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1 **Adjustment of the PIF7-HFR1 transcriptional module activity controls**
2 **plant shade adaptation**

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24 Running title, PIF-HFR1 role in plant shade adaptation

25

26 **ABSTRACT**

27 Shade caused by the proximity of neighboring vegetation triggers a set of
28 acclimation responses to either avoid or tolerate shade. Comparative analyses
29 between the shade avoider *Arabidopsis thaliana* and the shade tolerant
30 *Cardamine hirsuta*, revealed a role for the atypical basic-helix-loop-helix LONG
31 HYPOCOTYL IN FR 1 (HFR1) in maintaining the shade-tolerance in *C. hirsuta*,
32 inhibiting hypocotyl elongation in shade and constraining expression profile of
33 shade induced genes. We showed that *C. hirsuta* HFR1 protein is more stable
34 than its *A. thaliana* counterpart, likely due to its lower binding affinity to
35 CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), contributing to enhance
36 its biological activity. The enhanced HFR1 total activity is accompanied by an
37 attenuated PHYTOCHROME INTERACTING FACTOR (PIF) activity in *C.*
38 *hirsuta*. As a result, the PIF-HFR1 module is differently balanced, causing a
39 reduced PIF activity and attenuating other PIF-mediated responses such as
40 warm temperature-induced hypocotyl elongation (thermomorphogenesis) and
41 dark-induced senescence. By this mechanism and that of the already-known of
42 phytochrome A photoreceptor, plants might ensure to properly adapt and thrive
43 in habitats with disparate light amounts.

44

45 Keywords, *Cardamine hirsuta* / HFR1 / PIFs / shade avoidance / shade
46 tolerance.

47

48 INTRODUCTION

49 Acclimation of plants to adjust their development to the changing
50 environment is of utmost importance. This acclimation relies on the plant's
51 ability to perceive many cues such as water, nutrients, temperature or light.
52 Conditions in nature often involve simultaneous changes in multiple light cues
53 leading to an interplay of various photoreceptors to adjust plant growth
54 appropriately (Ballare & Pierik, 2017; de Wit *et al*, 2016; Fiorucci & Fankhauser,
55 2017; Mazza & Ballare, 2015; Pierik & Testerink, 2014). Nearby vegetation can
56 impact both light quantity and quality. Under a canopy, light intensity is
57 decreased and its quality is changed as the overtopping green leaves strongly
58 absorb blue and red light (R) but reflect far-red light (FR). As a consequence,
59 plants growing in forest understories receive less light of a much lower R to FR
60 ratio (R:FR) than those growing in open spaces. In dense plant communities,
61 FR reflected by neighboring plants also decreases R:FR but typically without
62 changing light intensity. We refer to the first situation as canopy shade (very low
63 R:FR) and the second as proximity shade (low R:FR). In general, two strategies
64 have emerged to deal with shade: avoidance and tolerance (Gommers *et al*,
65 2013; Pierik & Testerink, 2014; Valladares & Niinemets, 2008). Shade avoiders
66 usually promote elongation of organs to outgrow the neighbors and avoid light
67 shortages, reduce the levels of photosynthetic pigments to cope to light
68 shortage, and accelerate flowering to ensure species survival (Casal, 2013).
69 The set of responses to acclimate to shade is collectively known as the shade
70 avoidance syndrome (SAS). In contrast, shade-tolerant species usually lack the
71 promotion of elongation growth in response to shade and have developed a

72 variety of traits to acclimate to low light conditions and optimize net carbon gain
73 (Smith, 1982; Valladares & Niinemets, 2008).

74 In *Arabidopsis thaliana*, a shade avoider plant, low R:FR is perceived by
75 phytochromes. Among them, phyA has a negative role in elongation, particularly
76 under canopy shade, whereas phyB inhibits elongation inactivating
77 PHYTOCHROME INTERACTING FACTORS (PIFs), members of the basic-
78 helix-loop-helix (bHLH) transcription factor family that promote elongation
79 growth. In particular, PIFs induce hypocotyl elongation by initiating an
80 expression cascade of genes involved in auxin biosynthesis and signaling [e.g.,
81 *YUCCA 8 (YUC8)*, *YUC9*, *INDOLE-3-ACETIC ACID INDUCIBLE 19 (IAA19)*,
82 *IAA29*] and other processes related to cell elongation [e.g., *XYLOGLUCAN*
83 *ENDOTRANSGLYCOSYLASE 7 (XTR7)*]. Genetic analyses indicated that PIF7
84 is the key PIF regulator of the low R:FR-induced hypocotyl elongation with PIF4
85 and PIF5 having important contributions. Indeed, *pif7* mutant responds poorly to
86 low R:FR compared to the *pif4 pif5* double or *pif1 pif3 pif4 pif5* quadruple (*pifq*)
87 mutants, but the triple *pif4 pif5 pif7* mutant is almost unresponsive to low R:FR
88 (de Wit *et al*, 2016; Li *et al*, 2012; Lorrain *et al*, 2008; van Gelderen *et al*, 2018).
89 PhyB-mediated shade signaling involves other transcriptional regulators, such
90 as *LONG HYPOCOTYL IN FR 1 (HFR1)*, *PHYTOCHROME RAPIDLY*
91 *REGULATED 1 (PAR1)*, *BIM1*, *ATHB4* or *BBX* factors, that either promote or
92 inhibit shade-induced hypocotyl elongation (Bou-Torrent *et al*, 2014; Cifuentes-
93 Esquivel *et al*, 2013; Gallemi *et al*, 2017; Roig-Villanova *et al*, 2007; Sasidharan
94 & Pierik, 2010; Sessa *et al*, 2005; Yang & Li, 2017). HFR1, a member of the
95 bHLH family, is structurally related to PIFs but lacks the phyB- and DNA-binding
96 ability that PIFs possess (Galstyan *et al*, 2011; Hornitschek *et al*, 2012). HFR1

97 inhibits PIF activity by heterodimerizing with them, as described for PIF1 (Shi *et*
98 *al*, 2013), PIF3 (Fairchild *et al*, 2000), PIF4 and PIF5 (Hornitschek *et al*, 2009),
99 Heterodimerization with HFR1 prevents PIFs from binding to the DNA and
100 altering gene expression. In this manner HFR1 acts as a transcriptional cofactor
101 that modulates SAS responses, e.g. it inhibits hypocotyl elongation in seedlings
102 in a PIF-dependent manner, forming the PIF-HFR1 transcriptional regulatory
103 module (Galstyan *et al*, 2011).

104 What mechanistic and regulatory adjustments in shade signaling are
105 made between species to adapt to plant shade is a topic that has not received
106 much attention until now. This question has been recently addressed
107 performing comparative analyses between phylogenetically related species. In
108 two related *Geranium* species that showed petioles with divergent elongation
109 responses to shade, transcriptomic analysis led to propose that differences in
110 expression of three factors, *FERONIA*, *THESEUS1* and *KIDARI*, shown to
111 activate SAS elongation responses in *A. thaliana*, might be part of the
112 adjustments necessary to acquire a shade-avoiding or tolerant habit (Gommers
113 *et al*, 2017). When comparing two related mustard species that showed
114 divergent hypocotyl elongation response to shade, *A. thaliana* and *Cardamine*
115 *hirsuta* (Hay *et al*, 2014), molecular and genetic analyses indicated that phyA,
116 and to a lesser extent phyB, contributed to establish this divergent response. In
117 particular, the identification and characterization of the *C. hirsuta* phyA-deficient
118 *slender in shade 1 (sis1)* mutant indicated that differential features of this
119 photoreceptor in *A. thaliana* and *C. hirsuta* could explain their differential
120 response to shade. Thus, stronger phyA activity in *C. hirsuta* wild-type plants
121 resulted in a suppressed hypocotyl elongation response when exposed to low

122 or very low R:FR (Molina-Contreras *et al*, 2019). These approaches indicated
123 that the implementation of shade avoidance and shade tolerance involved the
124 participation of shared genetic components. They also suggest that other
125 responses co-regulated by these shared components will be accordingly
126 affected.

127 With this frame of reference, we asked whether the phyB-dependent PIF-
128 HFR1 module was also relevant to shape the shade response habits in different
129 plant species. We found that *C. hirsuta* plants deficient in ChHFR1 gained a
130 capacity to elongate in response to shade. We also report that *AtHFR1* and
131 *ChHFR1* are expressed at different levels and encode proteins with different
132 protein stability, caused by their different binding affinities with CONSTITUTIVE
133 PHOTOMORPHOGENIC 1 (COP1), known to affect *AtHFR1* stability under
134 shade (Pacin *et al*, 2016). We propose that adaptation to plant shade in *A.*
135 *thaliana* and *C. hirsuta* relies on the PIF-HFR1 regulatory module. As PIFs
136 regulates several other processes, we hypothesized that a set of responses co-
137 regulated by the PIF-HFR1 module are also affected and associated with the
138 shade-avoidance and shade-tolerant habits. After exploring this possibility, we
139 found that thermoregulation of hypocotyl elongation and dark-induced
140 senescence, two well-known PIF-regulated responses (Koini *et al*, 2009;
141 Sakuraba *et al*, 2014; Stavang *et al*, 2009), are consistently affected in *C.*
142 *hirsuta*.

143

144 **RESULTS**

145 ***HFR1* is required for the shade tolerance habit of *C. hirsuta***

146 First, we wanted to determine if HFR1 has a role in the shade-tolerance
147 habit of *C. hirsuta*, i.e., whether *ChHFR1* contributes to inhibit hypocotyl
148 elongation when this species is exposed to shade. For this purpose, we
149 generated several *C. hirsuta* RNAi lines to downregulate *HFR1* expression
150 (RNAi-HFR1 lines). As expected, *ChHFR1* expression was attenuated in
151 seedlings of two RNAi-HFR1 selected lines (#01 and #21) compared to the wild
152 type (Ch^{WT}) (Fig EV1A). When growing under white light (W) of high R:FR
153 (>1.5), hypocotyl length of these two RNAi-HFR1 lines was undistinguishable
154 from Ch^{WT} (Fig 1A). By contrast, under W supplemented with increasing
155 amounts of FR (W+FR) resulting in moderate (0.09), low (0.05-0.06) and very
156 low (0.02) R:FR (that simulated proximity and canopy shade) (Martinez-Garcia
157 *et al*, 2014), the hypocotyl elongation of RNAi-HFR1 seedlings was significantly
158 promoted compared to Ch^{WT} , which was unresponsive (Fig 1A).

159 Using CRISPR-Cas9, we obtained two mutant lines of *ChHFR1* (named
160 *chfr1-1* and *chfr1-2*) with a single nucleotide insertion in their sequence leading
161 to a premature stop codon (Fig EV1C). These mutants showed a non-significant
162 decrease of *ChHFR1* expression in W-grown seedlings (Fig EV1B). Similar to
163 the RNAi-HFR1 lines, their hypocotyls were undistinguishable from Ch^{WT} under
164 W but elongated strongly in response to W+FR exposure (Fig 1B), showing a
165 *slender in shade (sis)* phenotype. Together, we concluded that HFR1 represses
166 hypocotyl elongation in response to shade in *C. hirsuta*.

167 Exposure of *A. thaliana* wild-type (At^{WT}) and Ch^{WT} seedlings to low R:FR
168 induces a rapid increase in the expression of various direct target genes of

169 PIFs, including *PIF3-LIKE 1 (PIL1)*, *YUC8* and *XTR7* (Fig 1C, D) (Ciolfi *et al*,
170 2013; Hersch *et al*, 2014; Molina-Contreras *et al*, 2019). The shade-induced
171 expression of these genes was significantly higher in RNAi-HFR1 and *chfr1*
172 mutant lines compared to Ch^{WT} (Fig 1C, D), indicating that ChHFR1 might
173 repress shade-triggered hypocotyl elongation in part by down-regulating the
174 rapid shade-induced expression of these genes in *C. hirsuta*, as it was
175 observed with AtHFR1 in *A. thaliana* seedlings (Hornitschek *et al*, 2009).

