

1 **RESEARCH ARTICLE**

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3 **Photoreceptor Activity Contributes to Contrasting Responses**
4 **to Shade in Cardamine and Arabidopsis Seedlings**

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33 **Short title:** Genetic regulation of shade tolerance

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35 **One-sentence summary:** The lack of a shade-induced hypocotyl elongation
36 response in *Cardamine hirsuta* results from the enhanced repressor activity of
37 the phytochrome A photoreceptor.

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46 **ABSTRACT**

47
48 Plants have evolved two major ways to deal with nearby vegetation or shade:
49 avoidance and tolerance. Moreover, some plants respond to shade in different
50 ways; for example, *Arabidopsis thaliana* undergoes an avoidance response to
51 shade produced by vegetation, but its close relative *Cardamine hirsuta* tolerates
52 shade. How plants adopt opposite strategies to respond to the same
53 environmental challenge is unknown. Here, using a genetic strategy, we
54 identified the *C. hirsuta slender in shade1 (sis1)* mutants, which produce
55 strongly elongated hypocotyls in response to shade. These mutants lack the
56 phytochrome A (phyA) photoreceptor. Our findings suggest that *C. hirsuta* has
57 evolved a highly efficient phyA-dependent pathway that suppresses hypocotyl
58 elongation when challenged by shade from nearby vegetation. This suppression
59 relies, at least in part, on stronger phyA activity in *C. hirsuta*; this is achieved by
60 increased *ChPHYA* expression and protein accumulation combined with a
61 stronger specific intrinsic repressor activity. We suggest that modulation of
62 photoreceptor activity is a powerful mechanism in nature to achieve
63 physiological variation (shade tolerance vs. avoidance) for species to colonize
64 different habitats.

65
66 **INTRODUCTION**

67 Understanding how plants colonize different habitats requires identifying
68 the genetic differences underlying physiological variation between species. In
69 this work, we focus on angiosperm responses to changes in light produced by
70 nearby vegetation, perception of which alerts the plant to potential resource
71 competition by other plants. Nearby vegetation is perceived as changes in light
72 parameters: whereas sunlight has a high red (R) to far-red light (FR) ratio
73 (R:FR, >1.1), proximity to vegetation lowers this ratio (Smith, 1982). Because
74 vegetation specifically reflects FR, proximity to other plants initially results in a
75 mild reduction in R:FR (<0.7) due to the FR enrichment. Eventually, when the
76 vegetation canopy closes, sunlight is filtered by photosynthetic tissues, strongly
77 reducing the intensity of the photosynthetic active radiation (PAR, between 400
78 to 700 nm, which includes blue and R) while marginally affecting FR. As a
79 result, R:FR resulting from natural canopy shade typically drops to lower values
80 (<0.05) (Casal, 2012; de Wit *et al.*, 2016; Martinez-Garcia *et al.*, 2014; Smith,
81 1982). In the laboratory, both vegetation proximity and canopy shade can be
82 simulated by providing plants grown under white light (W, high R:FR) varying
83 amounts of supplemental FR (W+FR; low or very low R:FR) while maintaining

84 total PAR, a treatment known as simulated shade (Casal, 2012; Roig-Villanova
85 and Martinez-Garcia, 2016).

86 Plants have two main strategies to acclimate to vegetation proximity and
87 shade: avoidance or tolerance. In the early stages of development, shade-
88 avoider species invest energy into promoting elongation to overgrow their
89 neighbors as part of the so-called shade avoidance syndrome (SAS). By
90 contrast, shade-tolerant plants adopt other physiological and metabolic
91 responses to adapt to a highly conservative utilization of resources, commonly
92 accompanied by very low growth rates, i.e., do not involve promotion of
93 elongation growth (Smith, 1982; Valladares and Niinemets, 2008).

94 Analyses of the shade-avoider *Arabidopsis thaliana* laid the basis for our
95 knowledge of the genetic components and mechanisms involved in the
96 regulation of the SAS (Casal, 2012; Martinez-Garcia *et al.*, 2010; Roig-Villanova
97 and Martinez-Garcia, 2016). The shade signal is perceived by the phytochrome
98 photoreceptors: phytochrome B (phyB) and phyA have major and antagonistic
99 roles (respectively) in hypocotyl elongation, the most conspicuous *A. thaliana*
100 response to low R:FR (Casal, 2012; Mathews, 2010). Lowering the R:FR to
101 resemble either vegetation proximity or canopy shading, deactivates phyB in
102 wild-type seedlings, resulting in the hypocotyl elongation promotion. By
103 contrast, phyA accumulates and is strongly activated under very low R:FR to
104 prevent excessive seedling elongation (Martinez-Garcia *et al.*, 2014; Yang *et*
105 *al.*, 2018). Consistent with this, *A. thaliana* phyB-deficient mutants display
106 constitutive shade responses under high R:FR whereas *phyA* mutant seedlings
107 show enhanced hypocotyl elongation only under very low R:FR conditions,
108 which indicates that phyA antagonizes phyB activity under these specific
109 canopy shade conditions (Casal *et al.*, 2014; Martinez-Garcia *et al.*, 2014; Yang
110 *et al.*, 2018; Yanovsky *et al.*, 1995).

111 SAS responses are mainly initiated because of the interaction of active
112 phytochromes with PHYTOCHROME INTERACTING FACTORs (PIFs),
113 eventually triggering rapid changes in the expression of dozens of genes that
114 implement the SAS responses. Genetic analyses in *A. thaliana* indicate that
115 PIFs, which are basic-helix-loop-helix transcription factors, have a role in
116 positively regulating the shade-triggered hypocotyl elongation. The active form
117 of phyB interacts with PIFs and inhibits their transcriptional activity (Casal,

118 2012; Martinez-Garcia *et al.*, 2010). After exposure to shade, the proportion of
119 active phyB decreases and PIF activity increases. Enhanced PIF binding to G-
120 boxes of auxin biosynthetic genes (e.g. *YUCCA* genes) then promotes their
121 expression, which results in a rapid (1-4 h) increase in free IAA that is required
122 for the promotion of shade-induced hypocotyl elongation (Bou-Torrent *et al.*,
123 2014; Hornitschek *et al.*, 2012; Li *et al.*, 2012; Tao *et al.*, 2008). In addition,
124 nuclear-pore complex components and chloroplast-derived signals also prevent
125 an excessive response to shade, providing additional regulatory levels of this
126 response (Gallemi *et al.*, 2016; Ortiz-Alcaide *et al.*, 2019).

127 There are, however, still major gaps in understanding the genetic and
128 molecular regulation of SAS and, by extension, shade-tolerance traits.
129 Comparative analyses using shade-avoiding and shade-tolerant species is
130 expected to identify regulators of traits associated with shade tolerance habits
131 (Gommers *et al.*, 2013). Indeed, a comparative transcriptomic approach using
132 two *Geranium* species with divergent petiole responses to shade unveiled
133 components that might suppress growth in the shade-tolerant species
134 (Gommers *et al.*, 2017; Gommers *et al.*, 2018). The use of related species but
135 amenable for genetic analyses is expected to push this effort further to find
136 regulatory components used in nature to modulate these divergent responses.
137 This is what we are addressing in this work.

138 Comparing *A. thaliana* and its close relative *Cardamine hirsuta* to
139 understand the genetic basis for trait diversification between species is a
140 powerful strategy to understand the evolution of morphological traits. Key to this
141 approach is the wide morphological and physiological diversity between these
142 species, such as differences in leaf morphology and seed dispersal mechanism
143 among others (Barkoulas *et al.*, 2008; Hay *et al.*, 2014; Hofhuis *et al.*, 2016;
144 Vlad *et al.*, 2014; Vuolo *et al.*, 2016). Like *A. thaliana*, *C. hirsuta* has a short
145 generation time, small size, inbreeding habit, abundant progeny and ease of
146 large scale cultivation (Hay and Tsiantis, 2016; Hay *et al.*, 2014). It is a diploid
147 species with a small genome and eight chromosomes that has been completely
148 sequenced (Gan *et al.*, 2016). Genetic transformation by floral dipping, a dense
149 genetic map and chemically mutagenized populations, provide the tools to
150 identify the genetic components and molecular mechanisms underlying
151 diversification or morphology and response to environment (Hay and Tsiantis,

152 2016). *C. hirsuta* is an invasive herbaceous plant that can grow in open sun but
153 it is often found in shaded or semi-shaded areas. Indeed, *C. hirsuta* does not
154 need much light to grow and their stems become purplish (likely to prevent
155 oxidative damage) in strong sun
156 (<http://edis.ifas.ufl.edu/pdffiles/EP/EP51100.pdf>;
157 http://practicalplants.org/wiki/Cardamine_hirsuta;
158 <http://www.asturnatura.com/especie/cardamine-hirsuta.html>;
159 [http://dnr.wi.gov/topic/Invasives/documents/classification/LR_Cardamine_hirsuta](http://dnr.wi.gov/topic/Invasives/documents/classification/LR_Cardamine_hirsuta.pdf)
160 [a.pdf](https://www.wildfooduk.com/edible-wild-plants/hairy-bittercress/); <https://www.wildfooduk.com/edible-wild-plants/hairy-bittercress/>). These
161 observations are consistent with *C. hirsuta* being shade-tolerant (Bealey and
162 Robertson, 1992). In agreement, whereas seedlings of *A. thaliana* elongate in
163 response to shade, those of *C. hirsuta* are unresponsive to the same stimulus
164 (Hay *et al.*, 2014).

165 The divergent hypocotyl response to shade of *A. thaliana* and *C. hirsuta*
166 species led us to take a comparative approach to understand the genetic basis
167 of the evolution of this physiological trait. We found that *C. hirsuta* has acquired
168 a highly-efficient phyA-dependent pathway that represses hypocotyl elongation
169 and other SAS-associated responses when exposed to simulated shade. After
170 complementing *A. thaliana phyA* mutant plants with endogenous or *C. hirsuta*
171 phyA molecules we concluded that these two photoreceptors are not
172 exchangeable. Differences in phyA intrinsic activity hence contribute to a
173 different response of *C. hirsuta* and *A. thaliana* to shade exposure.

174

175 RESULTS

176 ***C. hirsuta* seedlings perceive low R:FR but do not elongate**

177 A recent study revealed that different species of the *Tradescantia* genus
178 with divergent tolerance to shade showed clear differences in maximum
179 quantum efficiency of photosystem II (Fv/Fm) upon variations of the growth light
180 (Benkov *et al.*, 2019). In particular, the sun-resistant *T. sillamontana* (a
181 succulent growing in semi-desert regions of Mexico and Peru, hence adapted to
182 high light intensities) was more tolerant to changes in irradiation intensity (i.e.
183 showed a more constant Fv/Fm) than the shade-tolerant *T. fluminensis*
184 (habitant of tropical rainforests and other shaded areas in south-eastern Brazil
185 and hence adapted to grow under low light intensities).

186 Using a similar experimental system, we aimed to confirm whether *C.*
187 *hirsuta* is a shade-tolerant plant compared to *A. thaliana* (a broadly accepted
188 shade-avoider). Indeed, when wild-type seedlings of these two species (Ch^{WT}
189 and At^{WT}) were transferred from normal white light (W) to conditions in which
190 PAR was first increased 10-fold (high light, HL) and then reduced 5-fold relative
191 to W (low light, LL) or viceversa, Fv/Fm changes were much more pronounced
192 in Ch^{WT} (Supplemental Figure 1A). The lower capacity of Ch^{WT} to adapt to
193 intense irradiation was confirmed by the bleaching symptoms (e.g., lower
194 chlorophyll contents) observed in Ch^{WT} (but not in At^{WT}) upon transferring to HL
195 (Supplemental Figure 1B). Ch^{WT} seedlings only showed a better performance
196 than At^{WT} when transferred from W to LL. Rapid light curve (RLC) analysis
197 confirmed that Ch^{WT} was better able to maintain its level of photosynthetic
198 activity under LL conditions than At^{WT} (Supplemental Figure 1C), as expected
199 for a shade-tolerant plant (Han *et al.*, 2015).

200 Besides differentially responding to decreased light quantity, plant
201 species from open habitats show a stronger elongation response to reduced
202 R:FR (i.e. light quality) compared to those from woodland shade habitats
203 (Gommers *et al.*, 2017; Smith, 1982). Further supporting the conclusion that *C.*
204 *hirsuta* tolerates shade, Ch^{WT} failed to elongate their hypocotyls when exposed
205 to a range of low R:FR treatments (i.e., W+FR), that mimic vegetation proximity
206 (intermediate or low R:FR; 0.09 - 0.07) and canopy shade (very low R:FR; 0.02)
207 (Supplemental Figure 2, Figure 1). W-grown Ch^{WT} hypocotyls, as well as
208 cotyledons, are substantially longer than those of At^{WT} growing under the same
209 conditions. Ch^{WT} hypocotyls were also longer than those of At^{WT} when growing
210 in the dark (Figure 1C), indicating that *C. hirsuta* is overall bigger than *A.*
211 *thaliana*. More importantly, when treated with growth stimulants, such as
212 gibberellic acid (Hay *et al.*, 2014) or picloram (PIC, a synthetic auxin),
213 hypocotyls of both species elongate (Figure 1D). We therefore concluded that
214 the elongation of *C. hirsuta* hypocotyls is not generally compromised, arguing
215 against the possibility that this species displays a constitutive SAS phenotype.

