosystems where access to light is restricted (e.g. in forest understories)

62 show a shade-tolerant habit by adapting their light capture and utilization systems to low light

63 intensity conditions. By contrast, plants growing in open habitats are shade-avoiders (also referred

64 to as shade-intolerant or sun-loving). In shade-avoider plant species, such as *Arabidopsis thaliana*

65 and most sun-loving crops, perception of the low R:FR signal by the phytochrome photoreceptors

66 activates a signaling pathway that eventually triggers a set of responses known as the shade

67 avoidance syndrome (SAS). The most prominent phenotype following exposure to low R:FR is

68 elongation (e.g., of seedling hypocotyl, leaf petiole and stem internode tissues), intended to

69 overgrow neighboring competitors and outcompete them in the access to light. If the neighboring

70 individuals overgrow and eventually shade the plant, the consequent reduction in light quantity (i.e.,

71 in the amount of radiation available for photosynthesis) results in additional and stronger SAS

72 responses such as reduced leaf size, attenuated defense mechanisms and early flowering (Roig-

73 Villanova & Martinez-Garcia, 2016).

74   The most extensively studied SAS response by far is hypocotyl elongation in *A. thaliana*. In

75 this species, low R:FR inactivates phyB, releasing PHYTOCHROME INTERACTING FACTORs

76 (PIFs) that can then regulate gene expression and promote elongation growth. This response is also

77 repressed by negative SAS regulators such as ELONGATED HYPOCOTYL 5 (HY5), amongst

78 many others (Cifuentes-Esquivel *et al.*, 2013; Ciolfi *et al.*, 2013). Biological activity of these

79 transcription factors can be modulated by additional components of the SAS regulatory network

80 such as LONG HYPOCOTYL IN FAR-RED 1 (HFR1, which binds PIFs to prevent their binding to

81 target genes) and PHYTOCHROME A (phyA, which gets stabilized in shade and then promotes

82 HY5 accumulation) (Ciolfi *et al.*, 2013; Martinez-Garcia *et al.*, 2014; Yang *et al.*, 2018). Both

83 HFR1 and phyA hence act as additional SAS repressors that were recently found to be instrumental

84 for the adaptation to shade. Indeed, the shade-tolerant *Cardamine hirsuta*, a close relative of *A.*

85 *thaliana*, does not elongate when exposed to low R:FR unless the function of phyA or HFR1 is

86 genetically lost in mutant plants (Hay *et al.*, 2014; Molina-Contreras *et al.*, 2019; Paulisic *et al.*,

87 2020).

88 Differences between shade-avoider and shade-tolerant species are not restricted to changes in

89 elongation after exposure to low R:FR. *A. thaliana* and *C. hirsuta* also show a differential response

90 to low R:FR in terms of photosynthetic pigment accumulation. Chlorophyll and carotenoid levels

91 drop about 20 % in *A. thaliana* plants grown under low R:FR conditions, whereas the decrease is

92 attenuated in *C. hirsuta* plants (Molina-Contreras *et al.*, 2019). Photoacclimation (i.e., the ability of

93 plants to respond with specific phenotypic adjustments to changes in the incident light) also

94 diverges. The shade-avoider *A. thaliana* showed a lower capacity to acclimate to reduced

95 photosynthetically active radiation (low PAR) but a higher capacity to acclimate to intense light

96 (high PAR) compared to the shade-tolerant *C. hirsuta* (Molina-Contreras *et al.*, 2019). However, the

97 possible connections between low R:FR signaling and photoacclimation responses remain virtually

98 unknown. Here we explored natural and engineered genetic diversity to investigate this connection.

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100

101 **MATERIALS AND METHODS**

102 **Plant material and growth conditions**

103 *Arabis alpina* (*pep1-1* mutant) (Wang *et al.*, 2009), *Arabidopsis thaliana* (Col-0 accession),

104 *Cardamine hirsuta* (Oxford, Ox accession) (Molina-Contreras *et al.*, 2019)*, Capsella bursa-pastoris*

105 (accessions Strasbourg-1, Str-1 and Freiburg-1, Fre-1), *Capsella rubella* and *Sisymbrium irio* plants

106 were grown in the greenhouse under long-day photoperiods (16 h light and 8 h dark) to produce

107 seeds, as described (Gallemi *et al.*, 2017). Seeds of *C. bursa-pastoris* were collected by Ruben

108 Alcazar (University of Barcelona, Spain) from wild populations in Strasbourg (France, coordinates:

109 48.612436, 7.767881; Str-1) and Freiburg (Germany, coordinates: 47.994945, 7.861979; Fre-1).

110 Seeds of *Capsella rubella*, collected from wild populations in Crete (Greece, coordinates 35.29,

111 24.42; accession 879) were previously described (Koenig *et al.*, 2019). Seeds of *Sisymbrium irio*

112 were collected from wild populations in Bellaterra (Barcelona, Spain, coordinates: 41.497731,

113 2.109558). Seeds of *Nasturtium officinale* were provided by a seed company (www.semillasfito.es).

114 *A. thaliana* and *C. hirsuta* mutant and transgenic lines were previously available in our laboratories

115 (Molina-Contreras *et al.*, 2019; Ortiz-Alcaide *et al.*, 2019; Paulisic *et al.*, 2020).

116 For the light acclimation experiments seedlings were germinated and grown on medium

117 without sucrose: 2.2 g/L MS basal salt mixture (Duchefa), 1% (w/v) agar, 0.25 g/L 2-(*N*-

118 morpholino)ethanesulfonic acid -MES- (Sigma Aldrich), pH 5.7. Normal light conditions (W) refer

119 to light produced by cool-white vertical fluorescent tubes (PAR of 20-24 µmol m^{-2} s^{-1}) with a R:FR

120   of 1.5-3.3. Low light (L) and high light (H) conditions corresponded to PAR values of 4 and 200

121   µmol m^{-2} s^{-1}, respectively produced by horizontal fluorescent tubes. Low R:FR treatment was

122   produced by supplementing W with FR (W+FR). FR was emitted from a GreenPower LED module

123   HF far-red (Philips), providing a R:FR of 0.02 (Martinez-Garcia *et al.*, 2014). Light fluence rates

124   were measured with a Spectrosense2 meter (Skye Instruments Ltd); full spectra photon distribution

125   of W and W+FR treatments have been described elsewhere (Molina-Contreras *et al.*, 2019).

126

Measurement of hypocotyl length

128   For hypocotyl measurement, about 30 seeds of each genotype were germinated and grown on

129   plates. For quantification of hypocotyl length, at least 20 seedlings were analyzed with the FIJI-

130   ImageJ software (Schindelin *et al.*, 2012), as described (Roig-Villanova *et al.*, 2019). All

131   experiments were repeated at least three times with consistent results. Hypocotyl measurements

132   from all the different experiments were averaged.

133

Photosynthetic measurements and pigment quantification

135   Whole seedlings were harvested, ground in liquid nitrogen, and the resulting powder was used

136   for quantification of chlorophylls and carotenoids either spectrophotometrically or by HPLC as

137   described (Bou-Torrent *et al.*, 2015). Chlorophyll fluorescence measurements were carried out on

138   seedlings using a MAXI-PAM fluorometer (Heinz Walz GmbH) as described (Molina-Contreras *et*

139   *al.*, 2019). Briefly, for every measurement the whole cotyledons of 7 seedlings were considered.