176

177 ***HFR1* expression is higher in *C. hirsuta* than in *A. thaliana* seedlings**

178 To test if the lack of elongation of Ch^{WT} hypocotyls in response to shade
179 was caused by higher levels of *ChHFR1* expression in this species, we used
180 primer pairs that amplify *HFR1* (Fig EV2A) and three housekeeping genes
181 (*EF1 α* , *SPC25*, *YLS8*) in both species (Molina-Contreras *et al*, 2019). As
182 expected, expression of *HFR1* was induced in shade-treated seedlings of both
183 species, in agreement with the presence of canonical PIF-binding sites (G-box,
184 CACGTG) in the *HFR1* promoters (Hornitschek *et al*, 2009; Martinez-Garcia *et*
185 *al*, 2000) (Fig EV3A). More importantly, *ChHFR1* transcript levels were always
186 higher than those of *AtHFR1* during the whole period analyzed (from days 3 to
187 7) (Fig 2). Because HFR1 is part of the PIF-HFR1 regulatory module, we next
188 compared transcript levels of *PIF* genes in both species. *PIF7* expression was
189 significantly lower in *C. hirsuta* than in *A. thaliana* in either W or W+FR during
190 the period analyzed (Fig 2). By contrast, *PIF4* expression was higher in *C.*
191 *hirsuta* than in *A. thaliana*, whereas that of *PIF5* was similar in both species (Fig
192 EV2B). Together, these results indicated that whereas *HFR1* expression is
193 enhanced, that of *PIF7* is globally attenuated in Ch^{WT} compared to At^{WT}

194 seedlings. As a consequence, the PIF-HFR1 transcriptional module might be
195 differently balanced in these species, with HFR1 imposing a stronger
196 suppression on the PIF7-driven hypocotyl elongation in the shade-tolerant *C.*
197 *hirsuta* seedlings.

198

199 **ChHFR1 protein is more stable than AtHFR1**

200 A higher specific activity of ChHFR1 compared to its orthologue AtHFR1
201 might also contribute to the role of this transcriptional cofactor in maintaining the
202 shade tolerance habit of *C. hirsuta*. To test this possibility, we transformed *A.*
203 *thaliana hfr1-5* plants with constructs to express either *AtHFR1* or *ChHFR1*
204 fused to the 3x hemagglutinin tag (3xHA). These genes were expressed under
205 the transcriptional control of the 2 kb of the *AtHFR1* promoter (*pAt*), generating
206 *hfr1>pAt:ChHFR1* and *hfr1>pAt:AtHFR1* lines (Fig 3A). Fusion of *pAt* to the
207 *GUS* reporter gene resulted in GUS activity in cotyledons and roots of
208 transgenic lines, with increased levels in hypocotyls of seedlings exposed for 2-
209 4 h to W+FR (Fig EV3B). Several independent transgenic lines of each
210 construct were analyzed for hypocotyl length (Appendix Fig S1), *HFR1*
211 transcript levels and 3xHA-tagged protein abundance. In these lines, HFR1
212 biological activity was estimated as the difference in hypocotyl length of
213 seedlings grown under W+FR ($\text{Hyp}_{\text{W+FR}}$) and W (Hyp_{W}) ($\text{Hyp}_{\text{W+FR}} - \text{Hyp}_{\text{W}}$)
214 (Molina-Contreras *et al*, 2019). The potential to suppress the hypocotyl
215 elongation in shade below that of *hfr1-5* seedlings would depend on the
216 transcript level of *HFR1* and/or its protein levels. The *hfr1>pAt:ChHFR1* lines
217 had shorter hypocotyls in shade (i.e., stronger global HFR1 activity) compared
218 to *hfr1>pAt:AtHFR1* lines of similar *HFR1* expression levels (Figs 3B, C, EV3C),

219 suggesting that total HFR1 activity was higher in *hfr1>pAt:ChHFR1* than in
220 *hfr1>pAt:AtHFR1* lines. However we observed much higher abundance of
221 HFR1-3xHA protein after shade exposure in *hfr1>pAt:ChHFR1* lines than in
222 *hfr1>pAt:AtHFR1* lines with comparable levels of *HFR1* expression (Fig 3D),
223 suggesting that the ChHFR1 protein might be much more stable. Together,
224 these results point to differences in protein stability (rather than in specific
225 activity) as the main cause for the enhanced HFR1 total activity of ChHFR1
226 compared to AtHFR1 in complemented lines.

227 AtHFR1 stability is affected by light conditions. In etiolated seedlings,
228 exposure to W promotes stabilization and accumulation of AtHFR1, whereas in
229 W-grown seedlings, high intensity of W increases its abundance (Duek *et al*,
230 2004; Yang *et al*, 2005). Importantly, AtHFR1 stability has a strong impact on its
231 biological activity as overexpression of stable forms of this protein leads to
232 phenotypes resulting from enhanced HFR1 activity (Galstyan *et al*, 2011; Yang
233 *et al*, 2005). As AtHFR1 and ChHFR1 primary structures are globally similar
234 (Fig EV4A), we aimed to test if ChHFR1 stability is also light-dependent. We
235 first examined ChHFR1 protein accumulation in response to different W
236 intensities in seedlings of an *A. thaliana hfr1-5* line that constitutively express
237 *ChHFR1* (*hfr1>35S:ChHFR1*) (Fig EV4B). When grown in our normal W
238 conditions ($\sim 20 \mu\text{mol m}^{-2} \text{s}^{-1}$), these seedlings accumulated low but detectable
239 levels of ChHFR1; when transferred to higher W intensity ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$),
240 ChHFR1 levels increased 10-fold (Fig EV4C). As *ChHFR1* is expressed under
241 the constitutive 35S promoter, these results indicate that ChHFR1 protein
242 accumulation is induced by high W intensity, as it has been described for
243 AtHFR1 (Yang *et al*, 2005). This prompted us to pretreat W-grown seedlings

244 with 3 h of high W intensity in all our subsequent experiments to analyze
245 ChHFR1 levels.

246 Next, we exposed *hfr1>pAt:ChHFR1* (line #22) and *hfr1>pAt:AtHFR1*
247 (line #13) seedlings to W+FR (Fig 4A). Although *HFR1* expression in both lines
248 was similarly induced after 3 h of W+FR, *hfr1>pAt:ChHFR1* line displayed
249 higher levels of recombinant HFR1 protein compared to *hfr1>pAt:AtHFR1* line
250 after 3-6 h of W+FR exposure (Fig 4A), suggesting a higher stability of the *C.*
251 *hirsuta* protein compared to the *A. thaliana* orthologue. ChHFR1 protein is more
252 abundant than AtHFR1 also when transiently expressed to comparable levels in
253 *Nicotiana benthamiana* (tobacco) leaves (Fig 4B, C). This indicates that the
254 higher abundance of ChHFR1 is an intrinsic property of the protein that resides
255 in its primary structure.

256 AtHFR1 is known to be targeted for degradation via the 26S proteasome
257 in dark-grown seedlings. Shade also promotes AtHFR1 degradation compared
258 to non-shade treatments (Pacin *et al*, 2016). Hence, ChHFR1 abundance might
259 be similarly targeted, and the increased ChHFR1 protein stability might be due
260 to differences in degradation kinetics, likely by the 26S proteasome. We
261 addressed this possibility by treating tobacco leaf discs overexpressing
262 *ChHFR1* and *AtHFR1* with the protein synthesis inhibitor cycloheximide (CHX)
263 combined with shade (Fig 4D). This treatment resulted in a decrease in
264 ChHFR1 and AtHFR1 protein levels. However, ChHFR1 degradation was
265 significantly slower than that of AtHFR1 (Fig 4D), supporting that changes in
266 degradation kinetics likely contribute to the observed differences in stability
267 between ChHFR1 and AtHFR1.

268 Light- and shade-regulated degradation of AtHFR1 requires binding to
269 COP1 and the COP1 E3 ubiquitin ligase activity. Binding to COP1 results in
270 HFR1 ubiquitination, which targets HFR1 for degradation via the 26S
271 proteasome (Jang *et al*, 2005; Pacin *et al*, 2016; Yang *et al*, 2005). COP1-
272 interacting proteins harbor sequence-divergent Val-Pro (VP) motifs that bind the
273 COP1 WD40 domain with different affinities (Lau *et al*, 2019).

274 Inspection of the COP1 WD40 - AtHFR1 complex structure (Lau *et al*,
275 2019) revealed that sequence differences between AtHFR1 and ChHFR1 map
276 to the N-terminus of the VP peptide involved in the interaction with COP1 (Fig
277 5A). We hypothesized that these sequence variations between HFR1 species
278 may result in different COP1 binding affinities, affecting targeting and
279 subsequent degradation of the two HFR1 orthologues. We thus quantified the
280 interaction of synthetic AtHFR1 and ChHFR1 VP peptides with COP1 using
281 microscale thermophoresis (MST, see Methods). AtHFR1 bound the COP1
282 WD40 domain with a dissociation constant (k_D) of $\sim 120 \mu\text{M}$ (Fig 5B, EV5). The
283 ChHFR1 VP peptide showed only weak binding to COP1 WD40, with a k_D in the
284 millimolar range (Fig 5B, EV5). Importantly, a second putative VP sequence in
285 At/ChHFR1 showed no detectable binding, while the previously characterized *A.*
286 *thaliana* cryptochrome 1 (AtCRY1) and the human HsTRIB1 VP sequences
287 bound COP1 WD40 with a k_D in the $\sim 1 \mu\text{M}$ range, in good agreement with
288 earlier isothermal titration calorimetry binding assays (Figs 5B, EV5) (Lau *et al*,
289 2019). Taken together, AtHFR1 VP peptide interacted more strongly with COP1
290 WD40, suggesting that AtHFR1 may represent a better substrate for COP1 than
291 ChHFR1.

292 Next we aimed to explore if these differences in COP1 affinity had an
293 impact in the subsequent degradation of AtHFR1 and ChHFR1 proteins. To test
294 this possibility, we generated chimeric *HFR1* genes in which the VP region was
295 swapped, named as *ChHFR1** and *AtHFR1** (Fig 5C). *ChHFR1** differed from
296 *ChHFR1* in the VP region, that was substituted for the AtHFR1-VP1.
297 Reciprocally, *AtHFR1** contained the ChHFR1-VP region. Like the wild-type
298 versions, these *HFR1* derivative genes were fused to the 3xHA and placed
299 under the control of the 35S promoter (Fig 5C). When transiently expressed in
300 tobacco leaves, ChHFR1* was now less abundant than AtHFR1*, suggesting
301 that the VP regions contain enough information to determine the pattern of
302 stability of the resulting HFR1 protein (Fig 5D). Because AtHFR1-VP1 binds to
303 COP1 WD40 domain with higher affinity than ChHFR1-VP1, these results
304 indicate a negative correlation of the binding affinity to COP1 with the
305 accumulation (i.e., the higher the affinity the lower the accumulation). Hence,
306 we concluded that in the HFR1 context, a stronger binding to COP1 results in
307 lower abundance.

308

309 **HFR1 interacts with PIF7**

310 AtHFR1 has been shown to interact with all the members of the
311 photolabile AtPIF quartet (PIF1, PIF3, PIF4 and PIF5). Using a yeast two-hybrid
312 (Y2H) assay, we observed that AtHFR1 homodimerized, which indicated that its
313 HLH domain is functional in this assay (Fig 6A). In the same assay, AtHFR1
314 was also shown to interact with AtPIF7 (Fig 6A). These results agree with
315 recent data (Zhang *et al*, 2019). Because AtPIF7 is the main PIF in *A. thaliana*
316 promoting hypocotyl elongation in response to low R:FR (Li *et al*, 2012), we

317 aimed to address whether HFR1 also interacts genetically with PIF7. First, we
318 analyzed the genetic interaction between AtHFR1 and AtPIF7. After crossing *A.*
319 *thaliana hfr1-5* with *pif7-1* and *pif7-2* mutants, we analyzed the hypocotyl
320 response of the obtained double mutants in different low R:FR conditions. As
321 expected, *hfr1* hypocotyls were longer and those of *pif7* mutants were shorter
322 compared to At^{WT} under both W+FR conditions used (Fig 6B). In W and low
323 R:FR (0.06), double *pif7 hfr1* mutant seedlings behaved mostly as *pif7* single
324 mutants. However, under very low R:FR (0.02), they elongated similar to At^{WT}
325 hypocotyls (Fig 6B). Together, these results indicate that *pif7* is epistatic over
326 *hfr1* under low R:FR, whereas it seems additive under very low R:FR, two
327 conditions that we speculate as mimicking proximity and canopy shade,
328 respectively (Martinez-Garcia *et al*, 2014).