216 In *A. thaliana*, exposure to simulated shade also triggers the elongation
217 of leaf petioles. We quantified the elongation response of the petiole and rachis
218 in 2-week-old Ch^{WT} and At^{WT} plants subjected to 7 days of high (W) or low R:FR
219 (W+FR). In agreement with previous studies (de Wit *et al.*, 2015; Kozuka *et al.*,

220 2010; Sasidharan *et al.*, 2010), shade-treated *At*^{WT} leaves showed substantially
221 longer petioles than those of plants grown under W. Petiole and rachis length in
222 *Ch*^{WT}, however, was similar in leaves from plants grown under W or W+FR
223 (Figure 1E, Supplemental Figure 3). These results together suggest that
224 elongation responses to low R:FR are dramatically arrested in *C. hirsuta* plants.
225

226 ***C. hirsuta* shows other attenuated responses to shade**

227 Beyond elongation responses, low R:FR triggers a reduction in the levels
228 of photosynthetic pigments, i.e., carotenoids and chlorophylls (Bou-Torrent *et al.*
229 *et al.*, 2015; Cagnola *et al.*, 2012; Roig-Villanova *et al.*, 2007). While these
230 pigments were also significantly reduced in shade-treated *Ch*^{WT} seedlings
231 (Figure 1F), the decrease was less prominent than in *At*^{WT}. These results
232 indicated that not all SAS responses are equally compromised in *C. hirsuta*.

233 We next used RNA sequencing (RNA-seq) to compare the genome wide
234 expression patterns of 7-day-old *At*^{WT} and *Ch*^{WT} whole seedlings in W versus 1
235 h of simulated shade (W+FR) (Figure 2). Incorporating knowledge about gene
236 orthology, 432 differentially expressed genes (DEGs) were categorized as
237 rapidly regulated by shade in one species or in both. Plotting the W+FR vs. W
238 fold-change in *C. hirsuta* against the same ratio in *A. thaliana* resulted in a
239 linear regression equation with a slope of 0.54 (Figure 2B), which supported
240 that shade-modulated changes in gene expression are also attenuated in *C.*
241 *hirsuta* compared to *A. thaliana*. In *A. thaliana*, shade treatment induced 246
242 (fold change > 1.5, $p < 0.05$,) and repressed 58 genes (fold change < -1.5, $p < 0.05$,).
243 In *C. hirsuta*, this same treatment induced 181 and repressed 54 genes
244 (Supplemental Figure 4A, Supplemental Data Sets 1–4). From the set of
245 induced DEGs, 102 responded in both species. They included several of the
246 well-known shade-marker genes in *A. thaliana* and other species, such as
247 *ARABIDOPSIS THALIANA HOMEBOX PROTEIN 2* (*ATHB2*),
248 *BRASSINOSTEROID-ENHANCED EXPRESSION 1* (*BEE1*), *BES1-*
249 *INTERACTING MYC-LIKE1* (*BIM1*), *LONG HYPOCOTYL IN FR 1* (*HFR1*) or
250 *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7* (*XTR7*) (Cifuentes-Esquivel *et al.*
251 *et al.*, 2013; Karve *et al.*, 2012; Procko *et al.*, 2014; Ueoka-Nakanishi *et al.*, 2011).

252 Gene ontology (GO) and MapMan-Bin (MMB) functional prediction of
253 these up-regulated gene group indicated that terms related to auxin were

254 significantly overrepresented (Supplemental Data Sets 5 and 6), suggesting an
255 early role for auxins in both *A. thaliana* and *C. hirsuta*. Indeed, W+FR treatment
256 for 1 h increased auxin (IAA) levels not only in *At*^{WT}, as published (Bou-Torrent
257 *et al.*, 2014; Hersch *et al.*, 2014; Hornitschek *et al.*, 2012; Tao *et al.*, 2008), but
258 also in whole *Ch*^{WT} seedlings (Figure 2C).

259 Using public transcriptomic data, we identified a group of 13 genes
260 whose expression was induced in *A. thaliana* wild-type seedlings but not in
261 mutants that do not accumulate auxins (*shade avoidance 3-2*, (*sav3-2*) and *pif7-*
262 *1*) after 1 h of shade treatment (Bou-Torrent *et al.*, 2014; Li *et al.*, 2012; Tao *et*
263 *al.*, 2008). Based on our RNAseq data, the expression of these genes was
264 significantly upregulated in *At*^{WT} and, to a lower extent, *Ch*^{WT} seedlings
265 (Supplemental Figure 5), consistent with the observed increase in IAA content
266 in both species. Since only *A. thaliana* elongates in response to shade
267 exposure, either the observed early changes in gene expression and auxin
268 levels are not reflecting the differences in hypocotyl growth between these
269 species, or the elongation is consequence of differential later events.

270 In our RNAseq analyses, 55 and 49 DEGs were specifically repressed in
271 either *At*^{WT} or *Ch*^{WT} seedlings, respectively, and just 3 genes were repressed in
272 both species. Regarding up-regulated genes, 142 and 79 DEGs were
273 specifically induced either in *At*^{WT} or in *Ch*^{WT}, respectively (Supplemental Figure
274 4A). GO and MMB functional prediction of the 142 DEGs specific for *At*^{WT}
275 showed genes related to several aspects of plant development, whereas the 79
276 DEGs specifically induced in *Ch*^{WT} showed enrichment for genes related with
277 the photosynthetic machinery. Particularly, *C. hirsuta* rapidly responds by
278 inducing the expression of genes encoding components of both photosystems I
279 and II, the NADH dehydrogenase-like complex (involved in chlororespiration)
280 and both small and large subunits of plastidial ribosomes (Supplemental Figure
281 5B, Supplemental Data Sets 5 and 6). Whether these rapid changes are
282 maintained after prolonged exposure to shade or have any functional relevance
283 is unknown. Nonetheless, these transcriptome differences support that the two
284 mustard species employ alternative strategies to adapt to plant proximity and
285 shade that go further from the modulation of elongation growth.

286 Comparative approaches have been used before to investigate the
287 differential response to shade of related species. Transcriptomic analyses using

288 two *Geranium* species that display divergent shade-induced petiole elongation
289 (*G. pyrenaicum* as a shade avoider or responsive, and *G. robertianum* as a
290 shade tolerant) identified a series of 31 up-regulated genes that included a
291 number of candidate regulators of differential shade avoidance (Gommers *et al.*,
292 2017). In these two species, putatively orthologous transcript groups (OMCL)
293 were defined, and the best BLAST hit with the *A. thaliana* transcriptome was
294 used to name *Geranium* OMCL groups (Gommers *et al.*, 2017). When we
295 compared our lists of shade-regulated genes with the *Geranium* OMCLs
296 differentially regulated after 2 h of low R:FR in the petioles, we found that the
297 number of genes up-regulated in both shade-tolerant and shade-avoider
298 species was higher for the *At*^{WT}/*Ch*^{WT} pair than between the *Geranium* species
299 (Supplemental Figure 4C; Supplemental Data Sets 7 and 8). GO analyses did
300 not identify any function from the lists of genes specifically induced in either *G.*
301 *pyrenaicum* or *G. robertianum*. Overlap was very limited between the sets of
302 repressed genes. Together, the contrasting rapid shade-induced gene
303 expression changes might either support differences in the early molecular
304 mechanisms between the *Geranium* and mustard groups, or just reflect the
305 differences in tissues (whole seedlings vs. leaf petioles) and/or shade and
306 growth conditions (continuous light vs. photoperiod) between experiments.

307 We also analyzed the changes in gene expression of *PIF3-LIKE 1 (PIL1)*
308 and *ATHB2*, two typical shade-marker genes, in response to longer (up to 8 h)
309 exposure to low R:FR. Expression of *PIL1* and *ATHB2* were rapidly induced in
310 both mustard seedlings after simulated shade exposure. However, the relative
311 induction of the expression of these genes was attenuated in *Ch*^{WT} compared to
312 *At*^{WT} (Figure 2D). Together, our results indicate that *C. hirsuta* seedlings sense
313 plant proximity and respond molecularly and metabolically to it; however, this
314 signal does not promote hypocotyl elongation in *C. hirsuta* as it does in the
315 shade-avoider *A. thaliana*.

316

317 **Shade-induced elongation in *C. hirsuta* is repressed**

318 To explain the hypocotyl elongation differences between *A. thaliana* and
319 *C. hirsuta*, we hypothesized two mutually exclusive mechanisms: (i)
320 **uncoupling**: shade perception is specifically unplugged from the endogenous
321 mechanisms of control of hypocotyl elongation, or (ii) **suppression**: there are

322 mechanisms that strongly suppress the shade-induced elongation of
323 hypocotyls. To distinguish between these possibilities, a genetic screening
324 looking for *C. hirsuta* seedlings with long hypocotyls under simulated shade (> 6
325 mm long) was carried out, using an EMS-mutagenized population (Vlad *et al.*,
326 2014). If suppression mechanisms exist, then loss-of-function mutants that
327 unleash shade-induced hypocotyl elongation might be recovered. Indeed, from
328 the various long hypocotyl seedlings identified we focused in two *slender in*
329 *shade* (*sis*) mutants, shown to be recessive and allelic. After backcrossing these
330 mutants twice with the Ch^{WT} plants, homozygous mutants had slightly longer
331 hypocotyls in W than the wild type, and very long hypocotyls under W+FR. We
332 named the mutants as *sis1-1* and *sis1-2* (Figure 3). These results indicated that
333 (1) loss-of-function (recessive) mutations support the “suppression”
334 mechanisms in *C. hirsuta* to establish shade-tolerance; and (2) a single gene,
335 *SIS1*, is able to repress the elongation response to shade in *C. hirsuta*.

336 As a first step to explore *SIS1* identity, we determined whether light
337 perception was altered in *sis1* mutants by analyzing hypocotyl length after de-
338 etiolation under monochromatic lights. We noticed that Ch^{WT} seedlings were
339 quite hyposensitive to R compared to At^{WT} (Figure 3B), suggesting that an
340 attenuated phyB signaling might result in a constitutive SAS hypocotyl
341 response, causing the observed suppression of the shade-induced hypocotyl
342 elongation. Considering the relationship between the attenuated
343 responsiveness to R and the strength of the shade-induced hypocotyl
344 elongation of the weak *phyB-4* and strong *phyB-1 A. thaliana* mutant seedlings
345 (Figure 3C,D), the hyposensitivity to R observed in Ch^{WT} might contribute but is
346 not enough to fully suppress the shade-induced hypocotyl elongation in this
347 species. Therefore, additional components are required to establish the shade-
348 tolerant hypocotyl habit in *C. hirsuta*. Indeed, mutant *sis1* seedlings, although
349 slightly hyposensitive to R and blue light, were fully blind to FR compared to
350 Ch^{WT} seedlings (Figure 3B).

351 A very similar pattern of response was also shown by *A. thaliana* phyA-
352 deficient *phyA-501* seedlings (Figure 3B) (Li *et al.*, 2011), which suggested that
353 *sis1* seedlings might be deficient in phyA activity or signaling. Sequencing of the
354 *C. hirsuta* *PHYA* (*ChPHYA*) gene from *sis1-1* and *sis1-2* plants showed point
355 mutations (transitions) that introduced either a nonsense mutation in Gln935 (in

356 *sis1-1*) or a missense mutation in the conserved Gly913 (in *sis1-2*) (Figure 3E,
357 Supplemental Figure 6A). Immunoblot analyses using a specific monoclonal
358 antibody against phyA (073D), indicated that only *sis1-1* was lacking phyA
359 (Figure 3D). Consistent with this, *C. hirsuta* lines with reduced activity of phyA
360 by overexpressing an RNA interference construct directed towards the *ChPHYA*
361 gene (lines 35S:RNAi-ChPHYA) also resulted in a *sis* phenotype (Supplemental
362 Figure 6B-D). Together, these results indicated that *sis1* are *C. hirsuta phyA*
363 deficient mutants (for clarity, we will keep the *sis1* mutant name along the
364 manuscript to distinguish it from the *phyA* mutants from *A. thaliana*). They also
365 suggested that shade tolerance in *C. hirsuta* might be caused by the existence
366 of a phyA-dependent suppression mechanism that represses the hypocotyl
367 elongation response to shade.