140   Effective quantum yield of photosystem II (PSII) under growth light, ϕPSII, was measured as

141   ΔF/Fm', where ΔF corresponds to Fm'-F (the maximum minus the minimum fluorescence of light-

142   exposed plants). Maximum quantum yield of PSII, Fv/Fm, was calculated as (Fm-Fo)/Fm, where

143   Fm and Fo are respectively the maximum and the minimum fluorescence of dark-adapted samples.

144   For dark acclimation, plates were incubated for at least 30 minutes in darkness to allow the full

145   relaxation of photosystems. Light curves were constructed with 10 incremental steps of actinic

146   irradiance (E; 0, 20, 55, 110, 185, 280, 395, 530, 610, 700 µmol photons·m^{-2}·s^{-1}of PAR). For each

147   step, ϕPSII was monitored every minute and relative electron transport rate (rETR) was calculated

148   as E×ϕPSII. The light response and associated parameters ETRm (maximum electron transport rate)

149   and alpha (photosynthetic rate in light-limited region of the light curve) were characterized by

150   fitting iteratively the model of the rETR versus E curves using MS Excel Solver (Platt *et al.*, 1980).

151   The fit was very good in all the cases (r>0.98).

152

Microarray data analyses

154   Microarray data corresponding to *A. thaliana* Col-0 (At-WT) and At-*pifq* seedlings exposed

155   to low-R:FR for 0, 1, 3 and 24 h (Leivar *et al.*, 2012) were analyzed to select for differentially-

156   expressed genes (DEGs) specifically related to photosynthesis. The reported list of DEGs was

157   further filtered using cut-offs of FDR <0.05 and log2-transformed fold change (log2FC) higher than

158   0.585 for upregulated genes and lower than -0.599 for downregulated genes. Then, photosynthesis-

159   related genes were identified by using the KEGG (Kyoto Encyclopedia of Genes and Genomes)

160   Mapper tool (Kanehisa & Sato, 2020).

161

162   **Transmission electron microscopy**

163   Transmission electron microscopy (TEM) was carried out as described (Flores-Perez *et al.*,

164   2008). Chloroplast features in the pictures were quantified by using the FIJI-ImageJ software

165   (Schindelin *et al.*, 2012).

166

167

168   **RESULTS**

169   **Different Brassicaceae species present divergent photoacclimation responses**

170   We previously showed that, compared to sun-loving *A. thaliana* Col-0 (At), shade-tolerant *C.*

171   *hirsuta* Ox (Ch) exhibits a better ability to maintain photosynthesis after transfer to low PAR but a

172   stronger chlorophyll loss when light intensity increases (Molina-Contreras *et al.*, 2019). To better

173   characterize the photoacclimation responses of these two Brassicaceae species, both At and Ch were

174   germinated and grown for 7 days under control PAR conditions (W, 20-24 µmol m^{-2} s^{-1}) and then

175   transferred to either low PAR (L, 4 µmol m^{-2} s^{-1}) or high PAR (H, 200 µmol m^{-2} s^{-1}) for 7 more

176   days (Fig. 1). Light curve analysis at day 3 after the transfer showed a better photosynthetic activity

177   of Ch compared to At when grown under L and a better activity of At compared to Ch when grown

178   under H (Fig. 1a). Derived parameters such as ETRm (maximum electron transport rate) and alpha

179   (alpha, photosynthetic rate in light-limited region of the light curve) also illustrated that At

180   performed better than Ch after transfer to H but worst after transfer to L (Fig. 1b). Other

181   photosynthetic parameters such as maximum quantum efficiency of PSII (Fv/Fm) and light use

182   efficiency of PSII (ɸPSII) only showed differences between At and Ch at longer times of exposure

183   to either H or L (Fig. 1c). At day 7, Fv/Fm values were lower in Ch than in At after transfer to H,

184   while the opposite was observed when transferred to L. A similar trend was observed in the case of

185   ɸPSII (Fig. 1c). These results together indicate that Ch tolerates better the transfer to low PAR

186   (consistent with Ch being more tolerant to shade), while an increase in light irradiance compromises

187   photosynthetic efficiency in Ch more than in shade-avoider At. Based on these results, we used

6

188   light curve analysis to estimate photoacclimation to low PAR and Fv/Fm measurements to estimate

189   photoacclimation to high PAR.

190   Besides At and Ch, the Brassicaceae family (mustards) includes many food crops (e.g.,

191   cauliflower, broccoli, radish, cabbage, kale, and similar green leafy vegetables) and a diversity of

192   wild species from forested and open habitats. As a first step to explore the possible connection

193   between low PAR and low R:FR responses, we analyzed photoacclimation and hypocotyl

194   elongation in six different Brassicaceae species or accessions, including At and Ch as controls. The

195   selected wild mustards were *Arabis alpina* (Aa), two accessions of *Capsella bursa-pastoris*,

196   Freiburg-1 (Cb-F) and Strasbourg-1 (Cb-S), *Capsella rubella* (Cr), *Nasturtium officinale* (No), and

197   *Sisymbrium irio* (Si). Initially, we aimed to classify them as shade-avoider or shade-tolerant based

198   on photoacclimation responses. After germination and growth for 7 days under W, seedlings were

199   either kept under W or transferred to L for one additional day before performing light curve

200   analyses (Fig. 2). Similar to the shade-avoider At, seedlings of Cb-F, Cb-S and Cr showed a

201   lowering of the curve under L conditions, whereas those of Aa, No and Si behaved as the shade-

202   tolerant Ch and showed virtually identical light curves under W and L (Fig. 2a). ETRm and alpha

203   values also illustrated that the L treatment led to decreased photosynthetic performance in At, Cb-F,

204   Cb-S and Cr but not in Ch, Aa, No and Si (Fig. 2b). We next analyzed photoacclimation to

205   increased irradiation quantifying Fv/Fm before or after transferring 7-day-old W-grown seedlings to

206   H for 7 additional days. Again, At grouped together with the two accessions of Cb and with Cr as

207   they acclimated much better to high PAR compared to the group formed by Ch, Aa, No and Si (Fig.

208   2c). Together, these photoacclimation results led to classify the former group as shade-avoiders, and

209   the latter as shade-tolerant species.

210

211   Photoacclimation responses can be uncoupled from shade-driven hypocotyl elongation

212   Next, we investigated whether the classification of the selected mustard species as shade-

213   avoider or shade-tolerant based on their photoacclimation features correlated with their elongation

214   response to low R: FR. After germination and growth for 3 days under W (R:FR=1.5-3.3), seedlings

215   were either kept under W or transferred to FR-supplemented W (W+FR, R:FR=0.02) for 4

216   additional days, and then hypocotyl length was measured (Fig. 3). Similar to At, the Cb-F accession

217   showed a strong hypocotyl elongation response, whereas Cb-S, Cr and No elongated moderately in

218   response to low R: FR. By contrast, Ch, Aa and Si did not elongate in response to low R:FR (Fig.

219   3a). These results indicate that the elongation response to low R:FR cannot be fully predicted based

220   on the photoacclimation phenotype of a particular accession. Nonetheless, accessions classified as

221   shade-avoider based on their photoacclimation behavior (i.e. poor photoacclimation to decreased

222 PAR but good photoacclimation to increased PAR) exhibit a range of responses to low R:FR (i.e.
223 from moderate to strong elongation), whereas plant species with a shade-tolerant photoacclimation
224 responses display either no elongation or a mild shade-avoider phenotype in terms of hypocotyl
225 elongation when exposed to low R:FR (e.g. No). These data together suggest that low R:FR-
226 induced hypocotyl elongation and PAR photoacclimation responses do not fully correlate in nature.