329 To further analyze the HFR1-PIF7 interaction, we aimed to test if *HFR1*
330 overexpression will interfere with *PIF7* overexpression and impede its effects.
331 For HFR1, we used a line overexpressing a stable but truncated form of the
332 protein (missing the N-terminal, 35S:GFP- Δ Nt-HFR1, line #03) that strongly
333 inhibits shade-induced hypocotyl elongation in *A. thaliana* without affecting
334 other aspects of the seedling development (Galstyan *et al*, 2011) (Fig 6C, D).
335 For PIF7 we used two available 35S:PIF7-CFP lines (#1 and #2) (Leivar *et al*,
336 2008) that were almost unresponsive to W+FR (Fig 6C) and smaller and less
337 developed than the At^{WT} in W (Fig 6D). The inhibition of shade-induced
338 elongation observed in the 35S:PIF7-CFP lines contrasts with the positive effect
339 of growth observed by several other authors when overexpressing PIF7 fused
340 to smaller tags (Flash-tag peptide) (Li *et al*, 2012), likely caused by toxic or
341 squelching effects caused by high levels of the PIF7-CFP protein. In W,

342 35S:GFP-ΔNt-HFR1 35S:PIF7-CFP double transgenic seedlings (#1 and #2)
343 did not differ in hypocotyl length and general aspect with *At*^{WT}; interestingly they
344 did elongate clearly in low and very low R:FR (Fig 6C, D). The recovery of the
345 shade-induced hypocotyl elongation and size of the seedlings took place even
346 though *HFR1* transcript levels were significantly lower than in the 35S:GFP-ΔNt-
347 HFR1 parental line. *PIF7* transcript levels were not significantly different in the
348 double transgenic seedlings than in their respective parental lines (Appendix Fig
349 S2). Therefore, the inhibitory effect of *PIF7-CFP* overexpression appeared to be
350 counteracted by the overexpression of the truncated *HFR1*, further supporting
351 the genetic interaction between HFR1 and PIF7 (Fig 6C, D).

352 Altogether, these analyses support that HFR1 and PIF7 interaction is
353 important for the regulation of hypocotyl elongation in response to shade. These
354 results are consistent with HFR1 functioning as a suppressor of PIF7.

355

356 **HFR1 restrains PIF activity in *C. hirsuta***

357 The similarity between shade-induced and warm temperature-induced
358 hypocotyl elongation (thermomorphogenesis) suggests common underlying
359 mechanisms. In *A. thaliana*, the increased activity of HFR1 at warm
360 temperatures was previously shown to provide an important restraint on PIF4
361 action that drives elongation growth (Foreman *et al*, 2011). Similarly, we
362 hypothesized that the increased activity of HFR1 in *C. hirsuta* might restrain PIF
363 activity more efficiently and consequently alter thermomorphogenesis (Fig 7A).
364 We analyzed this response by growing seedlings constantly at 22°C, 28°C, or
365 transferred from 22°C to 28°C after day 2 (Fig 7B). Whereas warm temperature
366 promoted hypocotyl elongation of *At*^{WT} seedlings compared to those growing at

367 22°C, *pifq* and *pif7-2* mutant seedlings were almost unresponsive to 28°C, in
368 accordance with the role of PIF4, PIF5 and PIF7 in thermomorphogenesis
369 (Fiorucci *et al*, 2020; Franklin *et al*, 2011; Stavang *et al*, 2009). Unlike the *hfr1-5*
370 mutant, which was slightly but significantly more responsive than *At*^{WT}, *A.*
371 *thaliana* seedlings that overexpress a stable form of HFR1 (35S:GFP-ΔNt-
372 HFR1, ΔNtHFR1) were almost unresponsive to 28°C (Fig 7C), indicating that
373 HFR1 activity impacts this PIF-dependent response. A lack of hypocotyl
374 elongation was also observed in *Ch*^{WT} at 28°C, a response that was recovered
375 in the *C. hirsuta chfr1* mutant seedlings (Fig 7C). These results support our
376 hypothesis that a strong suppression of PIFs by the enhanced HFR1 activity is
377 responsible for the lack of hypocotyl elongation at 28°C of *Ch*^{WT} seedlings (Fig
378 7A). Together, our results suggest that the activity of the PIF-HFR1 regulatory
379 module might be a general mechanism to coordinate the hypocotyl elongation in
380 response to both W+FR exposure and 28°C.

381 We also studied dark-induced senescence (DIS), another PIF-dependent
382 process (Fig 7D). In *A. thaliana*, DIS can be induced by transferring light grown
383 seedlings to complete darkness, a process in which PIF4 and PIF5 have major
384 roles (Liebsch & Keech, 2016; Sakuraba *et al*, 2014; Song *et al*, 2014). DIS
385 results in a degradation of chlorophylls, which can be quantified as markers of
386 senescence progression (Sakuraba *et al*, 2014; Song *et al*, 2014). To examine
387 DIS, we transferred light-grown *At*^{WT}, *pifq* and *Ch*^{WT} seedlings to total darkness
388 for up to 20 days (Fig 7E). After DIS was activated, *At*^{WT} seedlings became pale
389 and eventually died. After just 5 days of darkness, chlorophyll levels dropped,
390 and longer dark treatments resulted in pronounced differences between the
391 three genotypes. *At*^{WT} seedlings became visibly yellow at day 10, accompanied

392 by a strong reduction of chlorophyll levels that dropped to less than 10% (Fig
393 7F). DIS was delayed in 35S:GFP- Δ Nt-HFR1 seedlings, supporting that a stable
394 HFR1 form can interfere with PIF activity in regulating this trait. However, DIS in
395 was not advanced in *hfr1* mutants (Fig 7E). In Ch^{WT} seedlings, chlorophyll
396 levels declined more slowly and seedlings were still green after 20 days of
397 darkness, just like *pifq* (Fig 7E). The observed delay in the DIS in *C. hirsuta* was
398 not affected in *chfr1* mutants, suggesting that HFR1 does not regulate this trait
399 in any of the two species. It also pointed to a reduced PIF activity as the main
400 cause for the delayed DIS in this species (Fig 7D-F). As HFR1 is very unstable,
401 particularly in dark-grown conditions (Duek *et al*, 2004; Yang *et al*, 2005), it
402 seems plausible that HFR1 does not accumulate in seedlings when transferred
403 to the dark. Despite this attenuation of PIF activity, Ch^{WT} seedlings showed an
404 etiolated phenotype similar to that of At^{WT} when grown in the dark, in contrast to
405 *A. thaliana pifq* and 35S:GFP- Δ Nt-HFR1 seedlings (Fig 7G), suggesting the PIF
406 activity is high enough in *C. hirsuta* to induce the normal skotomorphogenic
407 development.

408

409 **DISCUSSION**

410 It is currently unknown whether the switch between shade avoidance and
411 tolerance strategies is an easily adjustable trait in plants. The existence of
412 closely related species with divergent strategies to acclimate to shade provides
413 a good opportunity to study the genetic and molecular basis for adapting to this
414 environmental cue. To this goal, we performed comparative analyses of the
415 hypocotyl response to shade in young seedlings of two related Brassicaceae: *A.*
416 *thaliana* and *C. hirsuta*. *A. thaliana*, a model broadly used to study the SAS

417 hypocotyl response, is well characterized on a physiological, genetic and
418 molecular level. *C. hirsuta* was previously described as a shade tolerant species
419 whose hypocotyls are unresponsive to simulated shade (Hay *et al*, 2014;
420 Molina-Contreras *et al*, 2019). Recent work showed that phyA is a major
421 contributor to the suppression of hypocotyl elongation of *C. hirsuta* seedlings in
422 response to shade, mainly due to the stronger phyA activity in this species
423 compared to the shade-avoider *A. thaliana* (Molina-Contreras *et al*, 2019).
424 Importantly, an enhanced phyA activity was not enough to explain the lack of
425 shade-induced hypocotyl elongation in *C. hirsuta*, pointing to additional
426 components that contribute to this response. Our aim to fill this gap led us to
427 uncover a role for HFR1 in this response.

428 In *C. hirsuta*, removal of HFR1 function resulted in a strong *slender in*
429 *shade* (*sis*) phenotype but milder than that of *sis1* plants, deficient in the phyA
430 photoreceptor (Molina-Contreras *et al*, 2019), providing genetic evidence for the
431 role of *HFR1* in restraining the *C. hirsuta* hypocotyl elongation in shade (Fig 1A,
432 B). This indicates that, like phyA, HFR1 contributes to implement the shade
433 tolerant habit in *C. hirsuta* seedlings. Because of the *sis* phenotype of *chfr1* and
434 RNAi-HFR1 seedlings (Fig 1) we hypothesized that HFR1 activity is higher in *C.*
435 *hirsuta* than in *A. thaliana*. Consistently, transcript levels of *HFR1* were
436 significantly higher in Ch^{WT} than At^{WT} seedlings in both W and W+FR (Fig 2).
437 Higher HFR1 levels in *C. hirsuta* may not be relevant in W because of the
438 expected lower abundance and activity of PIFs, but a higher pool of ChHFR1
439 ready to suppress early ChPIF action in shade could provide a fast and
440 sustained repression of the elongation response. Indeed, the shade-induced
441 expression of *PIL1*, *YUC8* and *XTR7*, known to be direct PIF target genes in *A.*

442 *thaliana*, was strongly and rapidly enhanced in *chfr1* and RNAi-HFR1 seedlings
443 (Fig 1C, D). More importantly, rapid shade-induced expression was globally
444 attenuated in Ch^{WT} compared to At^{WT} seedlings (Molina-Contreras *et al*, 2019).

445 In addition to changes in gene expression, a higher HFR1 activity in *C.*
446 *hirsuta* could also result from post-translational regulation affecting protein
447 stability. Our immunoblot analyses indicated that HFR1 proteins rapidly
448 accumulate in response to simulated shade (W+FR), likely as a consequence of
449 the strong shade-induced responsiveness of the promoter (Fig 4A). These
450 results support that regulation of HFR1 protein abundance in low R:FR occurs
451 mainly at the transcriptional level, as suggested (de Wit *et al*, 2016). More
452 importantly, ChHFR1 accumulates significantly more when expressed under the
453 control of a constitutive promoter either under W or W+FR (Fig 4B-D)
454 suggesting that intrinsic differences in post-translational stability between these
455 proteins play a role in their contrasting accumulation.

456 AtHFR1 protein abundance is modified post-translationally by
457 phosphorylation (Park *et al*, 2008) and ubiquitination in a light- and COP1-
458 dependent manner (Jang *et al*, 2005; Yang *et al*, 2005). Canopy shade
459 promotes nuclear accumulation of COP1 (Pacin *et al*, 2013; Pacin *et al*, 2016)
460 allowing it to directly interact with and polyubiquitinate AtHFR1, leading to its
461 degradation by the 26S proteasome (Huang *et al*, 2014; Jang *et al*, 2005; Yang
462 *et al*, 2005). AtHFR1, like ChHFR1, contains two putative COP1 binding sites
463 (VP motifs) on its N-terminal half (Fig EV4A), although only one binds COP1
464 (Figs 5A, EV5) (Lau *et al*, 2019). Deletion of AtHFR1 Nt leads to its stabilization
465 in the dark and light (Duek *et al*, 2004), and results in a stronger biological
466 activity (Galstyan *et al*, 2011; Jang *et al*, 2005; Yang *et al*, 2005), highlighting

467 the importance of the COP1-interacting domain for light regulation of AtHFR1
468 stability. Our MST binding assays showed that AtHFR1 binds to COP1 about
469 100 times more weakly than other plant COP1 substrates do (Lau *et al*, 2019),
470 and ChHFR1 even more weakly than AtHFR1 (Fig 5A, B). AtHFR1 and
471 ChHFR1 primary structures are similar, including the putative COP1-interacting
472 domain (Jang *et al*, 2005), except for the addition of 30 amino acids at the N-
473 terminal part of ChHFR1 and a 9-amino acid insertion in the C-terminal part of
474 AtHFR1 (Fig EV4A). We cannot discount the possibility that protein sequence
475 and/or structural differences other than the VP motifs could also contribute to
476 the affinity of the full-length HFR1 orthologues for COP1 and account for the
477 difference in abundance between *C. hirsuta* and *A. thaliana* HFR1. However,
478 the strong impact of swapping the VP region between ChHFR1 and AtHFR1 on
479 the abundance of the resulting HFR1* proteins (Fig 5C, D) further points to the
480 binding affinity of COP1 for its substrates as a main determinant of the stability
481 of the two HFR1 orthologues. Together, our results point to (1) the regulation of
482 affinity for COP1 as impacting HFR1 stability; and (2) HFR1 stability as a
483 mechanism to control global HFR1 activity to modulate adaptation of different
484 plant species to vegetation proximity and shade.