368 Molecular analyses showed that the relative induction of *PIL1* and
369 *ATHB2* expression was enhanced in both *sis1* mutants compared to Ch^{WT}
370 seedlings after more than 4 h of simulated shade exposure (Figure 4). This
371 relatively late effect of *ChPHYA* absence (*sis1*) on gene expression is
372 consistent with what was observed in *A. thaliana phyA* mutants (Ciolfi *et al.*,
373 2013). We also measured the levels of photosynthetic pigments (carotenoids
374 and chlorophylls) after long-term exposure to low R:FR, in wild-type and phyA-
375 deficient *A. thaliana* and *C. hirsuta* seedlings. Simulated shade triggered a
376 stronger decrease in the accumulation of these pigments in *phyA-501*, *sis1-1*
377 and *sis1-2* seedlings compared to wild-type controls (Figure 4B), hence
378 indicating that phyA represses this trait in both species, likely to avoid
379 exaggerated losses of photosynthetic pigments in response to vegetation
380 proximity and shade.

381 Phytochrome A represses the shade-induced hypocotyl elongation in *A.*
382 *thaliana* caused by the deactivation of phyB only under conditions that mimic
383 closed canopies, i.e., under very low R:FR (Casal *et al.*, 2014; Martinez-Garcia
384 *et al.*, 2014; Yanovsky *et al.*, 1995). Indeed, *A. thaliana phyA* deficient mutants
385 behaved almost like At^{WT} seedlings under various shade mimicking conditions
386 except for the lowest R:FR tested (Figure 4C). By contrast, *C. hirsuta sis1*
387 mutants behaved differently than its Ch^{WT} under all the low R:FR applied
388 (Figure 4D), indicating that phyA has a broader role in suppressing the shade-
389 induced hypocotyl elongation in *C. hirsuta* than in *A. thaliana*.

390

391 ***C. hirsuta* has higher phyA activity than *A. thaliana***

392 Our results suggested the possibility that phyA activity is higher in the
393 shade-tolerant *C. hirsuta* than in the shade-avoider *A. thaliana*. Higher phyA
394 activity can be achieved by at least two alternative and non-exclusive ways:
395 higher phyA levels and/or higher specific (intrinsic) activity of the photoreceptor.
396 To analyze these possibilities, we first aimed to compare *PHYA* expression
397 levels in *At*^{WT} and *Ch*^{WT} seedlings. Data extracted from our RNAseq experiment
398 indicated that the expression of several commonly-used reference genes, such
399 as *EF1α* or *YLS8* (Gallemi *et al.*, 2017b; Hornitschek *et al.*, 2009; Kohnen *et al.*,
400 2016), was within the same range (Supplemental Table 1). Then, we quantified
401 *PHYA* expression levels in *At*^{WT} and *Ch*^{WT} seedlings growing under W or W+FR
402 (Figure 5) using primers that recognize the sequence of the target gene (*PHYA*)
403 and three normalizer genes (*EF1α*, *SPC25*, *YLS8*) in both species
404 (Supplemental Figure 7). Expression of *PHYA* was significantly higher in *C.*
405 *hirsuta* than in *A. thaliana* seedlings (two-way ANOVA tests, $p < 0.05$) in
406 seedlings of different ages grown under W or W+FR conditions (Figure 5B).

407 Higher expression of *PHYA* in *C. hirsuta* might result in higher phyA
408 protein levels, contributing to an increased phyA activity in this species. Our
409 immunoblot analyses showed that *PHYA* protein levels were significantly higher
410 in *C. hirsuta* than *A. thaliana* etiolated seedlings (Figure 5D). More importantly,
411 whereas *PHYA* levels almost disappear after 6 h of W exposure in both species,
412 *C. hirsuta* seedlings maintained higher *PHYA* levels than *A. thaliana* when
413 exposed to W+FR for 6-10 h (Figure 5C-D). Together, these results support that
414 *PHYA* levels in *C. hirsuta* are generally higher than in *A. thaliana* seedlings,
415 even under shade conditions. This observation is consistent with the strongest
416 difference in hypocotyl length under W of wild-type and phyA-deficient seedlings
417 from *C. hirsuta* compared to *A. thaliana* (Figure 4C-D, Supplemental Figure 6D)
418 (Martinez-Garcia *et al.*, 2014). Furthermore, transgenic overexpression of *PHYA*
419 has been shown to attenuate shade-triggered hypocotyl elongation in *A.*
420 *thaliana* seedlings and stem elongation in other species (Heyer *et al.*, 1995;
421 Robson *et al.*, 1996; Roig-Villanova *et al.*, 2006).

422 To compare *AtphyA* and *ChphyA* specific (intrinsic) activities,
423 complementation analyses of the *A. thaliana phyA-501* mutant were carried out

424 with the *AtPHYA* or *ChPHYA* genes under the control of the endogenous
425 promoter of *AtPHYA* (*pAtPHYA:AtPHYA* or *pAtPHYA:ChPHYA*, respectively).
426 The resulting lines were named as *phyA>AtPHYA* and *phyA>ChPHYA* (Figure
427 6). We obtained a total of 5 independent *phyA>AtPHYA* lines and 7
428 independent *phyA>ChPHYA* lines with different transcript and protein levels
429 (Supplemental Figure 8). To estimate PHYA protein levels we used etiolated
430 seedlings, as *phyA* is photolabile. Because *PHYA* expression is repressed by
431 light via *phyA* and *phyB* (Canton and Quail, 1999), RNA was extracted from
432 seedlings either grown in the dark or under W+FR (Supplemental Figure 8A).
433 *PHYA* expression in seedlings grown in these two conditions correlated
434 positively in both *phyA>AtPHYA* ($R^2=0.79$) and *phyA>ChPHYA* lines ($R^2=0.79$)
435 (Supplemental Figure 8B). The slope of these equations, however, was
436 significantly higher ($p<0.05$) for *phyA>AtPHYA* (7.49) than *phyA>ChPHYA*
437 (2.81) lines. Specifically, *phyA>AtPHYA* and *phyA>ChPHYA* lines with
438 comparable *PHYA* expression levels in the dark showed lower *PHYA*
439 expression under simulated shade when complemented by *ChPHYA*
440 (*phyA>ChPHYA*) compared to *AtPHYA* (*phyA>AtPHYA*). These results pointed
441 to a stronger activity for the ChphyA protein in repressing its own (*PHYA*)
442 expression.

443 For the comparison of AtphyA and ChphyA activities, we initially studied
444 their effect on the promotion of the shade-induced hypocotyl elongation in
445 transgenic lines. *At*^{WT} and *phyA-501* seedlings were incorporated as controls. In
446 these experiments, the difference in hypocotyl length between seedlings grown
447 under W+FR vs. W ($\text{Hyp}_{\text{W+FR}}-\text{Hyp}_{\text{W}}$) provided values indicative of the
448 complementation level (or *phyA* biological activity) for the response analyzed.
449 Consequently, in these analyses, the lower the $\text{Hyp}_{\text{W+FR}}-\text{Hyp}_{\text{W}}$ value, the higher
450 the *phyA* activity. Opposite to that observed with transcript levels (Supplemental
451 Figure 8C), $\text{Hyp}_{\text{W+FR}}-\text{Hyp}_{\text{W}}$ correlated well with *ChPHYA* but not with *AtPHYA*
452 protein levels (Supplemental Figures 8D). These results together indicate that
453 the two photoreceptors are not fully exchangeable and suggest different intrinsic
454 qualities (i.e., biological activity) between the *phyA* receptors of *A. thaliana* and
455 *C. hirsuta*.

456 When lines with comparable *PHYA* protein levels were selected (Figure
457 6B), the response to shade ($\text{Hyp}_{\text{W+FR}}-\text{Hyp}_{\text{W}}$) was more strongly attenuated by

458 ChPHYA (Figure 6C-D). As an additional way to test for phyA activity, we
459 estimated hypocotyl elongation in seedlings etiolated (Hyp_D) and deetiolated
460 under monochromatic FR (Hyp_{FR}). In this case, the higher the difference
461 between these two values (Hyp_D-Hyp_{FR}), the stronger the activity of phyA.
462 Similar to the shade response analyses, ChphyA showed a stronger activity
463 than AtphyA in deetiolating seedlings under FR (Figure 6E-F). A good
464 correlation between these two phyA-mediated responses was also found when
465 all the lines were considered together (Supplemental Figure 8E), reinforcing our
466 interpretation that ChphyA is intrinsically more active than AtphyA.

467 The expression of dozens of auxin-responsive genes is repressed by
468 phyA after just 1 h of very low R:FR treatment (Yang *et al.*, 2018). As an
469 additional and complementary test of phyA biological activity different from
470 hypocotyl elongation we evaluated the repressive effect of AtphyA and ChphyA
471 on the expression of these genes. First, we selected *1-AMINO-*
472 *CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8 (ACS8)*, *GRETCHEN*
473 *HAGEN 3.3 (GH3.3)*, *INDOLE-3-ACETIC ACID INDUCIBLE 19 (IAA19)* and
474 *IAA29*, four auxin-responsive genes described as repressed phyA targets (Yang
475 *et al.*, 2018). As expected, the shade-induced expression of these genes was
476 attenuated in At^{WT} compared to *phyA-501* seedlings, but under our shade
477 conditions the differences were most obvious after long exposure to W+FR
478 (Figure 7).

479 The expression of the same genes was next quantified in seedlings from
480 the various *phyA>AtPHYA* and *phyA>ChPHYA* lines grown for 24 h under
481 W+FR. When plotting transcript levels of phyA target genes as a function of
482 *PHYA* expression in these lines, the clouds of data corresponding to
483 *phyA>ChPHYA* lines (red) were separated from that of *phyA>AtPHYA* lines
484 (blue) (Figure 7B). Importantly, the expression of all phyA target genes tested
485 was overall lower in *phyA>ChPHYA* than *phyA>AtPHYA* lines, indicating that
486 ChphyA repressed more efficiently gene expression than AtphyA (Figure 7B).
487 Consistent with this conclusion, the expression of these and other phyA target
488 genes (Yang *et al.*, 2018) was attenuated in shade-induced seedlings of Ch^{WT}
489 compared to At^{WT} (Supplemental Figure 9). Together, these data further support
490 that ChphyA is intrinsically more active than AtphyA.

491

492 DISCUSSION

493 Currently, the genetic basis of shade tolerance is poorly understood. To
494 address this open question, we have focused on comparative analyses of the
495 hypocotyl response to shade in young seedlings of two related mustards, *A.*
496 *thaliana* and *C. hirsuta*. Shade avoidance and tolerance are ecological concepts
497 originated from the natural habitats of plants species (Callahan *et al.*, 1997).
498 Hence, defining the shade habit of a species is difficult because shade
499 tolerance is not an absolute value but a relative concept; indeed, plants may
500 exhibit different strategies during the juvenile and adult phases of their lives
501 (Valladares and Niinemets, 2008). Despite the uncertainty, *A. thaliana* is
502 generally considered as shade avoider and it is a model broadly used to study
503 the SAS hypocotyl response, but there is little information referring to its
504 physiological shade-responsiveness habit. *C. hirsuta*, by contrast, has been
505 previously described as a shade tolerant species whose hypocotyls are
506 unresponsive to shade (Bealey and Robertson, 1992; Hay *et al.*, 2014), but little
507 is known about other shade response mechanisms. Here we confirm that, as
508 expected for a shade-tolerant species, *C. hirsuta* showed a much better
509 capacity to acclimate to LL than to HL compared to *A. thaliana* (Supplemental
510 Figure 1). Most strikingly, *C. hirsuta* seedlings failed to elongate in response to
511 simulated proximity or canopy shade (Figure 1). Such a dramatic hypocotyl
512 elongation response compared to *A. thaliana* makes these two related species
513 good candidates for comparative analyses of divergent responses to shade.

514 Our comparative and genetic analyses suggest that the absence of a
515 shade-induced hypocotyl elongation in *C. hirsuta* is not caused by defects on
516 the rapid biosynthesis of auxin in seedlings (Figure 2). Although we cannot
517 exclude local defects in auxin biosynthesis (e.g., in hypocotyls) that might be
518 masked by collecting whole seedlings, our conclusion is consistent with the lack
519 of effect of phyA on the rapid shade-induced biosynthesis of auxin (Yang *et al.*,
520 2018). On the contrary, we favor that the differences in hypocotyl elongation
521 between these species is the result of a suppression mechanisms sustained by
522 the stronger activity of the ChphyA photoreceptor, likely enhanced by the
523 attenuated ChphyB activity (Figure 3B). A stronger intrinsic (specific) repressor
524 activity of ChphyA would result in a strong suppression of the elongation of *C.*
525 *hirsuta* seedlings when exposed to shade (Figure 8). The underlying

526 mechanism likely relies, at least partly, upon suppression of auxin signaling via
527 phyA directly binding and stabilizing AUX/IAA proteins, as it has been shown in
528 *A. thaliana* (Yang *et al.*, 2018). In this scenario, ChphyA seems to suppress not
529 auxin biosynthesis but signaling more strongly than AtphyA, as deduced from
530 the results with transgenic lines (Figure 7B) but also from the stronger
531 repression in shade of auxin-responsive genes with a putative role in auxin-
532 signaling (e.g., several *IAA* and *SAUR* genes) detected in Ch^{WT} compared to
533 At^{WT} (Supplemental Figure 9).