227   The shade-avoider or shade-tolerant elongation phenotype in response to low R:FR can be
228 reversed by manipulating the levels of specific SAS regulators. Previous results have shown that At
229 lines overexpressing *HY5* (At-*HY5ox*) display an attenuated hypocotyl response to low R:FR (Ortiz-
230 Alcaide *et al.*, 2019), whereas a similar but weaker response was observed in a quadruple mutant
231 defective in all members of the photolabile PIF quartet (At-*pifq*) (Fig. 3b). Despite the different
232 degrees of elongation response to low R:FR, these two lines showed photoacclimation responses to
233 low PAR very similar to those of wild-type (At-WT) controls (Fig. 4). Both light curves (Fig. 4a)
234 and ETRm values (Fig. 4b) were almost identical in At-WT plants and mutants hyposensitive to
235 low R:FR (Fig. 3b). In the case of *C. hirsuta*, lines deficient in phyA (Ch-*sis1*) or HFR1 (Ch-*hfr1*)
236 gain the ability to elongate when exposed to low R:FR (Molina-Contreras *et al.*, 2019; Paulisic *et*
237 *al.*, 2020) (Fig. 3b). In contrast to the shade-hyposensitive At mutants, the hypersensitive Ch mutant
238 lines appeared to gain a partial shade-avoider phenotype in terms of photoacclimation to low PAR,
239 as a trend towards lower values of light curves (Fig. 4a) and ETRm (Fig. 4b) were observed under L
240 compared to W. However, photoacclimation to increased PAR estimated from Fv/Fm values and
241 also from chlorophyll levels (Molina-Contreras *et al.*, 2019) was similar for Ch-WT, Ch-*sis1* and
242 Ch-*hfr1* plants (Fig. 4c). We therefore concluded that manipulation of the plant ability to elongate
243 in response to proximity shade hardly impacts their photoacclimation capacity, at least when plants
244 are growing in the absence of the low R:FR signal.

245

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

248   Low R:FR signals not only influence hypocotyl elongation but they are also known to reduce
249 the contents of photosynthetic pigments (chlorophylls and carotenoids) in many plant species (Roig-
250 Villanova *et al.*, 2007; Cagnola *et al.*, 2012; Patel *et al.*, 2013; Bou-Torrent *et al.*, 2015; Molina-
251 Contreras *et al.*, 2019). The reduction is observed in both elongating (At-WT) and non-elongating
252 (Ch-WT) seedlings, but it is stronger in the former (Fig. 5). *C. hirsuta* mutants that gained the
253 ability to elongate in response to shade, such as Ch-*sis1* and Ch-*hfr1*, also displayed stronger
254 reductions in photosynthetic pigment contents relative to Ch-WT after low R:FR exposure (Fig. 5a)
255 (Molina-Contreras *et al.*, 2019). Conversely, *A. thaliana* mutants with a reduced ability to elongate

256   in response to shade, such as At-*pifq* and At-*HY5ox* (Fig. 3b), showed attenuated reduction of

257   pigment contents relative to At-WT when exposed to low R:FR (Fig. 5a).

258   To test whether decreases in photosynthetic pigment levels driven by simulated shade

259   exposure might affect photosynthetic activity, we next measured Fv/Fm and ɸPSII in seedlings

260   grown either under W or under W+FR (Fig. 5b). Indeed, low R:FR was found to result in decreased

261   photosynthetic activity in the lines with strong pigment loss responses independently on the species

262   (At-WT, Ch-*sis1* and Ch-*hfr1*). ETRm and alpha parameters also tended to be lower in W+FR-

263   exposed At-WT, Ch-*sis1* and Ch-*hfr1* seedlings compared to W controls (Fig. 5c). The effect of low

264   R:FR on photosynthesis was much less dramatic in the rest of the lines (At-*pifq*, At-*HY5ox* and Ch-

265   WT), which consistently displayed a reduced impact of W+FR exposure on their photosynthetic

266   pigment levels (Fig. 5).

267   Proximity shade signals have also been found to impact photosynthesis at the level of gene

268   expression. Analyses of low R:FR-triggered transcriptomic changes showed reduced levels of

269   transcripts encoding photosynthesis-related proteins (e.g. enzymes involved in chlorophyll and

270   carotenoid biosynthesis, components of the photosynthetic apparatus, and/or members of the carbon

271   fixation process) in several species, including alfalfa (Lorenzo *et al.*, 2019), maize (Shi *et al.*, 2019),

272   tomato (Cagnola *et al.*, 2012) and *A. thaliana* (Leivar *et al.*, 2012). Interestingly, the changes in the

273   expression of photosynthesis-related genes triggered by low R:FR are attenuated in the At-*pifq*

274   mutant compared to At-WT seedlings (Fig. 6). This is particularly evident in the case of low R:FR-

275   repressed photosynthetic genes (Fig. 6), suggesting that the PIF-mediated regulation of gene

276   expression in response to low R:FR is instrumental for the observed changes in photosynthesis (Fig.

277   5).

278

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

280   The observation that exposure of low R:FR caused a decreased in photosynthetic activity of

281   At-WT seedlings and shade-hypersensitive Ch mutants prompted us to analyze whether this light

282   signal may also cause changes in chloroplast ultrastructure. Cotyledons from At-WT seedlings

283   germinated and grown for 2 days under W and then either kept in W or transferred to W+FR for 5

284   additional days were collected and used for transmission electron microscopy (TEM). Chloroplasts

285   from low R:FR-exposed samples were found to exhibit larger grana stacks and contain less and

286   smaller plastoglobules compared to W-grown controls (Fig. 7). Interestingly, similar changes are

287   associated to low PAR photoacclimation (Rozak *et al.*, 2002; Lichtenthaler, 2007; Wood *et al.*,

288   2018). We therefore reasoned that exposure to low R:FR in the absence of any light intensity

289   change might trigger responses to anticipate a foreseeable shading involving a decrease in PAR. To

290 test this hypothesis, we analyzed light curves of WT and mutant seedlings grown in either W or

291 W+FR and then transferred to low PAR for 3 days (Fig. 8). Pre-exposure of At-WT seedlings to

292 low R:FR (W+FR) resulted in a strongly attenuated reduction in ETRm and alpha after their transfer

293 to low PAR (Fig. 8a). By contrast, At mutants with reduced SAS elongation responses also lost the

294 response to low R:FR in terms of improved photoacclimation to L (Fig. 8a). Pre-treatment with

295 W+FR had virtually no effect on the photoacclimation of Ch-WT seedlings to low PAR but caused

296 a slight improvement of ETRm in shade-hypersensitive Ch mutants at day 1 after transfer to L (Fig.

297 8a). When analyzing photoacclimation to high PAR, pre-exposure of At-WT or Ch-WT seedlings to

298 W+FR resulted in no improvement compared to W-grown controls (Fig. 8b). If anything, Ch-WT

299 seedlings grown under W+FR photoacclimated worse than W-grown seedlings when exposed to

300 higher light intensity (Fig. 8b). Based on these data we conclude that detection and transduction of

301 low R:FR signals not only allows plants to overgrow their neighbors but also to pre-adapt their

302 photosynthetic machinery to foreseeable conditions of actual shading involving reduced PAR. By

303 contrast, shade-tolerant plants have a better adapted photosynthetic capacity to grow under reduce

304 PAR and do not seem to use the low R:FR signal.

305

306 **DISCUSSION**

307   Plants have been traditionally classified as shade avoider and tolerant based mostly on their

308 natural habitat, although virtually all plants are exposed to at least some degree of shade during

309 their lifetime. As an ecological concept, shade tolerance refers to the capacity of a given plant to

310 tolerate low light levels, but it is also associated with a wide range of traits, including phenotypic

311 plasticity to optimize light capture (Valladares & Niinemets, 2008). Analyzing a range of caulescent

312 herbs, it was suggested that the elongation response upon exposure to low R:FR was dependent on

313 the shade habit, the shade-avoiders elongating the most and the shade-tolerant showing a mild or no

314 elongation response (Smith, 1982). Indeed, elongation might not be the best solution for plants that

315 spend all their lives under a canopy or permanently shaded by other plants. Another important

316 parameter to ascertain the degree of shade tolerance of a plant is photoacclimation capacity, which

317 is essential for plant fitness in environments with changing light input conditions (e.g., those where

318 the growth of nearby plants may suddenly compromise access to light). By taking into account both

319 parameters (the hypocotyl elongation response and the capacity to acclimate to low or high PAR),

320 here we analyzed the shade tolerance of several Brassicaceae species, including the closely related

321 mustard model systems *A. thaliana* and *C. hirsuta*. As a rule of thumb, we observed that *C. hirsuta*

322 and other species showing a good photoacclimation response to low PAR (and badly performing

323 after transfer to high PAR) showed a poor or null elongation response to low R:FR (Fig. 2, 3).