485 AtHFR1 was previously shown to interact with all the AtPIFQ members
486 and to form non-DNA-binding heterodimers (Fairchild *et al*, 2000; Hornitschek *et*
487 *al*, 2012; Shi *et al*, 2013). Our genetic and Y2H experiments extended the list of
488 AtHFR1 interactors to AtPIF7, the major SAS-promoting PIF (Fig 6). If ChHFR1
489 maintains similar PIF-binding abilities, the reduced expression of *ChPIF7* (Fig 2)
490 might further contribute to imbalance the PIF-HFR1 module in favor of the
491 negative HFR1 activity in *C. hirsuta* compared to *A. thaliana*. Because of the

492 higher stability of ChHFR1 over AtHFR1 in shade (Fig 4), an even stronger
493 repression of global PIF activity in *C. hirsuta* would contribute to the
494 unresponsiveness of hypocotyls to shade. The attenuation of the warm
495 temperature-induced hypocotyl elongation in *C. hirsuta*, which is a PIF-
496 regulated process in *A. thaliana* (Fiorucci *et al*, 2020; Hayes *et al*, 2017; Koini *et*
497 *al*, 2009; Stavang *et al*, 2009) and HFR1-dependent in both species (Figs 7A-
498 C), further agrees with our proposal of an enhanced activity of HFR1 in *C.*
499 *hirsuta* compared to *A. thaliana*. On the other hand, the delayed DIS observed
500 in *C. hirsuta*, shown to be PIF-regulated in *A. thaliana* (Sakuraba *et al*, 2014;
501 Song *et al*, 2014) but unaffected by HFR1 in the two species analyzed (Figs 7D,
502 E), suggests that PIF activity is globally lower *per se* in *C. hirsuta* than in *A.*
503 *thaliana*. Together, our results indicate that PIF-HFR1 module is balanced
504 differently in *C. hirsuta* by the combination of (1) an attenuated global PIF
505 activity and *PIF7* expression compared to *A. thaliana*, and (2) the increased
506 levels of ChHFR1 in light and shade conditions, resulting in the repression of
507 PIF-regulated processes in *C. hirsuta* (Fig 8). Importantly, although attenuated,
508 PIF activity in *C. hirsuta* is enough to provide a functional and effective etiolation
509 response (Fig 7G) for seedlings survival during germination in the dark.

510 Activity of HFR1 and phyA (Molina-Contreras *et al*, 2019) appears to be
511 increased in *C. hirsuta* to maintain unresponsiveness of hypocotyls to shade.
512 An aspect shared by both negative regulators is that their expression and/or
513 stability are strongly affected by light conditions. Expression of both *PHYA* and
514 *HFR1* is induced by simulated shade in de-etiolated seedlings. By contrast,
515 whereas the stability of the photolabile phyA is reduced by light but enhanced
516 by shade, that of AtHFR1 is promoted by light and decreased by shade (Casal

517 *et al*, 2014; Ciolfi *et al*, 2013; Duek *et al*, 2004; Kircher *et al*, 1999; Martinez-
518 Garcia *et al*, 2014; Pacin *et al*, 2016; Park *et al*, 2008; Yang *et al*, 2018).
519 Although expression of both *PHYA* and *HFR1* is higher in *C. hirsuta* than in *A.*
520 *thaliana*, different mechanisms might contribute to their increased activity in *C.*
521 *hirsuta*. Indeed, enhanced ChphyA repression was achieved by its stronger
522 specific intrinsic activity (Molina-Contreras *et al*, 2019). By contrast, enhanced
523 ChHFR1 repression was accomplished through its higher gene expression and
524 protein stability coupled with an attenuated PIF7 activity. Altogether this could
525 provide a more repressive state of the *C. hirsuta* PIF-HFR1 module. Because of
526 the temporal differences downregulating many of the shade marker genes
527 between phyA (observed after 4-8 hours of shade exposure) (Molina-Contreras
528 *et al*, 2019) and HFR1 (rapidly detected after just 1 h of shade exposure) (Fig
529 1C, D), it seems likely that ChHFR1 and ChphyA suppressor mechanisms of
530 shade response in *C. hirsuta* act independently, as it was reported for *A.*
531 *thaliana* (Ciolfi *et al*, 2013; Ortiz-Alcaide *et al*, 2019). Therefore, the concerted
532 activity of these two independent suppressor mechanisms seems to
533 coordinately prevent the shade-induced hypocotyl elongation in *C. hirsuta*.
534 Whether other shade tolerant species employ the same adaptive principles is
535 something we aim to explore in the future.

536

537 **MATERIALS AND METHODS**

538 **Plant material and growth conditions**

539 *Arabidopsis thaliana* *hfr1-5*, *pif7-1*, *pif7-2* and *pifq* mutants, 35S:PIF7-
540 CFP and 35S:GFP-ΔNt-HFR1 lines (in the Col-0 background, At^{WT}) and
541 *Cardamine hirsuta* (Oxford ecotype, Ox, Ch^{WT}) plants have been described

542 before (Galstyan *et al*, 2011; Hay *et al*, 2014; Leivar *et al*, 2008; Yang *et al*,
543 2005). Plants were grown in the greenhouse under long-day photoperiods (16 h
544 light and 8 h dark) to produce seeds, as described (Gallemi *et al*, 2016; Gallemi
545 *et al*, 2017; Martinez-Garcia *et al*, 2014). For transient expression assays,
546 *Nicotiana benthamiana* plants were grown in the greenhouse under long-day
547 photoperiods (16 h light and 8 h dark).

548 For hypocotyl assays, seeds were surface-sterilized and sown on solid
549 growth medium without sucrose (0.5xGM–). For gene expression analyses,
550 immunoblot experiments and pigment quantification, seeds were sown on a
551 sterilized nylon membrane placed on top of the solid 0.5xGM– medium. After
552 stratification (dark at 4°C) of 3-6 days, plates with seeds were incubated in plant
553 chambers at 22°C under continuous white light (W) for at least 2 h to break
554 dormancy and synchronize germination (Paulisic *et al*, 2017; Roig-Villanova *et*
555 *al*, 2019).

556 W was emitted from cool fluorescent tubes that provided from 20 to 100
557 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR) with a red (R) to far-red
558 light (FR) ratio (R:FR) from 1.3-3.3. The different simulated shade treatments
559 were produced by supplementing W with increasing amounts of FR (W+FR). FR
560 was emitted from GreenPower LED module HF far-red (Philips), providing R:FR
561 of 0.02-0.09. Light fluence rates were measured with a Spectrosense2 meter
562 (Skye Instruments Ltd), which measures PAR (400-700 nm), and 10 nm
563 windows of R (664-674 nm) and FR (725-735 nm) regions (Martinez-Garcia *et*
564 *al*, 2014). Details of the resulting light spectra have been described before
565 (Molina-Contreras *et al*, 2019).

566 Temperature induced hypocotyl elongation assays were done by placing
567 the plates with seeds under the indicated light conditions in growth chambers at
568 22°C or 28°C.

569

570 **Measurement of hypocotyl length**

571 Hypocotyl length was measured as described (Paulisic *et al*, 2017; Roig-
572 Villanova *et al*, 2019). Experiments were repeated at least three times with
573 more than 10 seedlings per genotype and/or treatment, providing consistent
574 results. Hypocotyl measurements from the different experiments were
575 averaged.

576

577 **Generation of transgenic lines, mutants and crosses**

578 *A. thaliana hfr1-5* plants were transformed to express *AtHFR1* and
579 *ChHFR1* under the promoters of 35S or *AtHFR1* (pAt). The obtained lines were
580 named as *hfr1>35S:ChHFR1*, *hfr1>pAt:AtHFR1* and *hfr1>pAt:ChHFR1*.
581 Transgenic RNAi-HFR1 and mutant *chfr1-1* and *chfr1-2* lines are in Ch^{WT}
582 background. Details of the constructs used for the generation of these lines
583 (Morineau *et al*, 2017) are provided as Appendix Supplementary Methods.

584

585 **Gene expression analyses**

586 Real-time qPCR analyses were performed using biological triplicates, as
587 indicated (Gallemi *et al*, 2017). Total RNA was extracted from seedlings, treated
588 as indicated, using commercial kits (Maxwell® SimplyRNA and Maxwell® RSC
589 Plant RNA Kits; www.promega.com). 2 µg of RNA was reverse-transcribed with
590 Transcriptor First Strand cDNA synthesis Kit (Roche, www.roche.com). The *A.*

591 *thaliana* *UBIQUITIN 10 (UBQ10)* was used for normalization in *A. thaliana hfr1-*
592 *5* lines expressing *AtHFR1* or *ChHFR1*. The *ELONGATION FACTOR 1 α*
593 (*EF1 α*), *YELLOW-LEAF-SPECIFIC GENE 8 (YLS8)* and *SPC25 (AT2G39960)*
594 were used for normalizing and comparing the levels of *HFR1* and *PIF7* between
595 *A. thaliana* and *C. hirsuta* (Molina-Contreras *et al*, 2019). Primers sequences for
596 qPCR analyses are provided in Appendix Table S1.

597

598 **Expression of *HFR1* derivatives in *Nicotiana benthamiana***

599 *N. benthamiana* plants were agroinfiltrated with *A. tumefaciens* strains
600 transformed with the plasmids to express the various *HFR1* derivatives, and
601 kept in the greenhouse under long-day photoperiods. Samples (leaf circles
602 obtained from infiltrated areas) were taken 3 days after agroinfiltration and
603 frozen immediately. In Fig 4D, prior freezing, leaf circles were incubated in Petri
604 dishes with 10 mL of the \pm CHX solution for the indicated times and conditions.
605 Each biological sample contained about 75 mg of leaf tissue from the same leaf.
606 Additional details of the preparation of the plasmids used are provided in
607 Appendix Supplementary Methods.

608

609 **Protein extraction and immunoblotting analyses**

610 To detect and quantify transgenic *AtHFR1* and *ChHFR1*, proteins were
611 extracted from ~50 mg of 7-day old seedlings (grown as indicated) or from 50-
612 75 mg of agroinfiltrated *N. benthamiana* leaves. Plant material was frozen in
613 liquid nitrogen, ground to powder and total proteins were extracted using an
614 SDS-containing extraction buffer (1.5 μ L per mg of fresh weight), as described
615 (Gallemi *et al*, 2017). Protein concentration was estimated using Pierce™ BCA

616 Protein Assay Kit (Thermo Scientific, www.thermofisher.com). Proteins (45 - 50
617 µg per lane) were resolved on a 10% SDS-PAGE gel, transferred to a PVDF
618 membrane and immunoblotted with rat monoclonal anti-HA (High Affinity, clone
619 3F10, Roche; 1:2000 dilution) or mouse monoclonal anti-GFP (monoclonal mix,
620 clones 7.1 + 13.1, Roche; 1:2000 dilution). Secondary antibodies used were
621 horseradish peroxidase (HRP) conjugated goat anti-rat (Polyclonal, A9037,
622 Sigma, www.sigmaaldrich.com; 1:5000 dilution) and HRP conjugated sheep
623 anti-mouse (Promega; 1:10000 dilution). Development of blots was carried out
624 in ChemiDoc™ Touch Imaging System (Bio-Rad, www.bio-rad.com) using ECL
625 Prime Western Blotting Detection Reagent (GE Healthcare, RPN2236). Relative
626 protein levels of three to four biological replicates were quantified using Image
627 Lab™ Software (Bio-Rad, www.bio-rad.com).

628

629 **Yeast 2 Hybrid (Y2H) assays**

630 For Y2H assays we employed a cell mating system, as described
631 (Gallemi *et al*, 2017). The leucine (Leu) auxotroph YM4271a yeast strain was
632 transformed with the AD-derived constructs and the tryptophan (Trp) auxotroph
633 pJ694α strain with the BD-derived constructs. Colonies were selected on
634 synthetic defined medium (SD) lacking Leu (SD-L) or Trp (SD-W), grown in
635 liquid medium and set to mate by mixing equal volumes of transformed cells.
636 Dilutions of the mated cells were selected on SD-LW and protein interactions
637 were tested on SD-LW medium lacking histidine (SD-HLW). Details of the yeast
638 constructs used are provided as Appendix Supplementary Methods.

639

640 **Expression of AtCOP1 WD40 protein and purification**

641 AtCOP1 WD40 (residues 349-675) was expressed in *Spodoptera*
642 *frugiperda* Sf9 cells (Thermofisher) and purified as described previously (Lau *et*
643 *al*, 2019). Details of the procedure are provided as Appendix Supplementary
644 Methods.