534 AtphyA and ChphyA might achieve different activities by changes in
535 particular residues that could alter susceptibility to post-translational
536 modifications. For instance, phyA stability, Pfr to Pr reversion rate upon shade
537 treatment or/and interaction with protein partners (e.g., PIF1/PIF3, FHY1/FHL,
538 AUX/IAA) affect phyA activity in *A. thaliana* (Dieterle *et al.*, 2005; Genoud *et al.*,
539 2008; Kim *et al.*, 2004; Oka *et al.*, 2012; Seo *et al.*, 2004; Sheerin *et al.*, 2015).
540 These intrinsic differences might be also enhanced by changes in protein
541 abundance of phyA or/and other components in its signaling pathway
542 specifically acting in light-grown seedlings (see below). Comparison of the
543 amino acid sequences of AtphyA and ChphyA, however, did not point to any
544 obvious specific residue or region that could be responsible for the observed
545 intrinsic differences in activity (Supplemental Figure 10). This is an issue that
546 would need future research.

547 The genetic mechanisms underlying physiological evolution remain
548 largely unknown, but changes in the timing, location and levels of gene
549 expression (i.e., *cis*-regulatory evolution of key genes) have caused much of
550 morphological evolution changes (Carroll, 2008). Our data on *PHYA* expression
551 and *PHYA* protein levels (Figure 5) agree with this view, but they go a step
552 beyond by showing that differences in protein (ChphyA and AtphyA) intrinsic
553 activities also contribute to differential responses to shade (Figure 6-7). As both
554 components (levels vs. intrinsic activity) are intimately connected (e.g., phyA
555 represses its own expression in a light-dependent manner), at this stage it is
556 difficult to quantify the specific contribution of each one. Moreover, additional
557 components might contribute: while we show that phyA is a central component
558 of a range of regulators that can be modulated in nature to implement shade
559 tolerance, the observation that none of the *phyA>ChPHYA* lines display a

560 shade-tolerant habit (Supplemental Figure 8D) strongly suggests that additional
561 downstream components of the shade-regulatory network are also participating
562 in suppressing this response in *C. hirsuta* (e.g., differences in phyB activity).
563 Indeed, it cannot be excluded that the mutant screen, despite identifying an
564 important regulator, did not establish the causal difference between the two
565 species in terms of shade-induced hypocotyl elongation. Nonetheless, our
566 results unveil the importance of modulating photoreceptor activity as a powerful
567 evolutionary mechanism in nature to achieve physiological variation between
568 species, hence enabling the colonization of new, different habitats. In addition,
569 searching for variability in phyA function could provide a suitable tool to modify
570 the impact of neighbors' cues in crops to minimize yield losses.

571

572 **MATERIALS AND METHODS**

573 **Plant material and plant growth conditions**

574 Plants of *Arabidopsis thaliana* Col-0 (At^{WT}) *phyA-501* (in Col-0 background),
575 *phyB-1*, *phyB-4* (both phyB deficient lines are in Ler background), and
576 *Cardamine hirsuta*, of the reference Oxford (Ox) accession (Ch^{WT}), have been
577 described (Hay *et al.*, 2014; Martinez-Garcia *et al.*, 2014; Reed *et al.*, 1993).
578 Plant growth conditions have been described elsewhere (Gallemi *et al.*, 2016;
579 Martinez-Garcia *et al.*, 2014). Normal light conditions refer to W produced by
580 cool-white vertical fluorescent tubes (PAR of 20-24 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Low and high
581 light conditions corresponded to PAR values of 4 and 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$,
582 respectively. Shade treatments in seedlings were provided by enriching W
583 (R:FR of 2.5) with different intensities of FR LEDs (730-nm peak; Philips
584 Greenpower Research modules) to produce the indicated R:FR (0.091 to 0.021)
585 without altering PAR. Light spectra are presented in Supplemental Figure 2. For
586 estimating petiole and rachis length, rosette plants were grown under long day
587 (LD, 16 h light, 8 h dark) photoperiods, in which W was generated by cool-white
588 horizontal fluorescent tubes (PAR of $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$, R:FR of 3.0); for shade
589 treatments, W was supplemented with FR (W+FR, PAR of $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$,
590 R:FR of 0.05). Fluence rates were measured with a Spectrosense2 meter
591 associated with a 4-channel sensor (Skye instruments Ltd.,
592 www.skyinstruments.com) which measures PAR (400-700 nm) and 10 nm
593 windows in the blue (464-473 nm), R (664-673 nm) and FR (725-734 nm)

594 regions (Gallemi *et al.*, 2017b). Light spectra were generated using a Flame
595 Model Spectrometer with Sony Detector (FLAME-S) (<https://oceanoptics.com>).

596

597 **Hypocotyl, petiole and rachis measurements**

598 For hypocotyl measurement, about 30 seeds of each genotype were
599 germinated on the plates for observing the seedling phenotype and at least 20
600 seedlings were measured for quantification of hypocotyl length. All experiments
601 were repeated at least three times with consistent results. Hypocotyl
602 measurements from all the different experiments were averaged. For petiole
603 measurement, about 30 seeds of each genotype were germinated under
604 continuous W. One week later, 20 seedlings in a similar stage of development
605 were transferred to individual pots and moved to a LD growth chamber (R:FR of
606 3.0). After one week, half of the rosette plants stayed under W and the other
607 half were moved to a W+FR shelf (R:FR of 0.05). After one week of differential
608 R:FR treatment, leaves were harvested and petiole was measured; in the case
609 of complex leaves from *C. hirsuta*, rachises were measured, covering the
610 distance from the base of the leaf until the base of the main leaflet
611 (Supplemental Figure 3). At least 8 leaves were measured for quantification of
612 petiole and rachis length for each leaf number. Experiments were repeated four
613 times with consistent results. Petiole and rachis measurements from all four
614 experiments were averaged.

615

616 **Photosynthetic pigment quantification and chlorophyll fluorescence.**

617 Whole 7-day-old seedlings of the indicated genotypes and grown under W or
618 W+FR (Figures 1 and 4) or transferred to HL conditions (Supplemental Figure
619 1B) were harvested, ground in liquid nitrogen, and the resulting powder was
620 used for quantification of chlorophylls and carotenoids spectrophotometrically or
621 by HPLC, as described (Bou-Torrent *et al.*, 2015).

622 Fluorescence measurements were carried out on seedlings grown under
623 different light regimes using a MAXI-PAM fluorometer (Heinz Walz GmbH). For
624 every measurement the whole cotyledons of 7 seedlings were considered.
625 Maximum quantum yield of photosystem II (PSII), F_v/F_m , was calculated as
626 $(F_m - F_o)/F_m$, where F_m and F_o are respectively the maximum and the minimum
627 fluorescence of dark-adapted samples. For dark acclimation, plates were

628 incubated for at least 30 minutes in darkness to allow the full relaxation of
629 photosystems. Rapid light curves (RLCs) were constructed with 10 incremental
630 steps of actinic irradiance (E; 0, 1, 21, 56, 111, 186, 281, 396, 531, 701 μmol
631 photons $\text{m}^{-2} \text{s}^{-1}$). For each step, the effective quantum yield of PSII ($\Delta F/F_m'$)
632 was monitored every min and relative electron transport rate (rETR) was
633 calculated as $E \times \Delta F/F_m'$. The light response was characterized by fitting
634 iteratively, using MS Excel Solver, the model of Platt (1980) to rETR versus E
635 curves. The fit was very good in all the cases ($r > 0.98$).

636

637 **Expression analyses by RT-qPCR and RNA-seq**

638 RNA was extracted from whole seedlings of *A. thaliana* and *C. hirsuta* (grown
639 as detailed in each experiment, three biological replicates per time point, each
640 biological replicate composed of 30-40 seedlings) using commercial kits
641 (RNAeasy Plant Mini kit; Qiagen; www.qiagen.com; or the semi-automatic
642 Maxwell SimplyRNA kit; Promega; www.promega.com). For real-time qPCR
643 analysis, two micrograms of RNA were reverse-transcribed using the M-MLV
644 Reverse Transcriptase (Invitrogen, www.lifetechnologies.com) or Transcriptor
645 First Strand cDNA synthesis (Roche, lifescience.roche.com). Reference genes
646 used were *UBQ10*, *EF1 α* , *SPC25* or/and *YLS8*.

647 For RNA-seq analyses, quantification of gene expression was performed
648 as indicated elsewhere (Gan *et al.*, 2016) and detailed as Supplemental
649 information. From the lists of genes, we selected as differentially expressed
650 those whose fold change was significantly (p adjusted < 0.05) and higher than
651 1.5 (Supplemental Data Sets 1 and 3) or lower than 0.67 (Supplemental Data
652 Sets 2 and 4) in seedlings treated for 1 h with W+FR compared to those grown
653 under W in either *C. hirsuta* (Supplemental Data Sets 1 and 2) or *A. thaliana*
654 (Supplemental Data Sets 3 and 4).

655

656 **Gene Ontology (GO) and MapMan analysis**

657 A strict synteny based approach was used to identify conserved orthologs
658 between the two species. The *A. thaliana* orthologs of the *C. hirsuta* genes were
659 used for getting the GO term annotations and MapMan-Bins. The GO term
660 annotations for *A. thaliana* genes, used as a reference, were obtained from The
661 Gene Ontology Consortium (<http://www.geneontology.org/>) (Ashburner *et al.*,

662 2000). The results are presented as Supplemental Data Set 5. For the
663 MapMan-Bin analyses, each list of genes were submitted to the “Mercator”
664 gene function prediction pipeline (Lohse *et al.*, 2014), that annotates the query
665 genes with the hierarchical ontology MapMan-Bins (Klie and Nikoloski, 2012;
666 Thimm *et al.*, 2004). Based on these MMB annotations, exact Fischer tests for
667 function enrichment within the six groups of differentially expressed genes were
668 carried out and interpreted (Supplemental Data Set 6).

669

670 **Protein extraction and immunoblot analysis**

671 Methods for extracting and detecting phyA protein levels in *A. thaliana* or *C.*
672 *hirsuta* seedlings (Gallemi *et al.*, 2017b; Martinez-Garcia *et al.*, 1999) are
673 detailed as follows. Protein extracts from *C. hirsuta* seedlings analyzed in
674 Figure 3 and Supplemental Figure 6 were prepared following the direct extract
675 protocol (Martinez-Garcia *et al.*, 1999) with the modifications described below.
676 Extracts were prepared from Ch^{WT}, *sis1* and RNAi-ChPHYA seedlings
677 germinated and grown in the dark for 4 days. Ten seedlings per genotype were
678 harvested in the dark and extracted in 1.5 mL microfuge tubes containing 300
679 μ L of Laemmli buffer supplemented with protease inhibitors (10 μ g/mL
680 Aprotinin, 1 μ g/mL E-64, 10 μ g/mL Leupeptin, 1 μ g/mL Pepstatin A, 100 μ M
681 PMSF). These extracts were prepared in duplicate and similar results were
682 observed. Plant material was ground using disposable grinders in the
683 Eppendorf tube at room temperature until the mixture was homogeneous
684 (usually less than 15 s). Once all the samples were prepared, tubes were
685 placed in boiling water for 3 minutes. Tubes were centrifuged in a microfuge at
686 maximum speed (13000 g, 10 min) immediately before loading. Fifteen μ L of
687 each extract, equivalent to about 0.5 seedlings, were loaded per lane in an SDS
688 - 8% PAGE.

689 Protein extracts analyzed in Figure 5 were prepared from At^{WT} and Ch^{WT}
690 seedlings grown as indicated in the figure legend. Extracts were obtained from
691 four biological replicates. Protein extracts analyzed in Figure 6 were prepared
692 from At^{WT}, *phyA-501*, *phyA>AtPHYA* and *phyA>ChPHYA* seedlings germinated
693 and grown in the dark for 4 days, as described (Gallemi *et al.*, 2017a). Extracts
694 were obtained from three biological replicates. Each biological replicate was
695 obtained from about 100 seedlings. Protein concentration in these extracts was

696 determined using the Pierce BCA Protein Assay kit (Cat no. 23225;
697 www.thermofisher.com). Five or 7.5 µg of each extract were loaded per lane in
698 an SDS - 8% PAGE.

699 Immunoblot analyses of PHYA and TUB were performed at the same
700 time with the antibodies (073D, commercial anti-TUB) and dilutions indicated
701 elsewhere (Martinez-Garcia *et al.*, 2014). Anti-mouse horseradish peroxidase-
702 conjugated antibody (www.promega.com) was used as a secondary antibody.
703 ECL or ECL-plus chemiluminescence kits (www3.gehealthcare.com) were used
704 for detection. Signal was visualized and quantified using the ChemiDoc Touch
705 Imaging System (www.bio-red.com).