324 Mustards such as *A. thaliana* that photoacclimated poorly to low PAR but better to high PAR
325 tended to more conspicuously elongate their hypocotyls in response to low R:FR, but there were
326 exceptions of poorly elongating species such as *Nasturtium officinale* (Fig. 2, 3). Furthermore,
327 mutation of genes encoding SAS regulators can dramatically change the elongation response to low
328 R:FR without improving the photoacclimation phenotype (Fig. 4). Besides confirming that the
329 capacity for photosynthetic acclimation to changing irradiance is species-specific (Bailey *et al.*,
330 2001), our results support the conclusion that measuring the hypocotyl elongation response to low
331 R:FR is not sufficient *per se* to classify a species as shade-tolerant or shade-avoider. Instead, the
332 photoacclimation response to low and high PAR appears to be a better indicator of shade tolerance.
333 A combination of both the shade-induced hypocotyl elongation response and the capacity to
334 acclimate to low and high PAR would be a good diagnostic to faithfully classify a plant as shade-
335 tolerant (bad adapted to high PAR exposure, well adapted to live under low PAR and poorly
336 responsive to low R:FR) or shade-avoider (well adapted to high PAR, poor performers under low
337 PAR that elongate when exposed to low R:FR).

338   Our results also unveiled that activation of low R:FR signaling in shade-avoider species such
339 as *A. thaliana* (At-WT) and shade-tolerant *C. hirsuta* plants with mutations causing low R:FR
340 hypersensitivity (Ch-*sis1* and Ch-*hfr1*) regulated photosynthesis at multiple levels. We confirmed
341 that exposure to W+FR caused a substantial decrease in the levels of photosynthetic pigments
342 (chlorophylls and carotenoids) in these lines (Roig-Villanova *et al.*, 2007; Bou-Torrent *et al.*, 2015;
343 Molina-Contreras *et al.*, 2019; Paulisic *et al.*, 2020) and proved that the changes had a direct impact
344 on decreasing phytosynthetic activity (Fig. 5). Low R:FR treatments are known to trigger changes
345 in gene expression within minutes (Kohnen *et al.*, 2016). These changes, which are often
346 instrumental for altering rapid growth responses, such as hypocotyl or petiole elongation, are
347 usually mediated by PIFs (Hornitschek *et al.*, 2009; Galstyan *et al.*, 2011; Cifuentes-Esquivel *et al.*,
348 2013; de Wit *et al.*, 2015; Gallemi *et al.*, 2017). PIFs were also found to regulate longer-term
349 changes in gene expression such as those affecting photosynthetic genes (Fig. 6). Because loss of
350 PIFQ function in the At-*pifq* mutant resulted in a much attenuated response to W+FR compared to
351 At-WT in terms of photosynthetic gene expression (Fig. 6) but it also prevented photosynthetic
352 pigment and activity loss (Fig. 5), we propose that stabilization of PIFQ proteins following low
353 R:FR exposure triggers a reprogramming of photosynthesis-related gene expression that eventually
354 results in lower pigment levels and reduced photosynthetic activity. Based on the results obtained
355 with other mutants (Fig. 5), we speculate that this signaling network is further influenced by factors
356 such as HFR1 and HY5, which prevent PIF binding to target genes by heterodimerization

357 (Hornitschek *et al.*, 2009) or competition for promoter binding sites (Toledo-Ortiz *et al.*, 2014),
358 respectively.

359   Concomitant with the described molecular and physiological changes, we discovered that low
360 R:FR treatment of At-WT seedlings triggered ultrastructural changes in the chloroplast
361 endomembrane systems resembling those occurring after transfer to low PAR (Fig. 7). Furthermore,
362 we demonstrated that pre-treatment with W+FR improved photoacclimation to low PAR of At-WT
363 seedlings but hardly had an effect in low R:FR-hypersensitive mutants of *C. hirsuta*, a shade-
364 tolerant species (Fig. 8). Based on these results, we conclude that the chloroplast ultrastructural
365 changes observed in At-WT plants grown under low R:FR are most likely aimed to acclimate their
366 photosynthetic machinery to perform better under low PAR by, for instance, allowing a more
367 efficient energy transfer. This low R:FR signal-mediated photosynthetic acclimation is likely part of
368 an anticipation mechanism for shade-avoider plants to prepare for the foreseeable reduction in PAR
369 associated with shading. Indeed, low R:FR signals are perceived before actual shading takes place
370 and light becomes limiting, and hence they are considered to act as a warning signal that shading
371 might occur (Martinez-Garcia *et al.*, 2010; Casal, 2013). When shade-avoider plants such as *A.*
372 *thaliana* and most crops (including tomato, cereals, or legumes) grow among taller plants or in a
373 forest understory, they will use the low R:FR signals coming from a closing canopy to elongate (to
374 overgrow its neighbors) but also to readapt its photosynthetic machinery to low PAR before actual
375 shading takes place. By contrast, shade-tolerant plants are adapted to grow under dim light and
376 hence photoacclimation to low PAR is hardly improved even when hypersensitive mutants that
377 show shade-avoider responses in terms of elongation (Fig. 3) and photosynthesis (Fig. 6) are pre-
378 exposed to low R:FR (Fig. 8).

379   While the observed decrease in photosynthetic pigment and activity levels in shade-avoider
380 plants appears to be part of the anticipation mechanism to an eventual reduction in PAR, a too
381 committed response might be detrimental if light conditions change (e.g., if shading does not occur
382 or shade plants become exposed again to direct sunlight). We have previously shown that a
383 compensation mechanism exist that represses the response to low R:FR when the photosynthetic
384 capacity of chloroplasts is compromised (Ortiz-Alcaide *et al.*, 2019). The retrograde (i.e.
385 chloroplast-to-nucleus) pathway that adapts low R:FR perception and signaling to the
386 photosynthetic status of the plant involves the antagonistic factors PIFs and HY5, which also
387 participate in retrograde signaling when underground seedlings are illuminated and start their
388 photomorphogenic (i.e. photosynthetic) development (Ruckle *et al.*, 2007; Martin *et al.*, 2016; Xu *et*
389 *al.*, 2016; Ortiz-Alcaide *et al.*, 2019). The balance of positive and negative regulators together with
390 the chloroplast-mediated control of SAS likely contribute to prevent an excessive response to shade,

12

391 hence preventing photooxidative damage (resulting from light intensity exceeding the
392 photosynthetic capacity of the plant) and facilitating the return to high R:FR conditions if the low
393 R:FR signal disappears (e.g. if a commitment to the shade-avoidance lifestyle is unnecessary).
394 Together, our work demonstrates that regulation of photosynthetic (chloroplast) performance is both
395 an output and an input of the response of plants to shade. Our results therefore contribute to a better
396 understanding of how plants respond to shade, a knowledge that will contribute to optimally grow
397 crop plants closer together or/and under canopies (e.g., in intercropping settings).