645

646 **Protein labeling and Microscale thermophoresis (MST)**

647 COP1 WD40 was labeled using Monolith Protein Labeling Kit RED-NHS
648 2nd Generation Amine Reactive kit (MO-L011, Nanotemper Technologies,
649 Munich, Germany). After the TEV cleavage, COP1 WD40 was in buffer A
650 containing 2 mM β -ME, which is incompatible with the labeling procedure.
651 Therefore, prior to labeling, the buffer was exchanged using labeling buffer NHS
652 provided in the kit. In the last step, the protein was purified from the free dye, in
653 the assay buffer 20 mM Hepes pH 7.5, 150 mM NaCl, 2 mM TCEP and 0.05%
654 [v/v] Tween-20 in 12 to 15 different fractions. The absorbance of each sample
655 was measured at 280 nm and 650 nm. The Degree of Labeling (DOL) was
656 calculated using the formula provided in the manual. Aliquots containing 2000 to
657 8000 nM concentration of proteins and DOL of >0.5 were flash frozen for the
658 use in the assay.

659 Peptide solutions were freshly prepared in the assay buffer at desired
660 concentrations. For each independent replicate, 10 μ L of peptide solution was
661 serially diluted 1:1 using assay buffer, in 16 PCR tubes. 10 μ L of solution was
662 discarded from the 16th tube, thus each tube contained 10 μ L of peptide
663 solution. Each dilution step was mixed with 10 μ L of 150 nM of COP1 WD40
664 and transferred into Monolith NT.115 Premium Capillaries (MO-K025). The
665 samples were measured with the Monolith NT.115 instrument at a 25% LED

666 Power and 20% MST power. The resulting thermophoresis data were analyzed
667 with the MOAffinityAnalysis software (Nanotemper Technologies).

668

669 **DATA AVAILABILITY**

670 This study includes no data deposited in external repositories.

671

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688

689 **AUTHOR CONTRIBUTIONS**

690 JFM-G conceived the original research plan, and directed and coordinated the
691 study. SP, WQ, CT, BA and FN designed and/or carried out experiments using
692 *A. thaliana* and *C. hirsuta*. MT and FN fundamentally contributed to design the
693 constructs to obtain *C. hirsuta* transgenic and mutant lines. HA-V and MH
694 designed and performed MST experiments and their analyses. SP and JFM-G
695 wrote the article with contributions and/or comments of all other authors.

696

697 **CONFLICT OF INTEREST**

698 The authors declare that they have no conflict of interest.

699

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885

886 **FIGURE LEGENDS**

887

888 **Figure 1. Hypocotyls of *C. hirsuta* seedlings with reduced levels of**
889 ***ChHFR1* strongly elongate in response to simulated shade.**

890 **A, B** Hypocotyl length of Ch^{WT} , **(A)** RNAi-*ChHFR1* transgenic and **(B)** *chfr1*
891 mutant seedlings grown under different R:FR. Seedlings were grown for 7 days
892 in continuous W (R:FR>1.5) or for 3 days in W then transferred to W
893 supplemented with increasing amounts of FR (W+FR) for 4 more days,
894 producing various R:FR. Aspect of representative 7-day old Ch^{WT} , RNAi-HFR1
895 and *chfr1-1* seedlings grown in W or W+FR (R:FR, 0.02), as indicated, is shown
896 in lower panel.

897 **C, D** Effect of W+FR exposure on the expression of *PIL1*, *YUC8* and *XTR7*
898 genes in seedlings of Ch^{WT} , **(C)** RNAi-HFR1 and **(D)** *chfr1* mutant lines.
899 Expression was analyzed in 7-day old W-grown seedlings transferred to W+FR
900 (R:FR, 0.02) for 0, 1, 4, 8 and 12 h. Transcript abundance is normalized to
901 *EF1 α* levels.

902 Data information: Values are the means \pm SE of three independent biological
903 replicates relative to Ch^{WT} value at 0 h. Asterisks mark significant differences
904 (Student *t*-test: ** p-value <0.01; * p-value <0.05) relative to Ch^{WT} value at the
905 same time point.

906

907 **Figure 2. Levels of *HFR1* transcript are higher in *C. hirsuta* than *A.***
908 ***thaliana* seedlings.**

909 Seedlings of *Ch*^{WT} and *At*^{WT} were grown for 3 days in W then either kept under
910 the same conditions or transferred to W+FR (R:FR, 0.02) for the indicated
911 times. Plant material was harvested every 24 h. Transcript abundance of *HFR1*
912 and *PIF7* was normalized to three reference genes (*EF1α*, *SPC25*, and *YLS8*).
913 Expression values are the means ± SE of three independent biological
914 replicates relative to the data of *At*^{WT} grown in continuous W at day 3. Asterisks
915 mark significant differences (2-way ANOVA: ** p-value <0.01, *** p-value
916 <0.001) between *Ch*^{WT} and *At*^{WT} when grown under W (black asterisks) or
917 W+FR (red asterisks).

918

919 **Figure 3. The activity of *ChHFR1* is higher than that of *AtHFR1* in *A.***
920 ***thaliana* seedlings.**

921 **A** Cartoon of constructs containing *ChHFR1* or *AtHFR1* under the *HFR1*
922 promoter of *A. thaliana* (*pAtHFR1*) used to complement *hfr1-5* mutant of *A.*
923 *thaliana* (*At hfr1-5*).

924 **B** Relative expression of *HFR1* in seedlings of *At*^{WT}, *At hfr1-5*,
925 *hfr1>pAt:ChHFR1* (in blue) and *hfr1>pAt:AtHFR1* (in red) lines grown under
926 W+FR (R:FR, 0.02). Expression values are the means ± SE of three
927 independent biological replicates relative to the data of 7 days old *At*^{WT}.
928 Transcript abundance is normalized to *UBQ10* levels.

929 **C** Elongation response of seedlings of the indicated lines grown for 7 days in
930 continuous W or 2 days in W then transferred for 5 days to W+FR (R:FR, 0.02).
931 The mean hypocotyl length in W (*Hyp_W*) and W+FR (*Hyp_{W+FR}*) of at least four

932 biological replicates was used to calculate $\text{Hyp}_{\text{W+FR}} - \text{Hyp}_{\text{W}}$. Error bars represent
933 SE.

934 **D** Relative HFR1 protein levels in seedlings of the indicated lines, normalized to
935 actin protein levels, are the means \pm SE of three independent biological
936 replicates relative to *hfr1>pAt:ChHFR1* line #22 that is taken as 1. Seedlings
937 were grown for 7 days in continuous W ($\sim 20 \mu\text{mol m}^{-2} \text{s}^{-1}$) after which they were
938 incubated for 3 h in high W ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$) and transferred to W+FR (R:FR,
939 0.06) for 3 h.

940 Data information: Different letters denote significant differences (one-way
941 ANOVA with Tukey test, p-value < 0.05) among means.

942

943 **Figure 4. ChHFR1 and AtHFR1 proteins show different stability in shade.**

944 **A** Expression of *HFR1* and protein levels of HFR1-3xHA in seedlings of
945 *hfr1>pAt:ChHFR1* (line #22) and *hfr1>pAt:AtHFR1* (line #13). Seedlings were
946 grown for 7 days in continuous W ($\sim 20 \mu\text{mol m}^{-2} \text{s}^{-1}$) after which they were
947 incubated for 3 h in high W ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$) and then either kept at high W or
948 transferred to W+FR (R:FR, 0.06) for 3 or 6 h, as indicated in the cartoon at the
949 top. Relative *HFR1* transcript levels, normalized to *UBQ10*, are the means \pm SE
950 of three independent biological replicates relative to *hfr1>pAt:ChHFR1* #22
951 grown for 3 h under W+FR. Relative protein levels, normalized to actin, are the
952 means \pm SE of three independent biological replicates relative to
953 *hfr1>pAt:ChHFR1* #22. Samples were collected at data points marked in the
954 cartoon with asterisks.

955 **B** Cartoon of constructs containing *ChHFR1* or *AtHFR1* under the 35S promoter
956 used for transient expression of transgenes in *N. benthamiana* leaves.

957 **C** Relative *HFR1* transcript levels transiently expressed in tobacco leaves,
958 normalized to the *GFP*, are the means \pm SE of three independent biological
959 replicates (left). Relative HFR1 protein levels, normalized to the GFP levels, are
960 the means \pm SE of four independent biological replicates (right). In **A** and **C**,
961 asterisks mark significant differences (Student *t*-test: * p-value <0.05, ** p-value
962 <0.01) between the indicated pairs.

963 **D** Degradation of ChHFR1 (*35S:ChHFR1*) and AtHFR1 (*35S:AtHFR1*) in
964 tobacco leaf discs treated with cycloheximide (CHX, 100 μ M) for the indicated
965 times. Tobacco plants were kept under high W (\sim 200 μ mol m⁻² s⁻¹) for 3 days
966 after agroinfiltration and then leaf circles were treated with W+FR (R:FR, 0.2)
967 and CHX. Relative HFR1 protein levels (ChHFR1, blue bars; AtHFR1, red bars),
968 normalized to the GFP levels, are the means \pm SE of four biological replicates
969 relative to data point 0, taken as 1 for each line. Asterisks mark significant
970 differences (2-way ANOVA: * p-value <0.05) between ChHFR1 and AtHFR1 at
971 the same time point.

972

973 **Figure 5. AtHFR1 interacts more strongly than ChHFR1 with the WD40**
974 **domain of COP1.**

975 **A** Overview of the COP1 WD40-AtHFR1 complex (PDB ID 6QTV). The COP1
976 WD40 domain and the AtHFR1 VP peptide are shown in surface representation
977 and colored in blue and orange, respectively. The N-terminus of HFR1 VP
978 peptide, the amino acid of which differs between AtHFR1 and ChHFR1, is
979 highlighted in magenta.

980 **B** Table summaries of the microscale thermophoresis binding assay (see **Fig**
981 **EV5**). The sequence of the respective synthetic peptides is indicated.

982 **C** Cartoon of constructs containing *ChHFR1*, *AtHFR1*, *ChHFR1** and *AtHFR1**
983 derivatives under the 35S promoter used for transient expression of transgenes
984 in *N. benthamiana* leaves.

985 **D** Relative HFR1 protein levels, normalized to the GFP levels, are the means \pm
986 SE of four independent biological replicates. Asterisks mark significant
987 differences (Student *t*-test: * p-value <0.05, ** p-value <0.01) between the
988 indicated pairs.

989

990 **Figure 6. AtHFR1 interacts with AtPIF7.**

991 **A** Y2H growth assay showing the interaction between AtHFR1 and AtPIF7. The
992 BD- and the AD- derivative constructs used in the assay are shown on the left
993 side of the panel. SD-LW or SD-HLW refer to the selective medium (plated as
994 drops in dilutions of 1, 1:10 and 1:100) indicative of transformed cells or
995 interaction between the hybrid proteins, respectively. Truncated forms of murine
996 p53 (BD-fused) and SV40 large T-antigen (AD-fused), known to interact, were
997 used as a positive control. Empty vectors (/) were used as negative controls.

998 **B, C** Hypocotyl length of seedlings of *At*^{WT}, **(B)** *pif7-1*, *hfr1-5*, *pif7-1 hfr1-5* (top
999 graph), *pif7-2*, *hfr1-5* and *pif7-2 hfr1-5* (bottom graph) mutants, and **(C)**
1000 transgenic 35S:GFP- Δ Nt-HFR1 (35S: Δ Nt-HFR1), two lines of 35S:PIF7-CFP
1001 (35S:PIF7 #1 and #2), and 35S:GFP- Δ Nt-HFR1 35S:PIF7-CFP double
1002 transgenic (35S: Δ Nt-HFR1 x 35S:PIF7 #1 and #2) seedlings grown under
1003 different R:FR. Seedlings were grown in W (R:FR > 1.5) for 7 days or for 2 days
1004 in W and then transferred to two W+FR treatments (R:FR 0.06 or 0.02) for 5
1005 additional days. Values of hypocotyl length are the means \pm SE of three
1006 independent biological replicates (at least 10 seedlings per replica).

1007 **D** Aspect of representative 7-day-old W-grown seedlings shown in **C**. Scale bar
1008 is 1 cm.

1009

1010 **Figure 7. *C. hirsuta* has an attenuated hypocotyl elongation at warm**
1011 **temperature and delayed dark-induced senescence (DIS).**

1012 **A** In At^{WT} , PIFs promote hypocotyl elongation as a response to warm
1013 temperature (28°C). High ChHFR1 activity is expected to inhibit this response
1014 by repressing PIFs more effectively in Ch^{WT} and attenuate hypocotyl elongation
1015 at 28°C.

1016 **B** Seedlings were grown for 7 days in W at either 22°C, 2 days at 22°C then
1017 transferred to 28°C for additional 5 days (22°C > 28°C) or for 7 days at 28°C, as
1018 represented in the panel.