706

707 **Hormone analyses**

708 Hormone extraction and analysis were carried out as described (Durgbanshi *et*
709 *al.*, 2005) with a few modifications. Briefly, 0.02 g of dry tissue (about 150 At^{WT}
710 seedlings and 100 Ch^{WT} seedlings) was extracted in 1 mL of ultrapure water
711 after spiking with 50 ng of [²H₂]-IAA, in a ball mill (MillMix20, Domel, Železniki,
712 Slovenija). After centrifugation at 4000 g at 4°C for 10 min, supernatants were
713 recovered and pH adjusted to 3 with 30% acetic acid. The water extract was
714 partitioned twice against 2 mL of diethyl ether and the organic layer recovered
715 and evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan,
716 Saint Herblain Cedex, France). Once dried, the residue was resuspended in a
717 10:90 MeOH:H₂O solution by gentle sonication. The resulting solution was
718 filtered through 0.22 µm polytetrafluoroethylene membrane syringe filters (Albet
719 S.A., Barcelona, Spain) and directly injected into an ultra performance LC
720 system (Acquity SDS, Waters Corp., Milford, MA, USA). Chromatographic
721 separations were carried out on a reversed-phase C18 column (Gravity, 50 ×
722 2.1 mm, 1.8-µm particle size, Macherey-Nagel GmbH, Germany) using a
723 MeOH:H₂O (both supplemented with 0.1% acetic acid) gradient at a flow rate of
724 300 µL min⁻¹. IAA was quantified with a TQS triple quadrupole mass
725 spectrometer (Micromass, Manchester, UK) connected online to the output of
726 the column through an orthogonal Z-spray electrospray ion source.

727

728 **Data availability**

729 The Illumina RNA-seq reads are available from the website
730 <http://chi.mpipz.mpg.de/assembly>. Source code of BAMLINK is available at
731 <http://chi.mpipz.mpg.de/software>. The data that support the findings of this
732 study are also available from the corresponding author on request.

733

734 **Accession Numbers**

735 Sequence data from this article can be found in the Arabidopsis Genome
736 Initiative or the *C. hirsuta* (<http://chi.mpipz.mpg.de/assembly>) databases under
737 the following accession numbers: *AtATHB2* (At4g16780), *ChATHB2*
738 (CARHR223400), *AtPIL1* (At2g46970), *ChPIL1* (CARHR142340), *AtUBQ10*
739 (At4g05320), *AtPHYA* (At1g09570), *ChPHYA* (CARHR009540), *ACS8*
740 (At4g37770), *GH3.3* (At2g23170), *IAA19* (At3g15540), *IAA29* (At3g15540),
741 *AtEF1 α* (At5g60390), *ChEF1 α* (CARHR274060 and CARHR274080), *SPC25*
742 (At2g39960), *ChSPC25* (CARHR134880 and CARHR134890), *YLS8*
743 (At5g08290) and *ChYLS8* (CARHR204840).

744

745 **Supplemental Data**

746 **Supplemental Figure 1.** Photosynthetic-related responses of *A. thaliana* and
747 *C. hirsuta* seedlings to changing light conditions.

748

749 **Supplemental Figure 2.** Light spectra of the treatments used in this study.

750

751 **Supplemental Figure 3.** Longitudinal length of *A. thaliana* and *C. hirsuta*
752 leaves respond differently to simulated shade.

753

754 **Supplemental Figure 4.** *A. thaliana* and *C. hirsuta* seedlings change gene
755 expression differently in response to simulated shade.

756

757 **Supplemental Figure 5.** The expression of a set of shade-induced but auxin-
758 dependent genes, identified in *A. thaliana*, is also shade-induced in *C. hirsuta*.

759

760 **Supplemental Figure 6.** Reduction of phyA activity in *C. hirsuta* seedlings
761 results in a *sis* phenotype.

762

763 **Supplemental Figure 7.** Partial alignment of *ChPHYA/AtPHYA*,
764 *ChEF1 α /AtEF1 α* , *ChSPC25/AtSPC25* and *ChYLS8/AtYLS8* sequences.

765

766 **Supplemental Figure 8.** Strategies to compare biological activity between
767 AtphyA and ChphyA in transgenic lines.

768

769 **Supplemental Figure 9.** The expression of a set of shade-induced phyA-
770 repressed genes, identified in *A. thaliana*, is attenuated in *C. hirsuta*.

771

772 **Supplemental Figure 10.** Alignment of *C. hirsuta* and *A. thaliana* phyA amino
773 acid sequences.

774

775 **Supplemental Table 1.** RPKM of eight genes commonly used for normalizing
776 in RT-qPCR analyses.

777

778 **Supplemental Table 2.** Primers used in this work.

779

780 **Supplemental Data Set 1.** Bioset of up-regulated genes in *C. hirsuta* seedlings
781 in response to simulated shade.

782

783 **Supplemental Data Set 2.** Bioset of down-regulated genes in *C. hirsuta*
784 seedlings in response to simulated shade.

785

786 **Supplemental Data Set 3.** Bioset of up-regulated genes in *A. thaliana*
787 seedlings in response to simulated shade.

788

789 **Supplemental Data Set 4.** Bioset of down-regulated genes in *A. thaliana*
790 seedlings in response to simulated shade.

791

792 **Supplemental Data Set 5.** Results of Venn Diagrams of the GO categorization.

793

794 **Supplemental Data Set 6.** Functional enrichment groups based on the
795 MapMan-Bin analyses.

796

797 **Supplemental Data Set 7.** Bioset of shade-regulated OMCL groups in
798 *Geranium pyrenaicum* petioles in response to simulated shade.

799

800 **Supplemental Data Set 8.** Bioset of shade-regulated OMCL groups in
801 *Geranium robertianum* petioles in response to simulated shade.

802

803 **Supplemental Data Set 9.** Summary of statistical tests.

804

805

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829

830 **AUTHOR CONTRIBUTIONS**

831 JFM-G conceived the original research plan, and directed and coordinated the
832 study. AH, HJ, XG and MT performed RNAseq and analyzed the data. AG-C
833 analyzed auxin levels; LM and MR-C measured and analyzed photosynthetic
834 parameters and pigment levels; MJM-C, SP, CT, JM-R, PP-A and IR-V
835 performed all the other experiments. All authors analyzed their data and
836 discussed the results. JFM-G wrote the paper with revisions of MR-C and
837 contributions or/and comments of all other authors.

838

839 **COMPETING INTERESTS**

840 The authors declare no competing interests.

841

842 **FIGURE LEGENDS**

843

844 **Figure 1. *A. thaliana* and *C. hirsuta* differ in the hypocotyl elongation**
845 **response to neighboring vegetation. (A)** Phenotype of representative
846 seedlings of wild-type *A. thaliana* (At^{WT}) and *C. hirsuta* (Ch^{WT}) after 3 days
847 grown in W and retained in W (left panels) or transferred to W+FR (R:FR of
848 0.02; right panels) until day 7 (d7). Scale bar, 5 mm. **(B)** Hypocotyl length of d7
849 At^{WT} and Ch^{WT} seedlings grown for the last 4 days under the indicated R:FR.
850 **(C)** Hypocotyl length of d4 At^{WT} and Ch^{WT} seedlings grown in darkness. **(D)**
851 Hypocotyl length of d7 At^{WT} and Ch^{WT} seedlings grown under W in media
852 supplemented with increasing concentrations of picloram (PIC). **(E)** Petiole and
853 rachis length of 3-week-old leaves of At^{WT} and Ch^{WT} plants grown for the last 7
854 days under the indicated R:FR. **(F)** Carotenoid (CRT) and chlorophyll (CHL)
855 levels of At^{WT} and Ch^{WT} seedlings grown in W and W+FR (as detailed in **A**).
856 Values are means and s.e.m. of three to five independent samples. Asterisks
857 indicate significant differences (** $p < 0.01$) relative to W-grown plants.

858

859 **Figure 2. *A. thaliana* and *C. hirsuta* seedlings respond to neighboring**
860 **vegetation by altering gene expression. (A)** RNA-seq was performed with
861 RNA extracted from At^{WT} and Ch^{WT} seedlings that were grown in W for 7 days
862 (d7) and then treated for 1 h with W+FR (R:FR = 0.02). White circles indicate
863 the moment of harvesting for RNA extraction. Three independent biological
864 replicates were used for each genotype and treatment. **(B)** Correlation between

865 log-transformed fold-change of 432 DEGs in *At*^{WT} and *Ch*^{WT}. The estimated
866 regression equation is shown at the top of the graph. **(C)** IAA content in *At*^{WT}
867 and *Ch*^{WT} seedlings grown and harvested as indicated in **A**. Whole seedlings
868 were collected and lyophilized to measure IAA levels. Data are presented as the
869 means and s.e.m. of three (*At*^{WT}) or four (*Ch*^{WT}) biological replicates. DW, dry
870 weight. **(D)** Effect of W+FR treatment on *PIL1* and *ATHB2* expression in *At*^{WT}
871 and *Ch*^{WT} seedlings (R:FR = 0.02). W-grown d7 seedlings of Col-0 and Ox were
872 treated for 0, 1, 4 and 8 h with W+FR. Transcript abundance, normalized to
873 EF1 α is shown. Values are means and s.e.m. of three independent RT-qPCR
874 biological replicates relative to values at 0 h for each species. In **C** and **D**,
875 asterisks indicate significant differences (**p<0.01, *p<0.05) relative to 0 h
876 samples.

877

878 **Figure 3. Mutant *sis1* seedlings of *C. hirsuta* are deficient in phyA activity.**

879 **(A)** Phenotype of representative seedlings of *Ch*^{WT}, *sis1-1* and *sis1-2* after 3
880 days grown in W and retained in W (white panels) or transferred to W+FR
881 (R:FR of 0.02; pink panels) until day 7 (d7). All panels are to the same scale.
882 **(B)** Hypocotyl length of *At*^{WT}, *phyA-501* (*A. thaliana*), *Ch*^{WT}, *sis1-1* and *sis1-2*
883 (*C. hirsuta*) lines grown for 4 days in darkness (Dark) or under monochromatic
884 FR (2.6 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), R (38.9 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and blue (B, 1.9 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light.
885 **(C)** Hypocotyl length of *A. thaliana* Ler, *phyB-4* and *phyB-1* seedlings grown for
886 4 days in darkness (Dark) or under monochromatic R (40.6 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light.
887 **(D)** Hypocotyl length of *A. thaliana* Ler, *phyB-4* and *phyB-1* seedlings under the
888 indicated R:FR. Seedlings were grown for 2 days in W (R:FR > 2.5) and then
889 kept in W (R:FR > 2.5) or transferred to W+FR (R:FR of 0.06 or 0.02) until day 7
890 (d7). **(E)** Schematic diagram of the lesions found in the *ChPHYA* gene in the
891 *sis1-1* and *sis1-2* alleles compared to the wild-type sequence (*Ch*^{WT}) and the
892 predicted changes in the amino acid sequence. **(F)** Immunoblot detection of
893 phyA and tubulin with mouse monoclonal anti-phyA (073D) and anti-TUB
894 antibodies in extracts of etiolated seedlings of *Ch*^{WT}, *sis1-1* and *sis1-2* lines.

895

896 **Figure 4. *C. hirsuta sis1* seedlings are impaired in their tolerance to plant**

897 **proximity. (A)** Effect of W+FR treatment on *PIL1* and *ATHB2* expression in
898 *Ch*^{WT} *sis1-1*- and *sis1-2* seedlings. Seedlings were grown as in Figure 2D.

899 Transcript abundance, normalized to EF1 α is shown. Values are means and
900 s.e.m. of three independent RT-qPCR biological replicates relative to values at
901 0 h for each genotype. Asterisks indicate significant differences (**p<0.01)
902 relative to 0 h samples. **(B)** Carotenoid (CRT) and chlorophyll (CHL) levels of
903 At^{WT} and phyA-501 *A. thaliana* and Ch^{WT}, *sis1-1* and *sis1-2* *C. hirsuta* seedlings
904 grown in W and W+FR (as detailed in Figure 1A). Values are means and s.e.m.
905 of five independent samples. Asterisks indicate significant differences
906 (**p<0.01) relative to W-grown plants. **(C,D)** Hypocotyl length of d7 At^{WT}, *phyA-*
907 *501* (*A. thaliana*) **(C)** and Ch^{WT}, *sis1-1*, *sis1-2* (*C. hirsuta*) **(D)** seedlings grown
908 for the last 4 days under the indicated R:FR. Asterisks indicate significant
909 differences (*p<0.05, **p<0.01) relative to the corresponding wild-type plant
910 grown under the same R:FR. In **D**, asterisks apply for both *sis1* mutants.