398
399

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410

AUTHOR CONTRIBUTIONS

411
412 MRC and JFMG conceived the original research plan, directed and coordinated the study. LM
413 and MR-C measured and analyzed photosynthetic parameters and pigment levels; SP, IR-V and
414 WQ performed all the other experiments. All authors analyzed their data and discussed the results.
415 MRC and JFM-G wrote the paper with revisions and contributions or/and comments of all other
416 authors.

417

COMPETING INTERESTS

418
419 The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. *Arabidopsis thaliana* **and** *Cardamine hirsuta* **show antagonistic photoacclimation responses to high and low PAR. (a)** Light curves of *A. thaliana* (At) and *C. hirsuta* (Ch) seedlings germinated and grown under normal white light (W) for 7 days and then either kept under W or transferred to either high light (H) or low light (L) for 3 more days. Values represent the mean and standard error of n=3 plants for treatment. **(b)** Maximum relative electron transport rate (ETRm) and photosynthetic rate in the light-limited region of the light curve (alpha) calculated from the curves shown in (a). **(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 days under W and then transferred to either H or L for more 7 days. Data were taken at 0, 3 and 7 days after the transfer.Values are mean and standard error of n=9 seedlings per treatment.

Figure 2. Brassicaceae plants can be grouped with either *Arabidopsis thaliana* **or** *Cardamine 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 imbrio* (Si) seedlings germinated and grown under normal white light (W) for 7 days and then either kept under W or transferred to low light (L) for 1 more day. Values represent the mean and standard error of n=3 plants for treatment. **(b)** ETRm and alpha values calculated from the curves shown in (a). **(c)** Fv/Fm values of seedlings grown for 7 days under W and then transferred to high light (H) for 7 more days. Mean and standard error of n=9 seedlings per treatment are represented.

Figure 3. The hypocotyl elongation response to low R:FR is plastic in Brassicaceae plants. (a) The indicated genotypes were germinated and grown under normal white light (W) for 3 days and then either kept under W or transferred to low R:FR (W+FR) for 4 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+FR relative to W (*t* test, * $P<0.05$ and ** $P<0.01$).

Figure 4. Mutations that alter sensitivity to low R:FR do not impact photoacclimation responses. (a) Light curves of *A. thaliana* and *C. hirsuta* wild-type and mutant seedlings germinated and grown under normal white light (W) for 7 days and then either kept under W or

454 transferred to low light (L) for 1 more day. Values represent the mean and standard error of n=3

455 plants for treatment. **(b)** ETRm values calculated from the curves shown in (a). **(c)** Fv/Fm values

456 and HPLC-determined chlorophyll levels of seedlings grown for 7 days under W and then

457 transferred to high light (H) for 7 more days. Mean and standard error of n=9 seedlings (Fv/Fm) or

458 n=3 independent pools (HPLC) per treatment are represented.

459

460 **Figure 5. Activation of low R:FR signaling reduces photosynthetic pigment levels and activity.**

461 **(a)** The indicated genotypes were germinated and grown under normal white light (W) for 3 days

462 and then either kept under W or transferred to low R:FR (W+FR) for 4 more days. Then, the levels

463 of photosynthetic pigments (carotenoids and chlorophylls) were quantified spectrophotometrically.

464 **(b)** Fv/Fm and ϕPSII values of seedlings germinated and grown as indicated in (a). Lower pictures

465 show false-color images of the indicated parameters in wild-type seedlings. **(b)** ETRm and alpha

466 values of seedlings germinated and grown as indicated in (a). In the plots, mean and standard error

467 of n=3 independent pools of seedlings (a) or n=9 seedlings (b, c) per treatment are represented.

468 Asterisks mark statistically significant changes in W+FR relative to W (*t* test, *P*<0.05).

469

470 **Figure 6. Exposure to low R/FR triggers changes in photosynthetic gene expression that are**

471 **attenuated in the hyposensitive At-*pifq* mutant.** Data were extracted from a publicly available

472 experiment (Leivar *et al.*, 2012). At-WT and At-*pifq* lines were germinated and grown under

473 normal white light (W) for 2 days and exposed to low R:FR (W+FR) for 0, 1. 3 or 24 h. Plots

474 represent the number of differentially expressed genes (DEGs) either up-regulated or down-

475 regulated in W+FR vs. W that are involved in photosynthetic pigment biosynthesis (KEGG

476 pathways ath00906 and ath00860), photosynthesis (ath00195 and ath00196), and carbon fixation

477 (ath00710).

478

479 **Figure 7. Low R:FR triggers ultrastructural changes in *A. thaliana* chloroplasts.** At-WT seeds

480 were germinated and grown under normal white light (W) for 2 days and then either kept under W

481 or transferred to low R:FR (W+FR) for 5 more days. Cotyledons were then used for TEM analysis

482 of chloroplast ultrastructure. Representative pictures at different scales (numbers indicate μm) are

483 shown. Boxplots show quantification of the indicated parameters from the images.

484

485 **Figure 8: Pre-exposure to low R:FR improves the photoacclimation to low PAR in shade-**

486 **avoider plants. (a)** The indicated genotypes were germinated and grown under normal white light

487 (W) for 3 days, transferred to either W or W+FR for 4 days, and then exposed to L for 3 more days.

488   Mean and standard error of ETRm and alpha values at 0, 1, 2 and 3 days after transfer to L are

489   shown (n=9 seedlings per treatment). **(b)** Wild-type *A. thaliana* and *C. hirsuta* lines were

490   germinated and grown under W for 2 days, transferred to either W or W+FR for 5 days, and then

491   exposed to H for 7 more days. Fv/Fm values and HPLC-determined chlorophyll levels were

492   determined. Mean and standard error of n=9 seedlings (Fv/Fm) or n=3 independent pools (HPLC)

493   per treatment are represented.