1019 **C** Hypocotyl length of seedlings of (left) At^{WT} , *pifq*, *pif7-2*, Ch^{WT} , (middle)
1020 35S:GFP- Δ Nt-HFR1 (Δ NtHFR1), *hfr1-5* and (right) *chfr1-1* and *chfr1-2* lines
1021 grown at warm temperatures. Hypocotyl lengths are the means \pm SE of three
1022 biological replicates. Asterisks mark significant differences (Student *t*-test: * p-
1023 value <0.05, ** p-value <0.01) relative to the same genotype grown at 22°C (left
1024 and right graphs, black asterisks), and between the indicated pairs (middle
1025 graph, red asterisks).

1026 **D** In At^{WT} , PIF-mediated DIS involves a reduction of chlorophyll levels. HFR1
1027 activity might inhibit DIS through repression of PIFs. If PIF activity is attenuated
1028 in Ch^{WT} , DIS would be delayed in this species compared to At^{WT} .

1029 **E** Seedlings were grown for 7 days in W and then transferred to total darkness
1030 for several days to induce senescence, as illustrated at the right panel.

1031 **F** Relative chlorophylls levels of (left) *At*^{WT}, *pifq*, *Ch*^{WT}, (middle) Δ NtHFR1, *hfr1-*
1032 *5* and (right) *chfr1-1* and *chfr1-2* lines after DIS was promoted for the indicated
1033 time. For each genotype, values are relative to pigment levels at time 0 (7 days
1034 in W). Data are the means \pm SE of four independent biological replicates.

1035 **G** Aspect of 4-day old dark-grown seedlings of *At*^{WT}, *pifq*, *pif7-2*, *hfr1-5* and Δ Nt-
1036 HFR1 (left panel), and *At*^{WT}, *Ch*^{WT} and *chfr1-1* (right panel).

1037

1038 **Figure 8. Model summarizing how PIF-HFR1 transcriptional module is**
1039 **differently balanced in *A. thaliana* and *C. hirsuta*.**

1040 Shade (low R:FR) displaces phytochrome photoequilibrium towards the inactive
1041 form, allowing PIFs to promote the expression of shade avoidance related
1042 genes, such as *HFR1*. PIF transcript or/and protein levels are induced in
1043 response to warm temperatures, resulting in enhanced expression of growth-
1044 promoting genes. HFR1 abundance is also increased by warm temperature.
1045 HFR1 modulates these responses by heterodimerizing with PIFs and inhibiting
1046 their DNA-binding ability. As a result, HFR1 attenuates hypocotyl elongation of
1047 *A. thaliana* seedlings in response to shade or warm temperature. In *C. hirsuta*,
1048 higher HFR1 activity inhibits more effectively PIF action than in *A. thaliana*. In
1049 addition, PIF abundance is attenuated in *C. hirsuta*. Both changes alter the PIF-
1050 HFR1 balance in *C. hirsuta*, resulting in lower PIF transcriptional activity. As a
1051 consequence, shade- and warm temperature-induced hypocotyl elongation are
1052 repressed and DIS is delayed in this species.

1053

1054 **EXPANDED VIEW FIGURE LEGENDS**

1055

1056 **Figure EV1. Characterization of RNAi-HFR1 and *chfr1* mutants in *C.***
1057 ***hirsuta*.**

1058 **A, B** Relative expression levels of *ChHFR1* gene, normalized to *EF1 α* in Ch^{WT} ,
1059 **(A)** two RNAi-HFR1 lines (#01 and #21) and **(B)** the two *chfr1* mutants of *C.*
1060 *hirsuta*. Seedlings were grown for 7 days in W. Expression values are the mean
1061 \pm SE of three independent biological replicates relative to Ch^{WT} . Asterisks mark
1062 significant differences (Student *t*-test: ** p-value <0.01) relative to Ch^{WT} .

1063 **C** The two identified *chfr1-1* and *chfr1-2* mutants have one nucleotide insertion
1064 at position 420 of the *ChHFR1* ORF, which leads to a frame shift and a
1065 premature stop codon.

1066

1067 **Figure EV2. Alignments of *HFR1*, *PIF4*, *PIF5* and *PIF7* partial DNA**
1068 **sequences in *A. thaliana* and *C. hirsuta***

1069 **A** Location of shared primers and amplicons used for comparison of expression
1070 levels by RT-qPCR between species.

1071 **B** Transcript abundance of *PIF4* and *PIF5*, normalized to *YLS8*, *SPC25* and
1072 *EF1 α* in Ch^{WT} and At^{WT} grown as in **Fig 2**. Expression values are the means \pm
1073 SE of three independent biological replicates relative to the data of At^{WT} grown
1074 in continuous W at day 3. Asterisks mark significant differences (2-way ANOVA:
1075 ** p-value <0.01, *** p-value <0.001) between Ch^{WT} and At^{WT} when grown
1076 under W (black asterisks) or W+FR (red asterisks).

1077

1078 **Figure EV3. *ChHFR1* and *AtHFR1* complement the *A. thaliana hfr1-5***
1079 **mutant long hypocotyl phenotype.**

1080 **A** Cartoon of *HFR1* promoters from *A. thaliana* (*pAtHFR1*) and *C. hirsuta*
1081 (*pChHFR1*). These promoters cover 2000 bp from the beginning of the
1082 translation start of the two *HFR1* genes. The positions of G-boxes (CACGTG)
1083 are indicated with arrows.

1084 **B** GUS staining of representative *A. thaliana* seedlings expressing *GUS* under
1085 the *pAtHFR1* (line #03). Seven-day-old W-grown seedlings were treated with
1086 W+FR for the indicated amount of time.

1087 **C** Correlation between $\text{Hyp}_{\text{W+FR}} - \text{Hyp}_{\text{W}}$ (means \pm SE of at least four biological
1088 replicates, data shown in **Fig 3C**) and relative levels of *ChHFR1* or *AtHFR1*
1089 expression (means \pm SE of three biological replicates, data shown in **Fig 3B**).
1090 The estimated regression equations and the R^2 values are shown for each plot.

1091

1092 **Figure EV4. ChHFR1 protein accumulates in high W.**

1093 **A** Alignment of *AtHFR1* and *ChHFR1* protein sequences. Putative COP1
1094 interacting motifs, defined in *AtHFR1*, are highlighted with a light grey box. VP
1095 motifs are highlighted with blue letters. Amino acid sequences inside the blue
1096 line rectangles correspond to the synthetic *AtHFR1*, *ChHFR1* and *At/ChHFR1*
1097 VP peptides used in the microscale thermophoresis assays (Appendix Table
1098 S3).

1099 **B** Cartoon representing the light treatments given to seedlings to estimate
1100 relative *HFR1*-3xHA levels. Seedlings grown for 7 d in low W ($\sim 20 \mu\text{mol m}^{-2} \text{s}^{-1}$,
1101 R:FR ≈ 6.4) were first moved to high W ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$, R:FR ≈ 3.9) for 3 h and
1102 then either transferred to high W (control) or high W+FR (R:FR ≈ 0.06) for 3 h.
1103 Seedling samples were collected at the time points indicated with asterisks.

1104 **C** Relative HFR1-3xHA protein levels of *hfr1>35S:ChHFR1* seedlings (line #16)
1105 grown as indicated in **B**, with a representative immunoblot in a lower panel.
1106 Relative protein levels are the mean \pm SE of three independent biological
1107 replicates relative to the data point of 0 h in high W (0 h W). Asterisks mark
1108 significant differences in protein levels (Student *t*-test: ** p-value <0.01; * p-
1109 value <0.05) relative to the 0 h W value.

1110

1111 **Figure EV5. Microscale thermophoresis (MST) experimental traces and**
1112 **analysis.**

1113 **A-F** Raw MST traces and analysis of AtCOP1 WD40 with different peptides in
1114 triplicates (duplicates for At/ChHFR1 VP). The concentration of AtCOP1 WD40
1115 is fixed at 0.15 μ M mixed with 16 serially diluted peptide concentrations at 1:1
1116 ratio. Panels **A**, **C** and **E** show the normalized MST traces. The blue box area
1117 illustrates the fluorescence before activation of the infrared- (IR-) laser and red
1118 box area illustrates average fluorescence after activation of the IR-laser.
1119 Average values \pm SD (error bars) were subsequently used for fluorescence
1120 normalization. k_D fit displaying fraction bound as a function of ligand
1121 concentration is shown in adjacent right panels **B**, **D** and **F**.

1122 **A** Raw MST traces for AtHFR1 (in blue) and ChHFR1 (in light-brown) VP
1123 peptides. Individual concentrations that showed slight aggregation or
1124 precipitation are shown in gray and were excluded from the k_D fit calculation.

1125 **B** Fitted data over a concentration range from 0.032 to 500 μ M for AtHFR1 VP
1126 (blue dots) and 0.032 to 1000 μ M for ChHFR1 VP (light-brown dots) were used
1127 to derive the corresponding dissociation constant k_D .

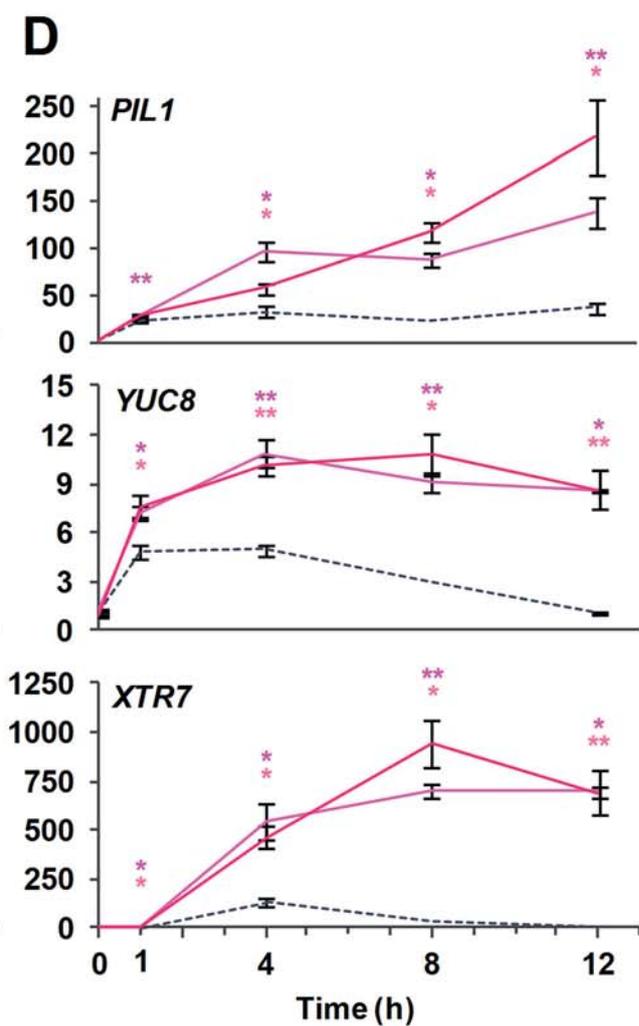
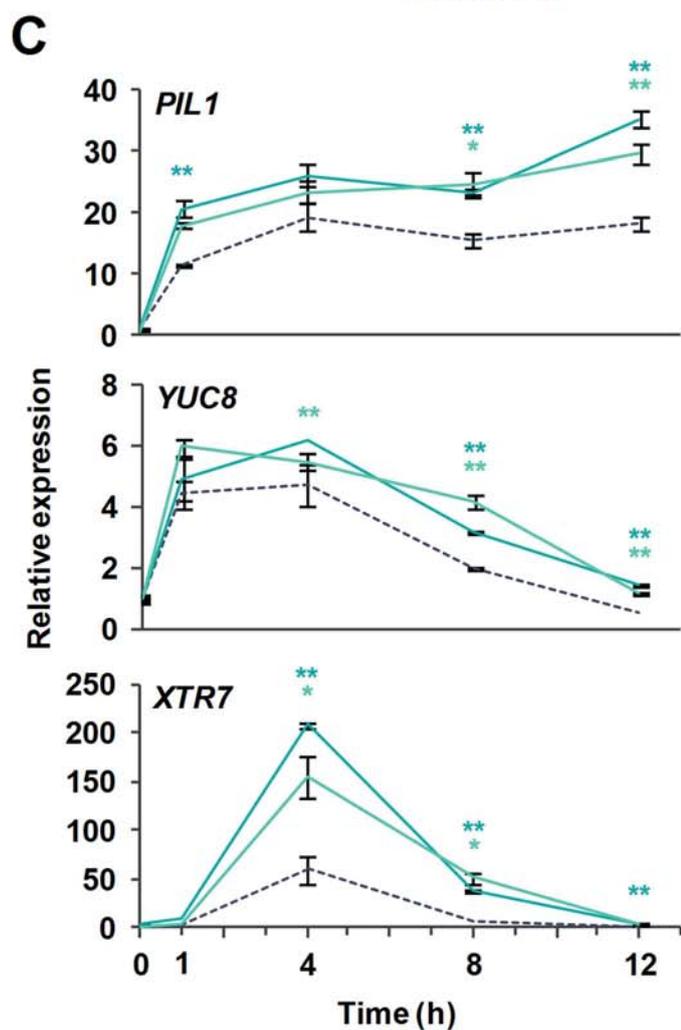
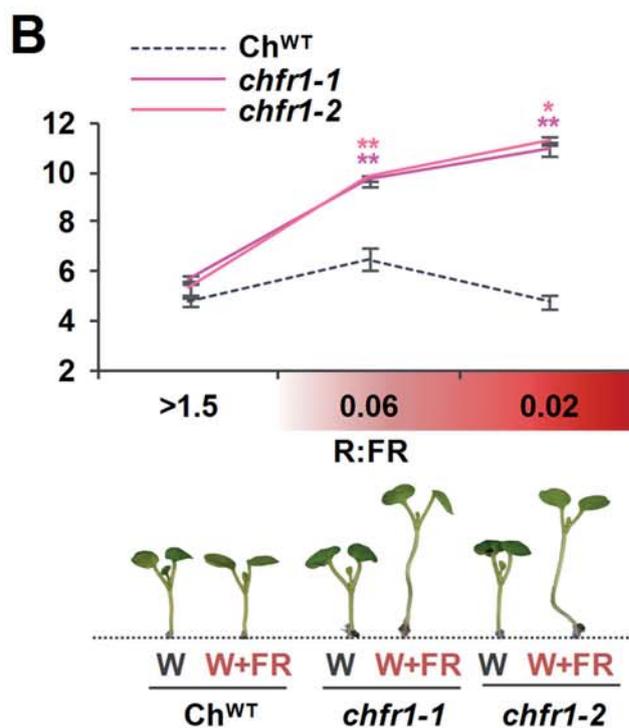
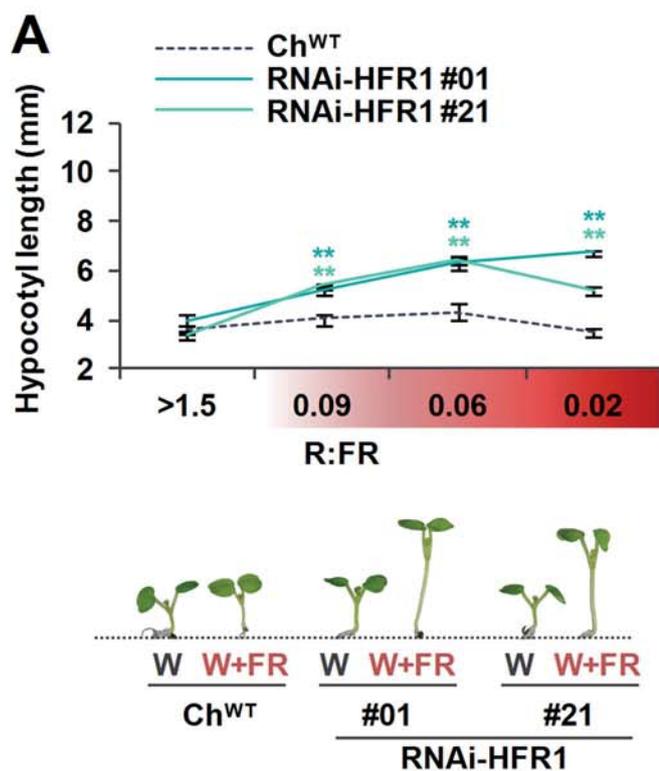
1128 **C** Raw MST traces for At/ChHFR1 VP peptide (in orange). One concentration
1129 that showed slight precipitation or aggregation is shown in gray. A concentration
1130 range of 0.0154 to 506 μM was used for the At/ChHFR1 VP.