911

912 **Figure 5. *C. hirsuta* seedlings have higher phyA levels than those of *A.***
913 ***thaliana*.** **(A)** Cartoon showing the design of the experiment. Wild-type
914 seedlings of *A. thaliana* (At^{WT}) and *C. hirsuta* (Ch^{WT}), grown as in Figure 1A,
915 were harvested at the indicated times of W or W+FR treatments (asterisks) for
916 RNA extraction. **(B)** Evolution of *PHYA* transcript levels in *A. thaliana* and *C.*
917 *hirsuta* wild-type seedlings grown as detailed in **A**. Primers used (Supplemental
918 Figure 8A) allow quantifying and comparing expression levels by RT-qPCR
919 between both species. *PHYA* transcript abundance was normalized to three
920 reference genes (*EF1 α* , *SPC25* and *YLS8*). Values are means and s.e.m. of
921 three independent RT-qPCR biological replicates relative to *PHYA* transcript
922 levels of d3 *A. thaliana* seedlings. Two-way ANOVA showed that *PHYA* levels
923 are significantly different (**p<0.01) between species under either W or W+FR.
924 **(C)** Immunoblot detection of phyA and tubulin with the antibodies indicated in
925 Figure 3C in extracts of At^{WT} and Ch^{WT} seedlings grown as detailed at the top of
926 the section: 5-day-old etiolated seedlings were exposed to W light and material
927 was harvested before and after 6 h of W-exposure (arrows). **(D)** Evolution of
928 relative phyA protein levels (PHYA:TUB) in At^{WT} and Ch^{WT} seedlings exposed to
929 simulated shade, as detailed at the top of the section: 5-day-old etiolated
930 seedlings were exposed to W+FR light and material was harvested before and
931 after 6, 8 and 10 h of simulated shade exposure (arrows). Values are means
932 and s.e.m. of four independent biological replicates relative to PHYA:TUB levels

933 of etiolated At^{WT} seedlings. Two way ANOVA showed that relative PHYA levels
934 under W+FR are significantly increased (** $p < 0.01$) in *C. hirsuta* than *A. thaliana*.

935

936 **Figure 6. ChphyA has a stronger activity than AtphyA in repressing**
937 **shade-induced hypocotyl elongation. (A)** Cartoon detailing the constructs

938 used to complement *A. thaliana phyA-501* mutant plants. **(B)** Relative

939 PHYA:TUB in etiolated seedlings of At^{WT} , *phyA-501*, and selected

940 *phyA>AtPHYA* (blue bars) and *phyA>ChPHYA* (red bars) complementation

941 lines. Seedlings were grown as indicated in Supplemental Figure 8. Values are

942 means and s.e.m. of four independent biological replicates relative to

943 PHYA:TUB levels of etiolated At^{WT} seedlings. **(C)** Cartoon illustrating how phyA

944 activity in simulated shade was established as differences in hypocotyl length

945 between simulated shade- and the W-grown seedlings ($Hyp_{W+FR} - Hyp_W$).

946 Seedlings were grown for 2 days under W then for 5 additional days under W or

947 W+FR (R:FR = 0.02), when hypocotyls were measured. **(D)** $Hyp_{W+FR} - Hyp_W$ in

948 seedlings of At^{WT} , *phyA-501*, and selected *phyA>AtPHYA* (blue bars) and

949 *phyA>ChPHYA* (red bars) complementation lines. **(E)** Cartoon illustrating how

950 phyA activity in de-etiolation was established as differences in hypocotyl length

951 between dark- and FR-grown seedlings ($Hyp_D - Hyp_{FR}$). Seedlings were grown as

952 indicated in Figure 3B. **(F)** $Hyp_D - Hyp_{FR}$ in seedlings of At^{WT} , *phyA-501*, and

953 selected *phyA>AtPHYA* (blue bars) and *phyA>ChPHYA* (red bars)

954 complementation lines. In **C** and **E**, mutant *phyA-501* seedlings have no phyA

955 activity.

956

957 **Figure 7. ChphyA has a stronger activity than AtphyA in repressing**
958 **shade-induced expression of ACS8, GH3.3, IAA19 and IAA29 genes. (A)**

959 Effect of phyA in the shade-induced expression of ACS8, GH3.3, IAA19 and

960 IAA29. W-grown d5 seedlings of At^{WT} and *phyA-501* were treated for 0, 1, 8 and

961 24 h with W+FR (R:FR = 0.02), when material was harvested for RNA

962 extraction, as indicated at the top of the panel. Transcript abundance,

963 normalized to EF1 α is shown. Values are means and s.e.m. of three

964 independent RT-qPCR biological replicates relative to values at 0 h for At^{WT} .

965 Asterisks indicate significant differences (** $p < 0.01$, * $p < 0.05$) between *phyA-501*

966 and At^{WT} seedlings exposed for the same time to W+FR. **(B)** Correlation

967 between *ACS8*, *GH3.3*, *IAA19* and *IAA29* expression and relative levels of
968 PHYA protein in the seedlings of *At*^{WT}, *phyA-501*, *phyA>AtPHYA* (blue lines and
969 dots) and *phyA>ChPHYA* (red lines and dots) complementation lines. Gene
970 expression was quantified in W-grown d5 seedlings exposed to W+FR
971 (R:FR=0.02) during 24 h, as indicated at the top of the panel. Transcript
972 abundance was normalized to *EF1α*. Relative phyA protein levels (PHYA:TUB,
973 data already shown in Supplemental Figure 8) were estimated in etiolated
974 seedlings. Values are means and s.e.m. of three independent RT-qPCR
975 biological replicates relative to values of *At*^{WT}. The estimated regression lines
976 for the *phyA>AtPHYA* (blue line) and *phyA>ChPHYA* (red line)
977 complementation lines are shown for each correlation.

978

979 **Figure 8. Model of how increased phyA activity in *C. hirsuta* might**
980 **implement the shade tolerance of hypocotyl elongation.** Increases in phyA
981 activity caused by the constitutive overexpression of *PHYA* also attenuate the
982 shade-induced hypocotyl elongation in transgenic plants, and it results in
983 partially tolerant *A. thaliana* seedlings.

984

985

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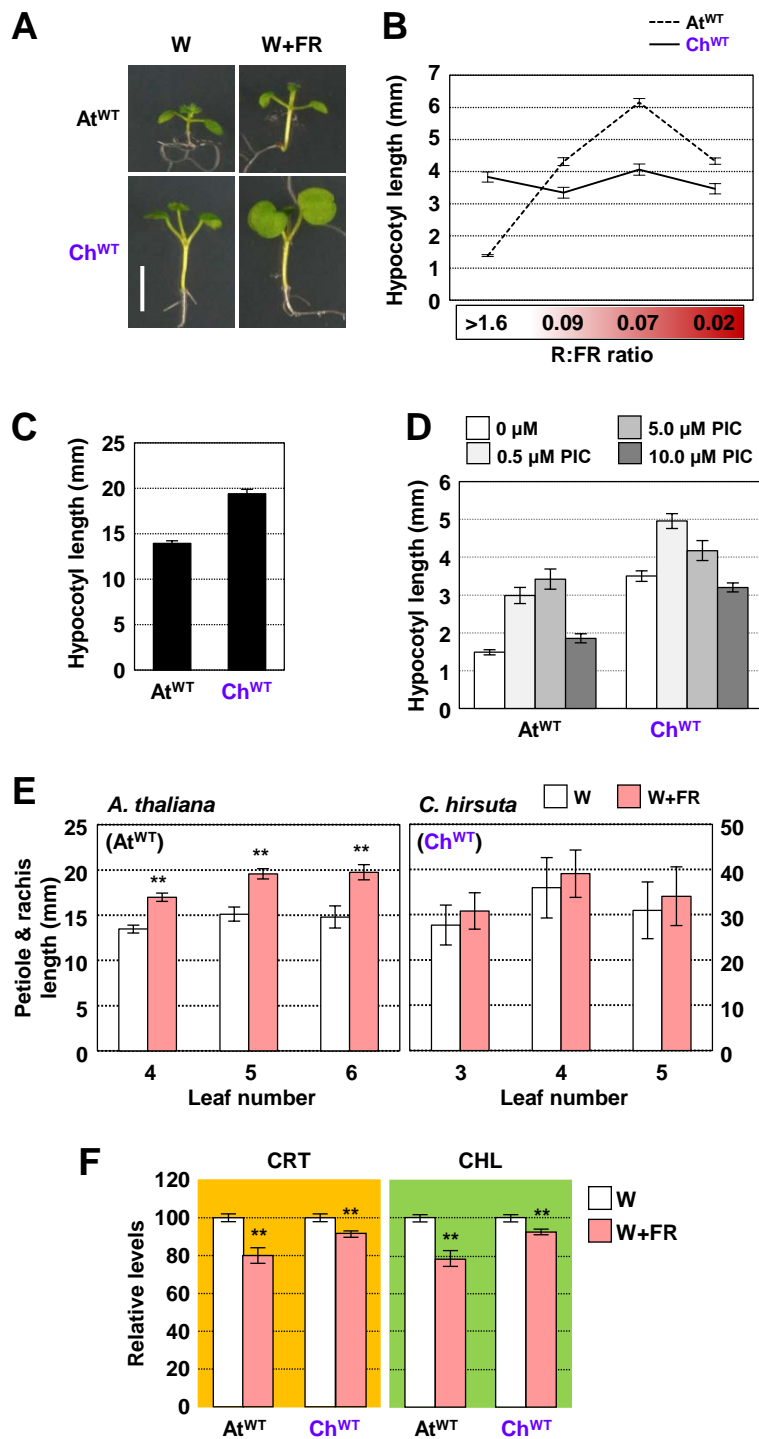


Figure 1. *A. thaliana* and *C. hirsuta* differ in the hypocotyl elongation response to neighboring vegetation. (A) Phenotype of representative seedlings of wild-type *A. thaliana* (*At*^{WT}) and *C. hirsuta* (*Ch*^{WT}) after 3 days grown in W and retained in W (left panels) or transferred to W+FR (R:FR of 0.02; right panels) until day 7 (d7). Scale bar, 5 mm. (B) Hypocotyl length of d7 *At*^{WT} and *Ch*^{WT} seedlings grown for the last 4 days under the indicated R:FR. (C) Hypocotyl length of d4 *At*^{WT} and *Ch*^{WT} seedlings grown in darkness. (D) Hypocotyl length of d7 *At*^{WT} and *Ch*^{WT} seedlings grown under W in media supplemented with increasing concentrations of picloram (PIC). (E) Petiole and rachis length of 3-week-old leaves of *At*^{WT} and *Ch*^{WT} plants grown for the last 7 days under the indicated R:FR. (F) Carotenoid (CRT) and chlorophyll (CHL) levels of *At*^{WT} and *Ch*^{WT} seedlings grown in W and W+FR (as detailed in A). Values are means and s.e.m. of three to five independent samples. Asterisks indicate significant differences (***p*<0.01) relative to W-grown plants.

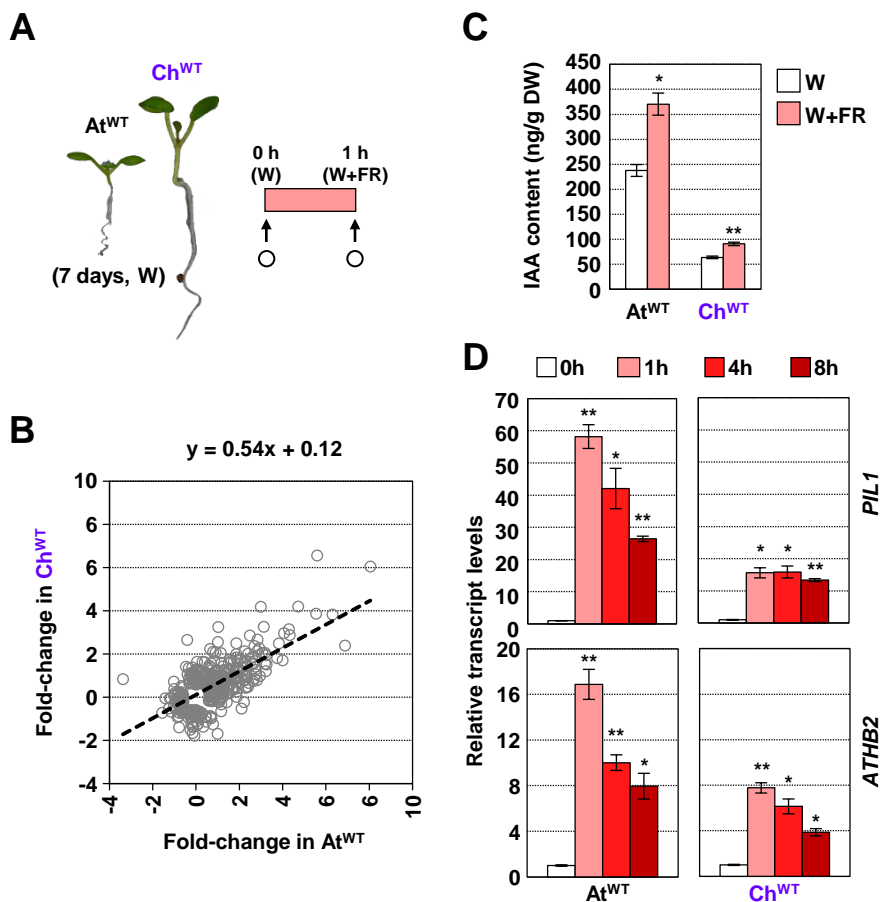


Figure 2. *A. thaliana* and *C. hirsuta* seedlings respond to neighboring vegetation by altering gene expression. (A) RNA-seq was performed with RNA extracted from At^{tWT} and Ch^{WT} seedlings that were grown in W for 7 days (d7) and then treated for 1 h with W+FR (R:FR = 0.02). White circles indicate the moment of harvesting for RNA extraction. Three independent biological replicates were used for each genotype and treatment. (B) Correlation between log-transformed fold-change of 432 DEGs in At^{tWT} and Ch^{WT}. The estimated regression equation is shown at the top of the graph. (C) IAA content in At^{tWT} and Ch^{WT} seedlings grown and harvested as indicated in A. Whole seedlings were collected and lyophilized to measure IAA levels. Data are presented as the means and s.e.m. of three (At^{tWT}) or four (Ch^{WT}) biological replicates. DW, dry weight. (D) Effect of W+FR treatment on *PIL1* and *ATHB2* expression in At^{tWT} and Ch^{WT} seedlings (R:FR = 0.02). W-grown d7 seedlings of Col-0 and Ox were treated for 0, 1, 4 and 8 h with W+FR. Transcript abundance, normalized to EF1 α is shown. Values are means and s.e.m. of three independent RT-qPCR biological replicates relative to values at 0 h for each species. In C and D, asterisks indicate significant differences (**p<0.01, *p<0.05) relative to 0 h samples.