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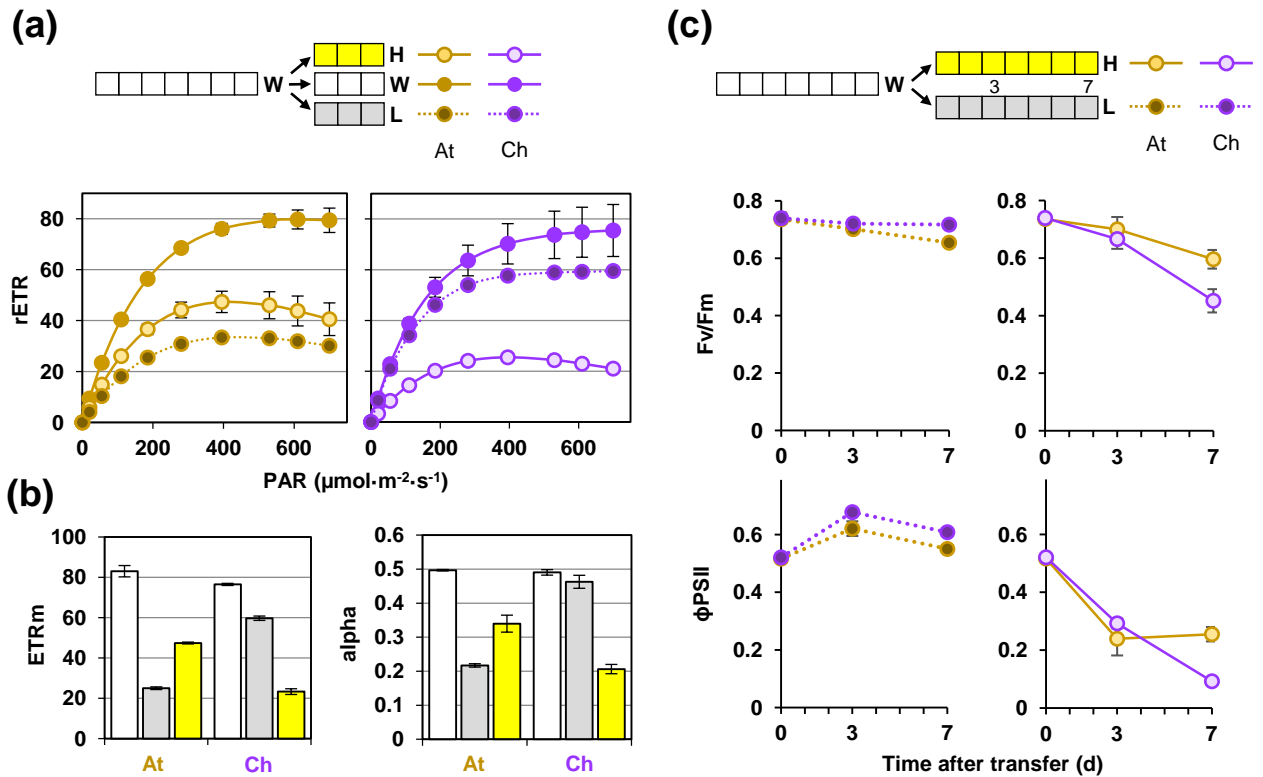611   *Cell* **44**(1): 29-41 e24.

612

Figure 1

Figure 1. *Arabidopsis thaliana* **and** *Cardamine hirsuta* **show antagonistic photoacclimation responses to high and low PAR. (a)** Light curves of *A. thaliana* (At) and *C. hirsuta* (Ch) seedlings germinated and grown under normal white light (W) for 7 days and then either kept under W or transferred to either high light (H) or low light (L) for 3 more days. Values represent the mean and standard error of n=3 plants for treatment. **(b)** Maximum relative electron transport rate (ETRm) and photosynthetic rate in the light-limited region of the light curve (alpha) calculated from the curves shown in (a). **(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 days under W and then transferred to either H or L for more 7 days. Data were taken at 0, 3 and 7 days after the transfer.Values are mean and standard error of n=9 seedlings per treatment.

Figure 2

Figure 2. Brassicaceae plants can be grouped with either *Arabidopsis thaliana* or *Cardamine 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 imbrio* (Si) seedlings germinated and grown under normal white light (W) for 7 days and then either kept under W or transferred to low light (L) for 1 more day. Values represent the mean and standard error of n=3 plants for treatment. **(b)** ETRm and alpha values calculated from the curves shown in (a). **(c)** Fv/Fm values of seedlings grown for 7 days under W and then transferred to high light (H) for 7 more days. Mean and standard error of n=9 seedlings per treatment are represented.

Figure 3

Figure 3. The hypocotyl elongation response to low R:FR is plastic in Brassicaceae plants. (a) The indicated genotypes were germinated and grown under normal white light (W) for 3 days and then either kept under W or transferred to low R:FR (W+FR) for 4 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+FR relative to W (*t* test, * $P<0.05$ and ** $P<0.01$).

Figure 4

Figure 4. Mutations that alter sensitivity to low R:FR do not impact photoacclimation responses. (a) Light curves of *A. thaliana* and *C. hirsuta* wild-type and mutant seedlings germinated and grown under normal white light (W) for 7 days and then either kept under W or transferred to low light (L) for 1 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 chlorophyll levels of seedlings grown for 7 days under W and then transferred to high light (H) for 7 more days. Mean and standard error of n=9 seedlings (Fv/Fm) or n=3 independent pools (HPLC) per treatment are represented.
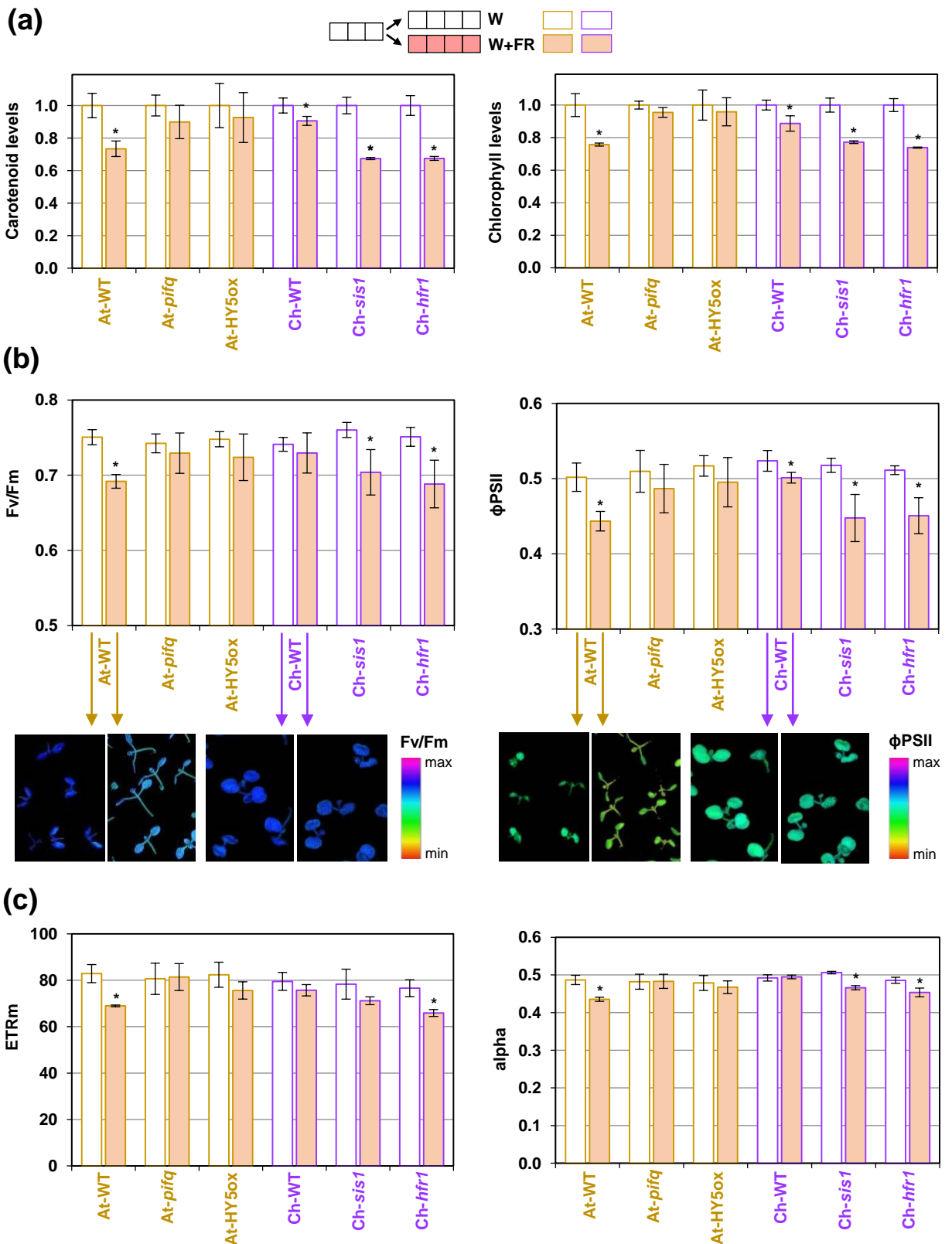
Figure 5