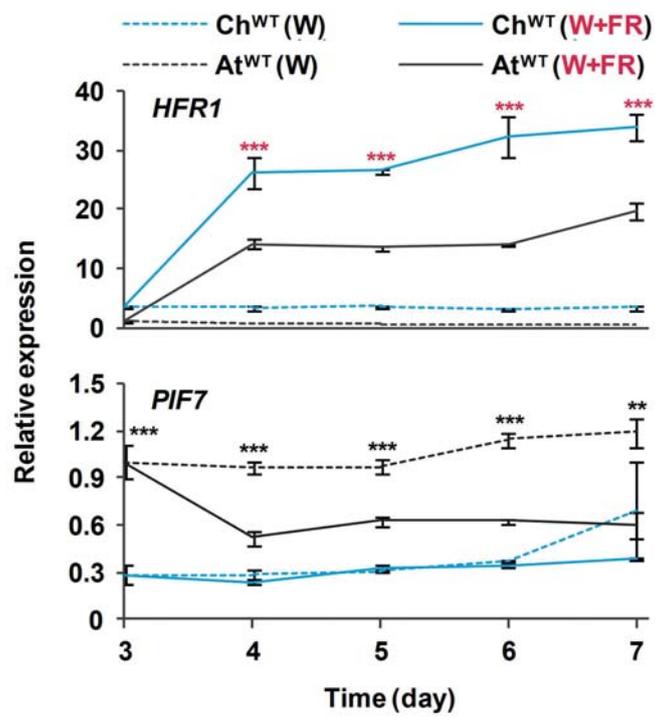
1131 **D** No k_D was determined, as no binding between COP1 WD40 and the
1132 At/ChHFR1 VP peptide (orange dots) was detected.

1133 **E** A concentration range from 0.0076 to 250 μM for HsTRIB1 (in red) and
1134 AtCRY1 (in green) peptides was used. Raw MST traces show no aggregation or
1135 precipitation effects during this binding. One AtCRY1 VP outlier is shown in
1136 gray.

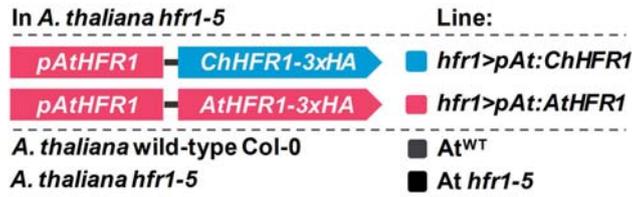
1137 **F** The k_D for HsTRIB1 (brown dots) and AtCRY1 (green dots) VP peptides was
1138 calculated using the normalized traces.

1139

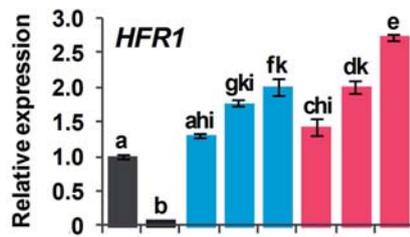




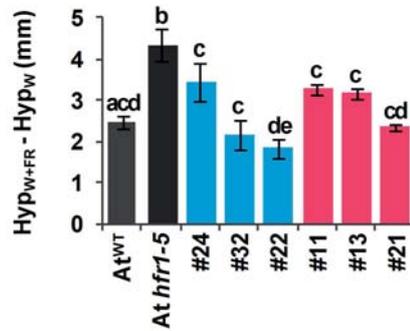
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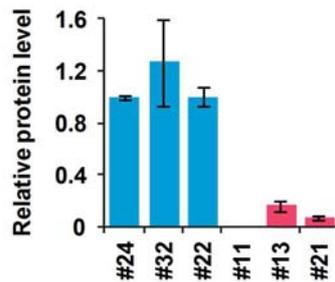
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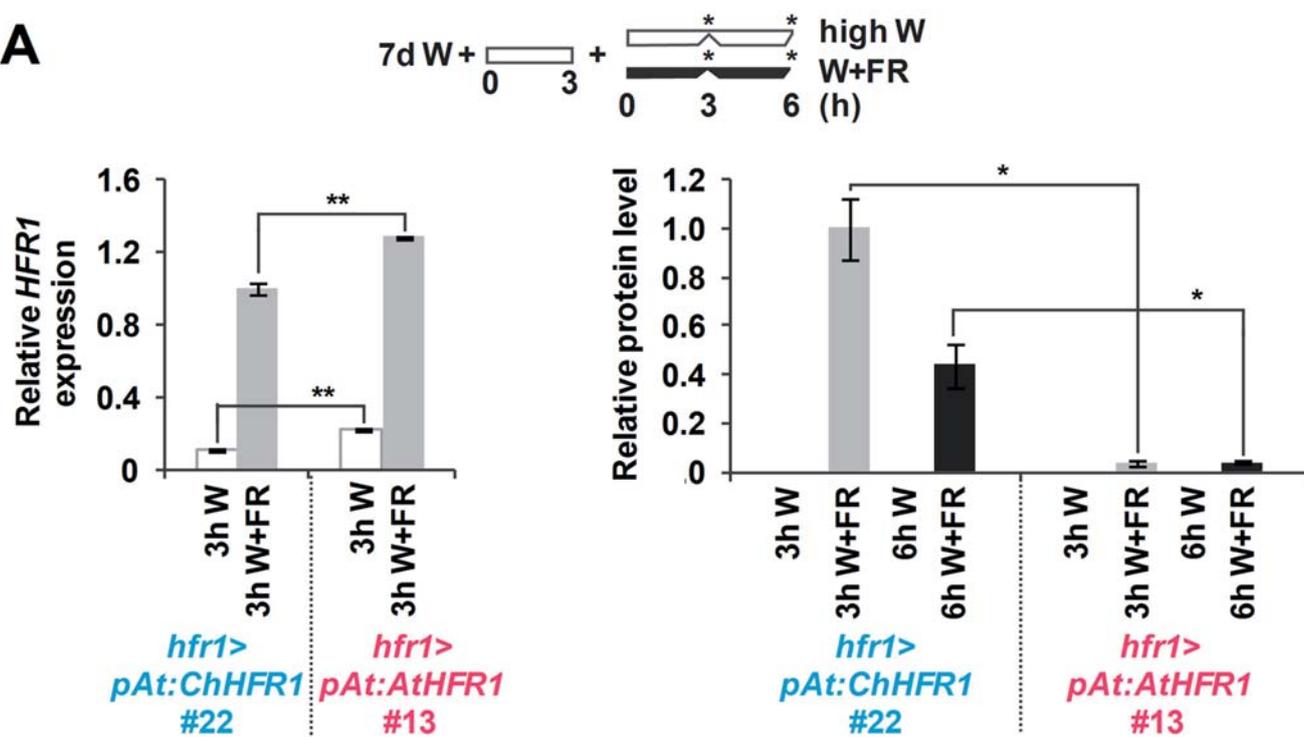
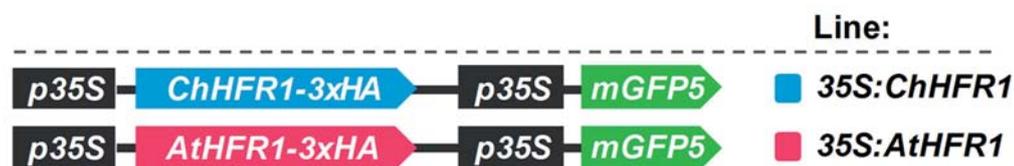
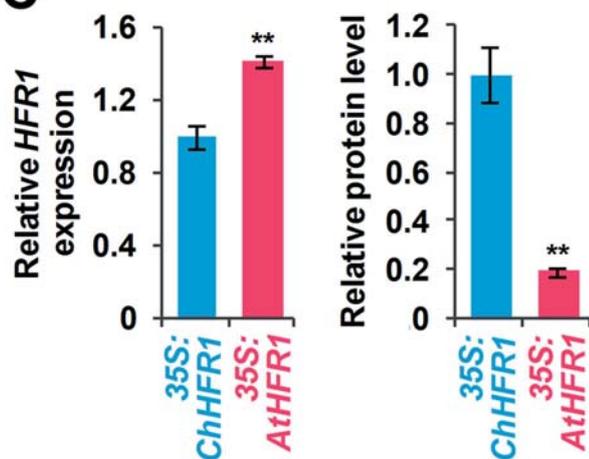
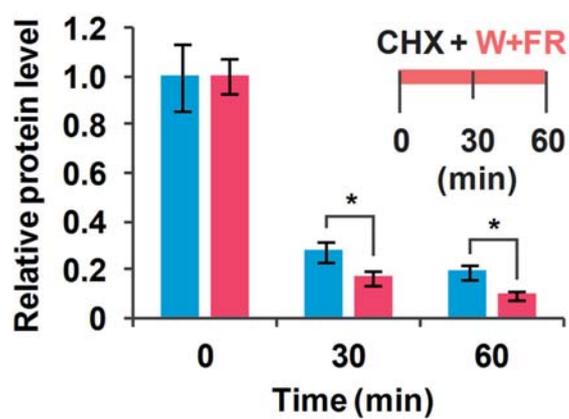