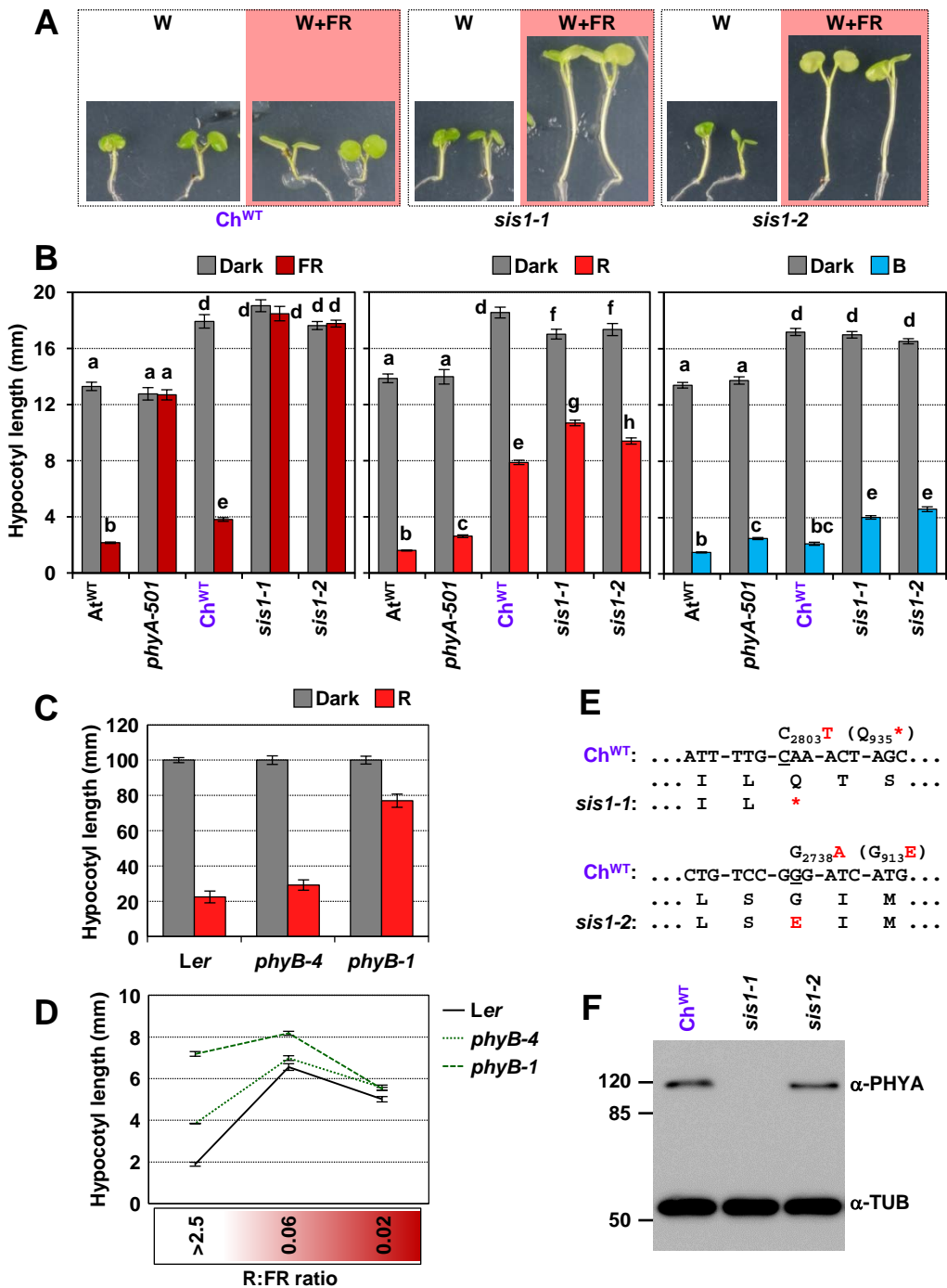


Figure 3. Mutant *sis1* seedlings of *C. hirsuta* are deficient in phyA activity. (A) Phenotype of representative seedlings of *Ch*^{WT}, *sis1-1* and *sis1-2* after 3 days grown in W and retained in W (white panels) or transferred to W+FR (R:FR of 0.02; pink panels) until day 7 (d7). All panels are to the same scale. **(B)** Hypocotyl length of *At*^{WT}, *phyA-501* (*A. thaliana*), *Ch*^{WT}, *sis1-1* and *sis1-2* (*C. hirsuta*) lines grown for 4 days in darkness (Dark) or under monochromatic FR (2.6 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), R (38.9 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and blue (B, 1.9 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light. **(C)** Hypocotyl length of *A. thaliana* Ler, *phyB-4* and *phyB-1* seedlings grown for 4 days in darkness (Dark) or under monochromatic R (40.6 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light. **(D)** Hypocotyl length of *A. thaliana* Ler, *phyB-4* and *phyB-1* seedlings under the indicated R:FR. Seedlings were grown for 2 days in W (R:FR > 2.5) and then kept in W (R:FR > 2.5) or transferred to W+FR (R:FR of 0.06 or 0.02) until day 7 (d7). **(E)** Schematic diagram of the lesions found in the *ChPHYA* gene in the *sis1-1* and *sis1-2* alleles compared to the wild-type sequence (*Ch*^{WT}) and the predicted changes in the amino acid sequence. **(F)** Immunoblot detection of phyA and tubulin with mouse monoclonal anti-phyA (073D) and anti-TUB antibodies in extracts of etiolated seedlings of *Ch*^{WT}, *sis1-1* and *sis1-2* lines.

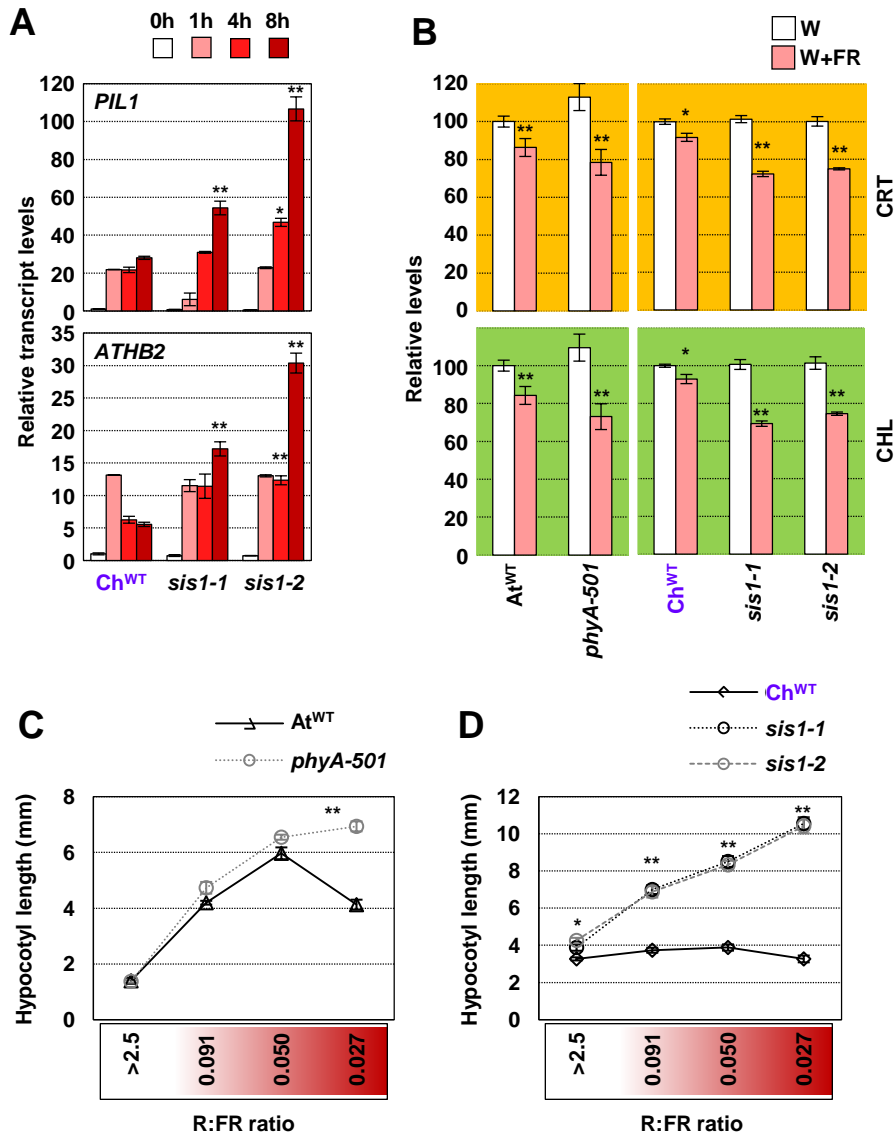


Figure 4. *C. hirsuta sis1* seedlings are impaired in their tolerance to plant proximity. (A) Effect of W+FR treatment on *PIL1* and *ATHB2* expression in Ch^{WT} *sis1-1*- and *sis1-2* seedlings. Seedlings were grown as in Figure 2D. Transcript abundance, normalized to EF1 α is shown. Values are means and s.e.m. of three independent RT-qPCR biological replicates relative to values at 0 h for each genotype. Asterisks indicate significant differences (** $p < 0.01$) relative to 0 h samples. **(B)** Carotenoid (CRT) and chlorophyll (CHL) levels of At^{WT} and *phyA-501* *A. thaliana* and Ch^{WT}, *sis1-1* and *sis1-2* *C. hirsuta* seedlings grown in W and W+FR (as detailed in Figure 1A). Values are means and s.e.m. of five independent samples. Asterisks indicate significant differences (** $p < 0.01$) relative to W-grown plants. **(C,D)** Hypocotyl length of d7 At^{WT}, *phyA-501* (*A. thaliana*) **(C)** and Ch^{WT}, *sis1-1*, *sis1-2* (*C. hirsuta*) **(D)** seedlings grown for the last 4 days under the indicated R:FR. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$) relative to the corresponding wild-type plant grown under the same R:FR. In **D**, asterisks apply for both *sis1* mutants.