Figure 5. Activation of low R:FR signaling reduces photosynthetic pigment levels and activity. (a) The indicated genotypes were germinated and grown under normal white light (W) for 3 days and then either kept under W or transferred to low R:FR (W+FR) for 4 more days. Then, the levels of photosynthetic pigments (carotenoids and chlorophylls) were quantified spectrophotometrically. (b) Fv/Fm and φPSII values of seedlings germinated and grown as indicated in (a). Lower pictures show false-color images of the indicated parameters in wild-type seedlings. (b) ETRm and alpha values of seedlings germinated and grown as indicated in (a). In the plots, mean and standard error of n=3 independent pools of seedlings (a) or n=9 seedlings (b, c) per treatment are represented. Asterisks mark statistically significant changes in W+FR relative to W (t test, $P<0.05$).
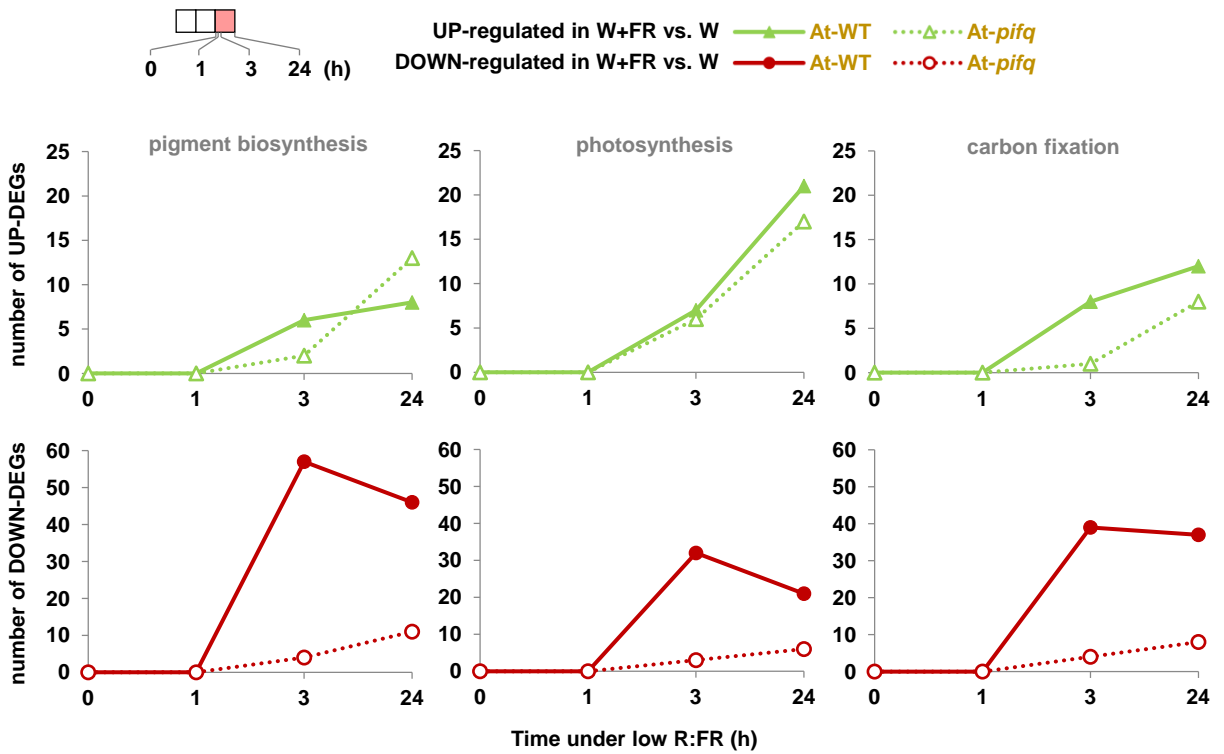
Figure 6