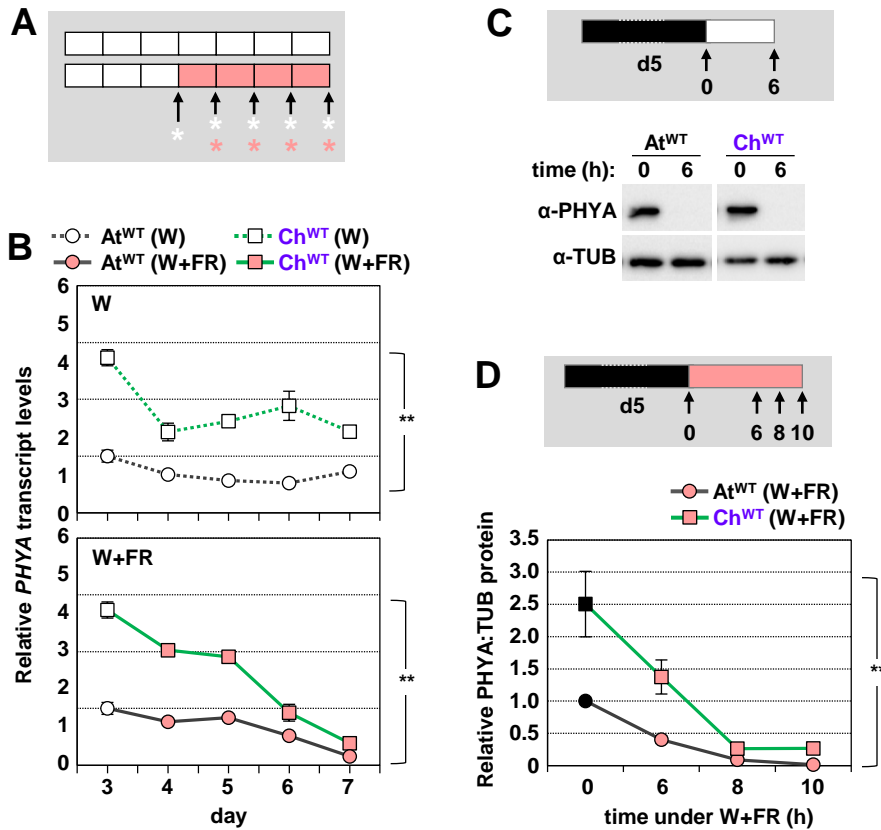


Figure 5. *C. hirsuta* seedlings have higher phyA levels than those of *A. thaliana*. (A) Cartoon showing the design of the experiment. Wild-type seedlings of *A. thaliana* (At^{WT}) and *C. hirsuta* (Ch^{WT}), grown as in Figure 1A, were harvested at the indicated times of W or W+FR treatments (asterisks) for RNA extraction. (B) Evolution of *PHYA* transcript levels in *A. thaliana* and *C. hirsuta* wild-type seedlings grown as detailed in A. Primers used (Supplemental Figure 6A) allow quantifying and comparing expression levels by RT-qPCR between both species. *PHYA* transcript abundance was normalized to three reference genes (*EF1*, *SPP25* and *YLS8*). Values are means and s.e.m. of three independent RT-qPCR biological replicates relative to *PHYA* transcript levels of d3 *A. thaliana* seedlings. Two-way ANOVA showed that *PHYA* levels are significantly different (** $p < 0.01$) between species under either W or W+FR. (C) Immunoblot detection of phyA and tubulin with the antibodies indicated in Figure 3C in extracts of At^{WT} and Ch^{WT} seedlings grown as detailed at the top of the section: 5-day-old etiolated seedlings were exposed to W light and material was harvested before and after 6 h of W-exposure (arrows). (D) Evolution of relative phyA protein levels (PHYA:TUB) in At^{WT} and Ch^{WT} seedlings exposed to simulated shade, as detailed at the top of the section: 5-day-old etiolated seedlings were exposed to W+FR light and material was harvested before and after 6, 8 and 10 h of simulated shade exposure (arrows). Values are means and s.e.m. of four independent biological replicates relative to PHYA:TUB levels of etiolated At^{WT} seedlings. Two way ANOVA showed that relative PHYA levels under W+FR are significantly increased (** $p < 0.01$) in *C. hirsuta* than *A. thaliana*.

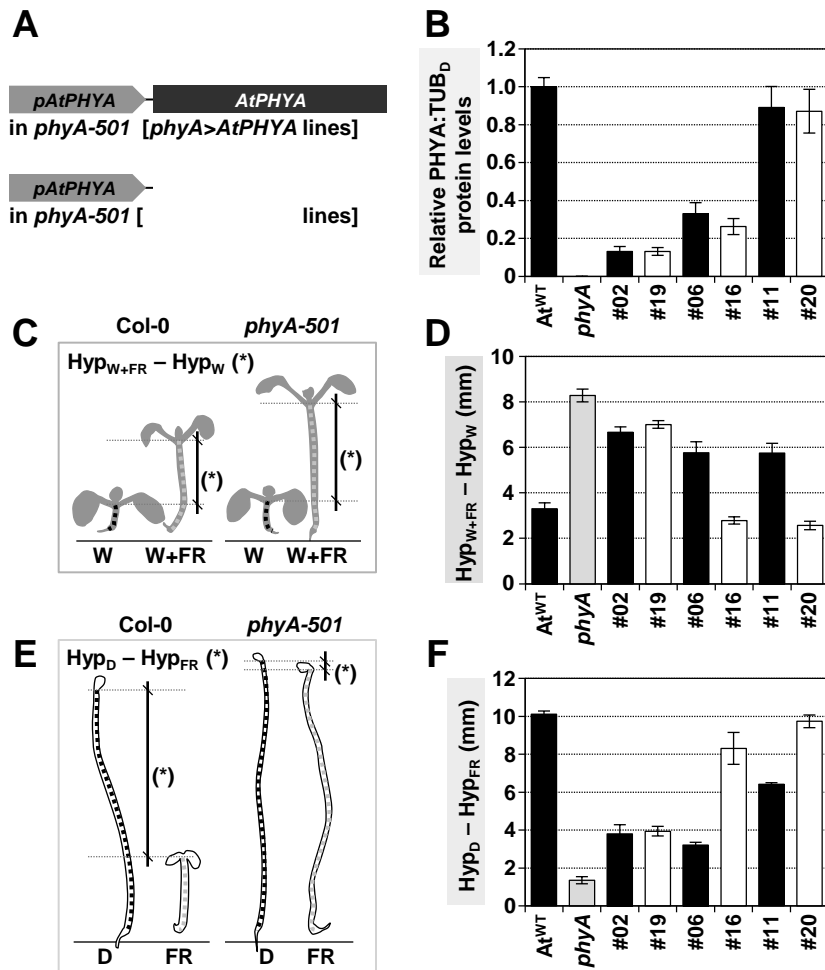


Figure 6. ChphyA has a stronger activity than AtpHYA in repressing shade-induced hypocotyl elongation. (A) Cartoon detailing the constructs used to complement *A. thaliana phyA-501* mutant plants. (B) Relative PHYA:TUB_D in etiolated seedlings of At^{WT}, *phyA-501*, and selected *phyA>AtPHYA* (blue bars) and *phyA>ChPHYA* (red bars) complementation lines. Seedlings were grown as indicated in Supplemental Figure 8. Values are means and s.e.m. of four independent biological replicates relative to PHYA:TUB_D levels of etiolated At^{WT} seedlings. (C) Cartoon illustrating how *phyA* activity in simulated shade was established as differences in hypocotyl length between simulated shade- and the W-grown seedlings (Hyp_{W+FR}-Hyp_W). Seedlings were grown for 2 days under W then for 5 additional days under W or W+FR (R:FR = 0.02), when hypocotyls were measured. (D) Hyp_{W+FR}-Hyp_W in seedlings of At^{WT}, *phyA-501*, and selected *phyA>AtPHYA* (blue bars) and *phyA>ChPHYA* (red bars) complementation lines. (E) Cartoon illustrating how *phyA* activity in de-etiolation was established as differences in hypocotyl length between dark- and FR-grown seedlings (Hyp_D-Hyp_{FR}). Seedlings were grown as indicated in Figure 3B. (F) Hyp_D-Hyp_{FR} in seedlings of At^{WT}, *phyA-501*, and selected *phyA>AtPHYA* (blue bars) and *phyA>ChPHYA* (red bars) complementation lines. In C and E, mutant *phyA-501* seedlings have no *phyA* activity.

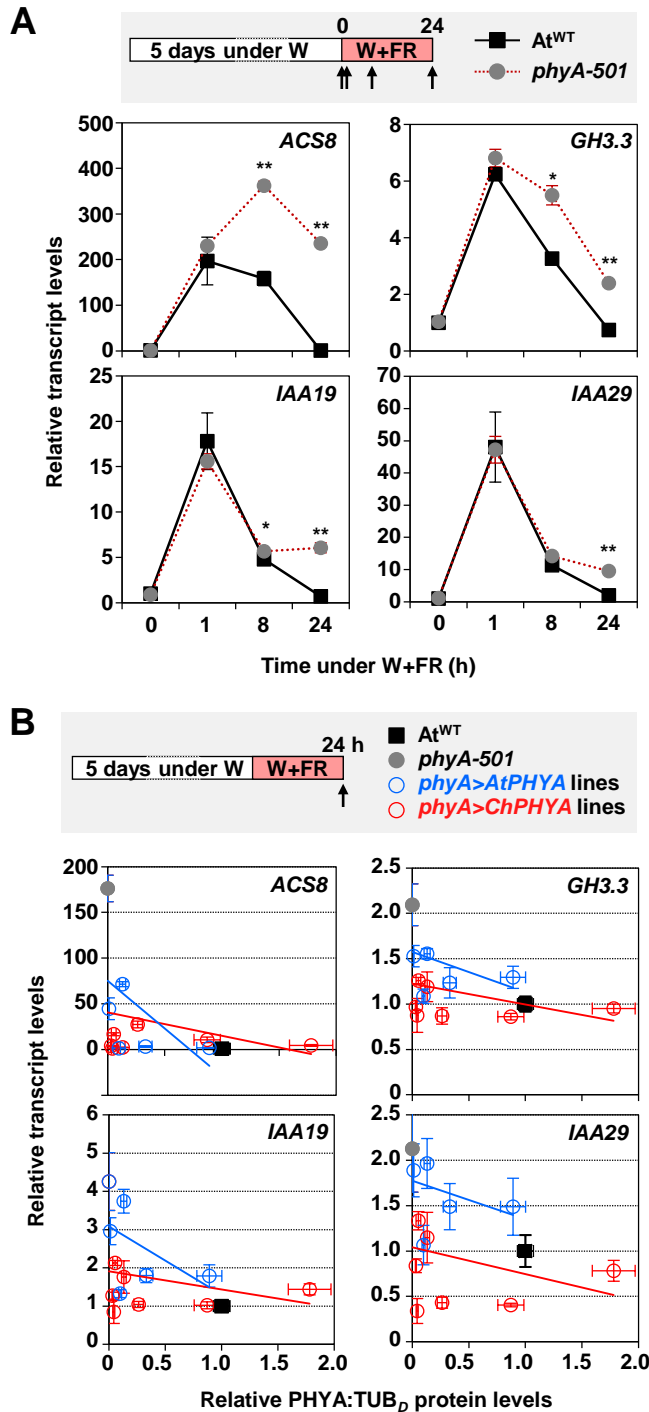


Figure 7. ChphyA has a stronger activity than AtpHYA in repressing shade-induced expression of ACS8, GH3.3, IAA19 and IAA29 genes. (A) Effect of phyA in the shade-induced expression of ACS8, GH3.3, IAA19 and IAA29. W-grown d5 seedlings of At^{WT} and $phyA-501$ were treated for 0, 1, 8 and 24 h with W+FR (R:FR = 0.02), when material was harvested for RNA extraction, as indicated at the top of the panel. Transcript abundance, normalized to EF1 α is shown. Values are means and s.e.m. of three independent RT-qPCR biological replicates relative to values at 0 h for At^{WT} . Asterisks indicate significant differences (** $p < 0.01$, * $p < 0.05$) between $phyA-501$ and At^{WT} seedlings exposed for the same time to W+FR. **(B)** Correlation between ACS8, GH3.3, IAA19 and IAA29 expression and relative levels of PHYA protein in the seedlings of At^{WT} , $phyA-501$, $phyA>AtPHYA$ (blue lines and dots) and $phyA>ChPHYA$ (red lines and dots) complementation lines. Gene expression was quantified in W-grown d5 seedlings exposed to W+FR (R:FR=0.02) during 24 h, as indicated at the top of the panel. Transcript abundance was normalized to EF1 α . Relative phyA protein levels (PHYA:TUB, data already shown in Supplemental Figure 8) were estimated in etiolated seedlings. Values are means and s.e.m. of three independent RT-qPCR biological replicates relative to values of At^{WT} . The estimated regression lines for the $phyA>AtPHYA$ (blue line) and $phyA>ChPHYA$ (red line) complementation lines are shown for each correlation.

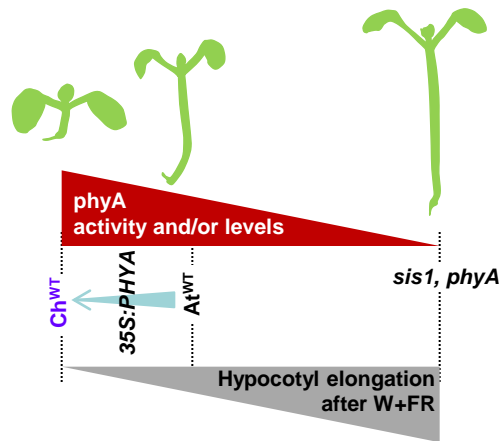


Figure 8. Model of how an increased phyA activity in *C. hirsuta* might implement the shade tolerance of hypocotyl elongation. Increases in phyA activity caused by the constitutive overexpression of *PHYA* also attenuate the shade-induced hypocotyl elongation in transgenic plants, and it results in partially tolerant *A. thaliana* seedlings.

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