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 normal white light (W) for 2 days and exposed to low R:FR (W+FR) for 0, 1. 3 or 24 h. Plots represent the number of differentially expressed genes (DEGs) either up-regulated or down-regulated in W+FR vs. W that are involved in photosynthetic pigment biosynthesis (KEGG pathways ath00906 and ath00860), photosynthesis (ath00195 and ath00196), and carbon fixation (ath00710).
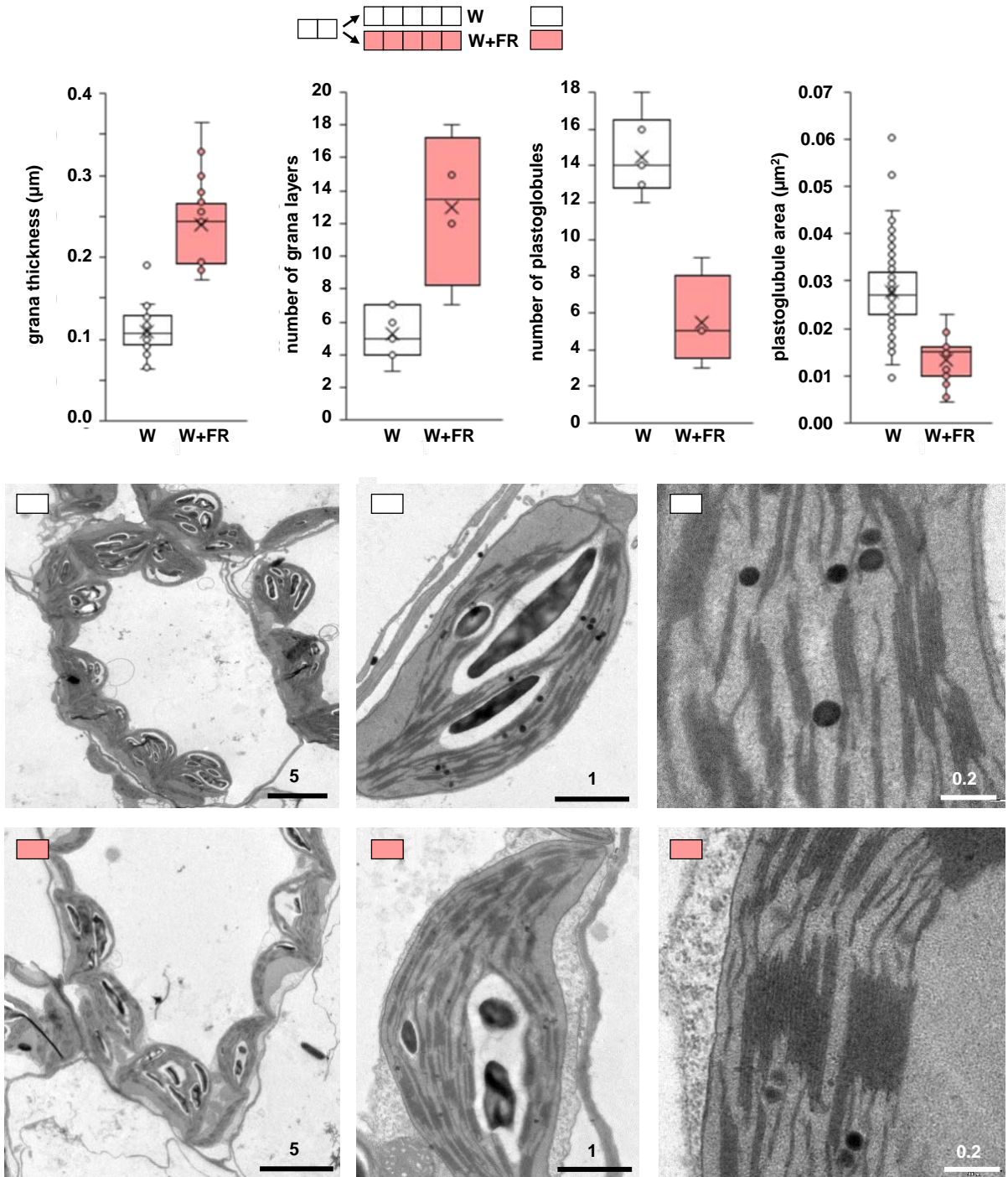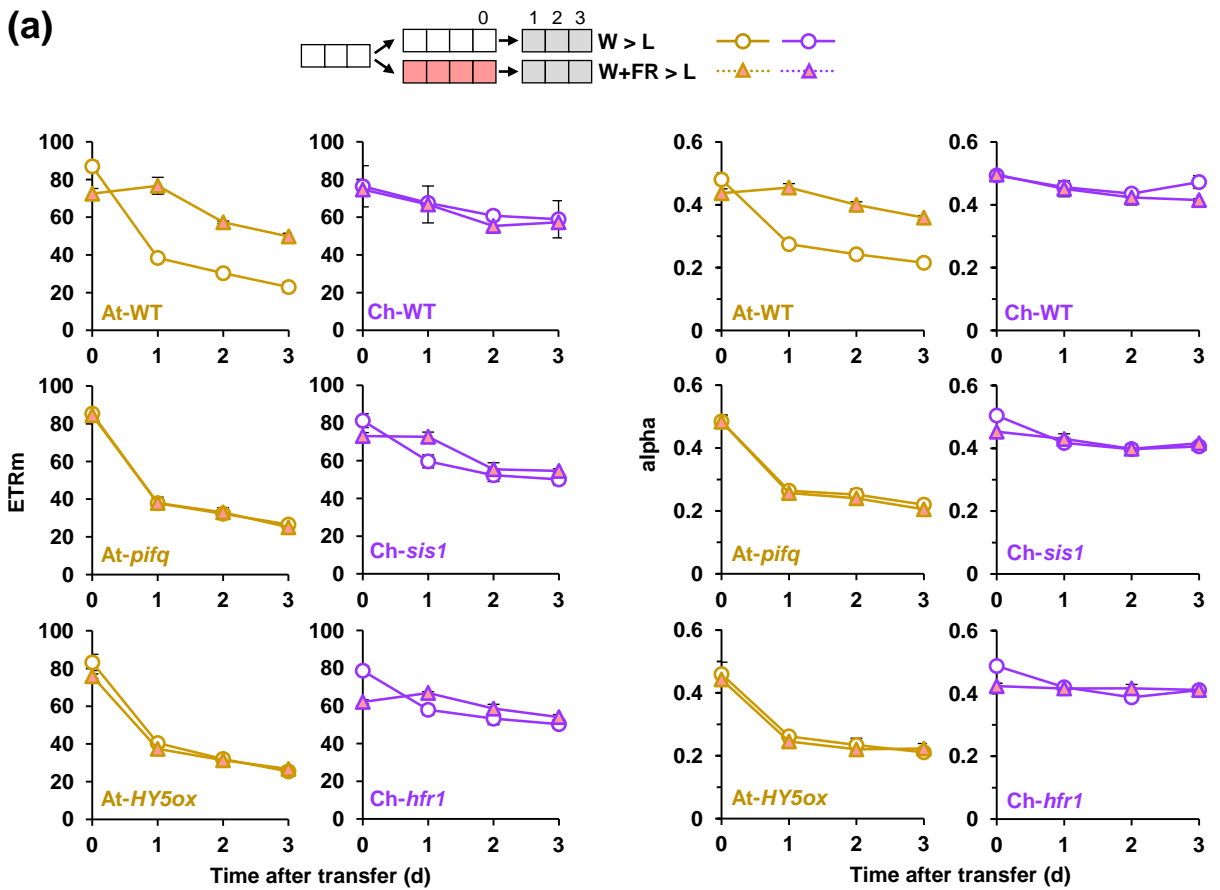
Figure 7

Figure 7. Low R:FR triggers ultrastructural changes in *A. thaliana* chloroplasts. At-WT seeds were germinated and grown under normal white light (W) for 2 days and then either kept under W or transferred to low R:FR (W+FR) for 5 more days. Cotyledons were then used for TEM analysis of chloroplast ultrastructure. Representative pictures at different scales (numbers indicate µm) are shown. Boxplots show quantification of the indicated parameters from the images.
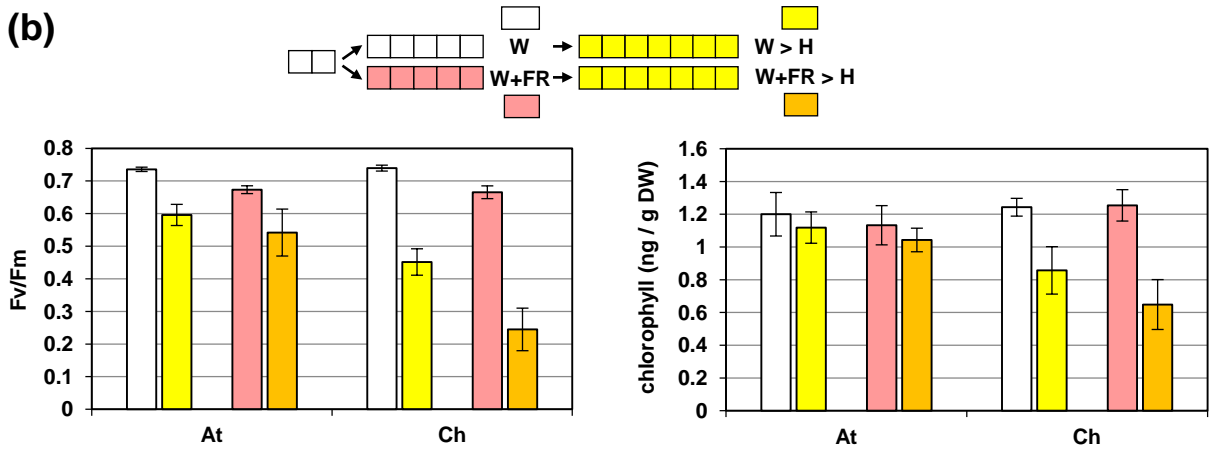
Figure 8

(a)

At-WT · **Ch-WT** · **At-pifq** · **Ch-sis1** · **At-HY5ox** · **Ch-hfr1**

ETRm (y-axis, 0–100) · alpha (y-axis, 0–0.6)

Time after transfer (d)

W > L · W+FR > L

(b)

W → W > H · W+FR → W+FR > H

Fv/Fm · chlorophyll (ng / g DW)

At · Ch

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 normal white light (W) for 3 days, transferred to either W or W+FR for 4 days, and then exposed to L for 3 more days. Mean and standard error of ETRm and alpha values at 0, 1, 2 and 3 days after transfer to L are shown (n=9 seedlings per treatment). **(b)** Wild-type *A. thaliana* and *C. hirsuta* lines were germinated and grown under W for 2 days, transferred to either W or W+FR for 5 days, and then exposed to H for 7 more days. Fv/Fm values and HPLC-determined chlorophyll levels were determined. Mean and standard error of n=9 seedlings (Fv/Fm) or n=3 independent pools (HPLC) per treatment are represented.