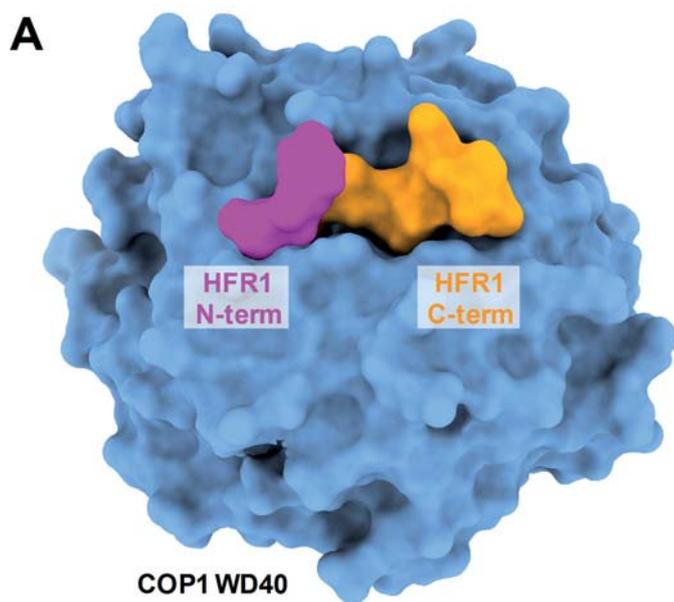
C



D



A**B****C****D**



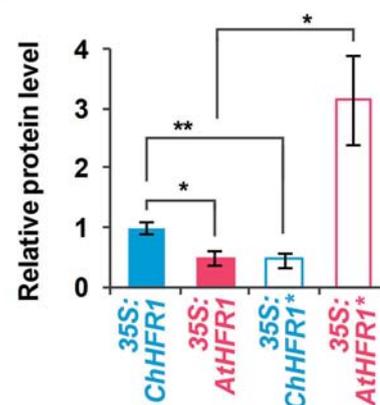
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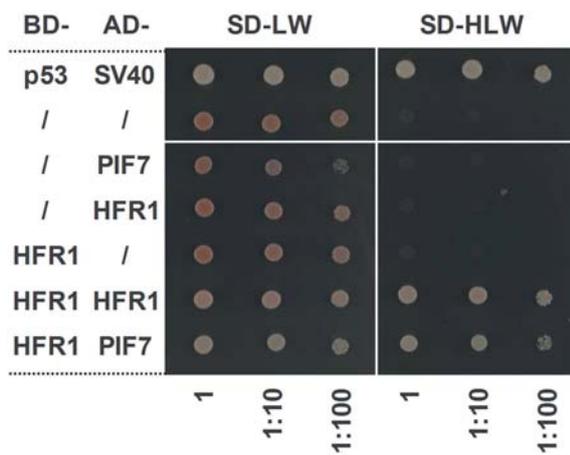
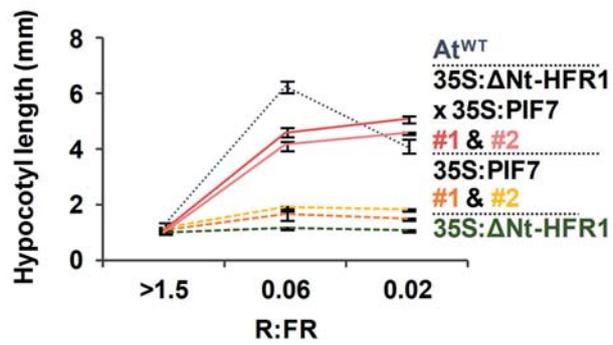
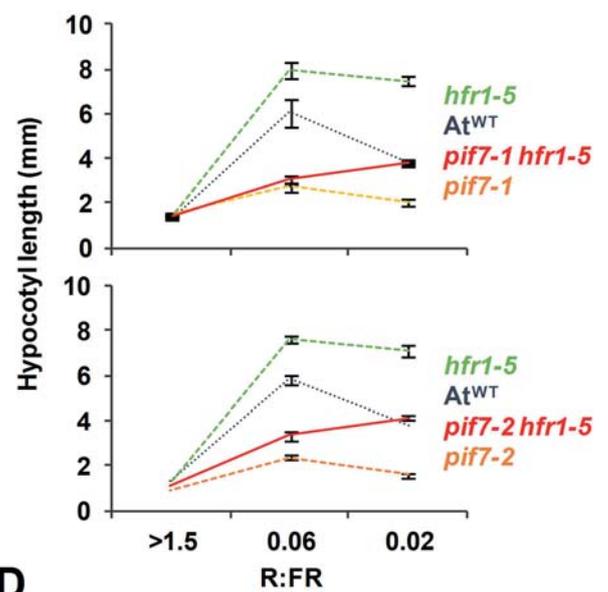
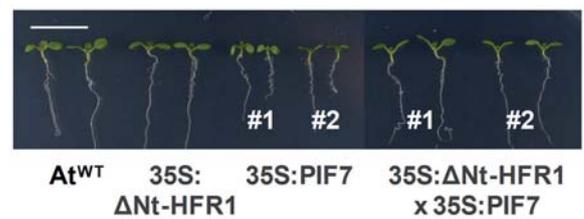
Protein	Peptide	k_D
AtHFR1	YLQIVPEI	~ 120 μ M
ChHFR1	HHQIVPEI	~ 2 mM
At/ChHFR1	LLWVPDE	n.d.
AtCRY1	EDQMVPSIT	~ 1 μ M
HsTRIB1	SDQIVPE	~ 1 μ M

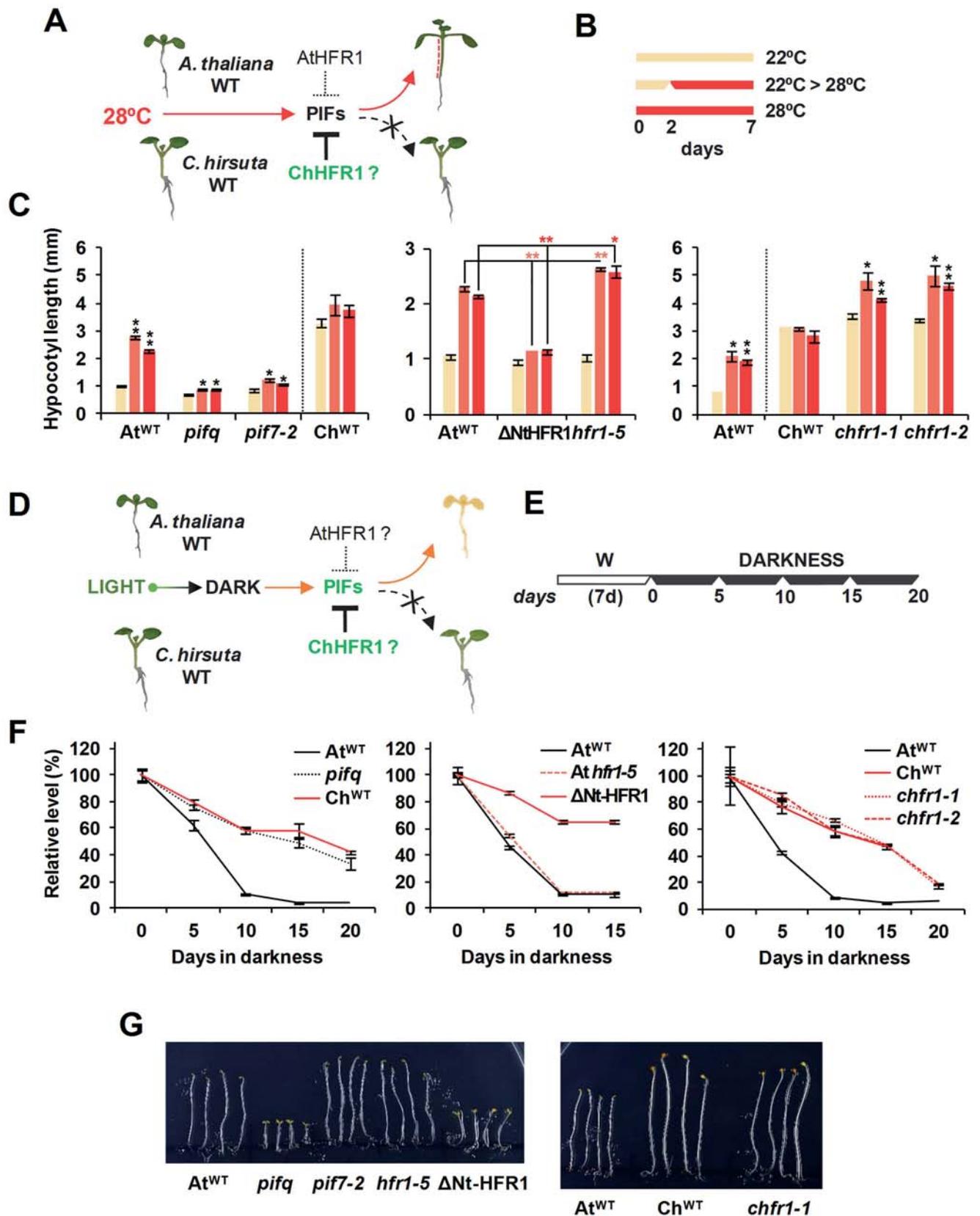
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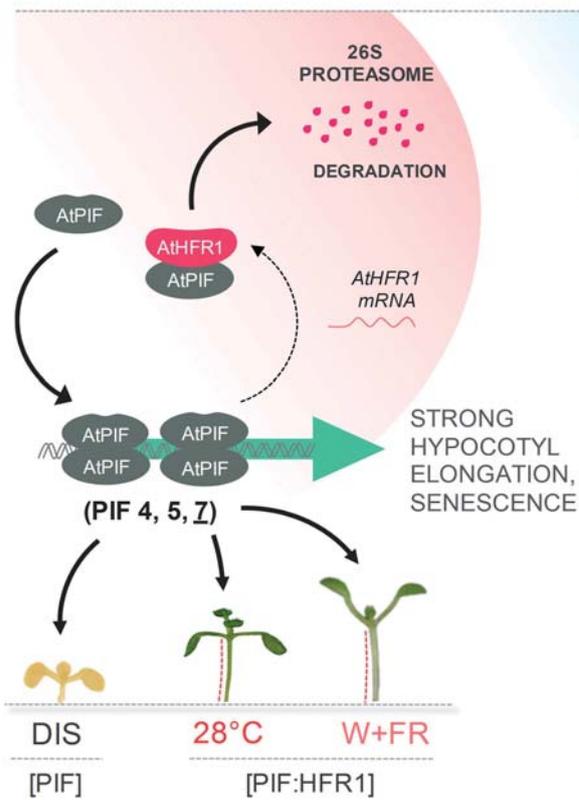


A**C****B****D**



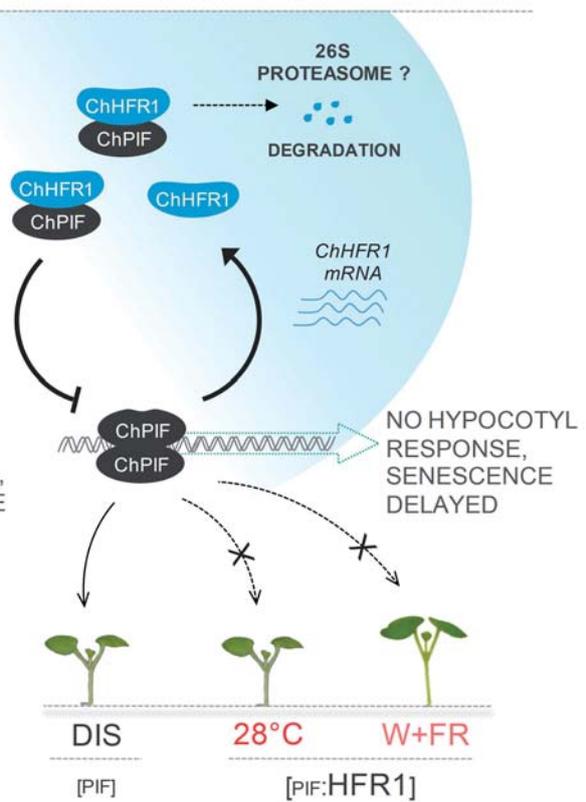
A. thaliana

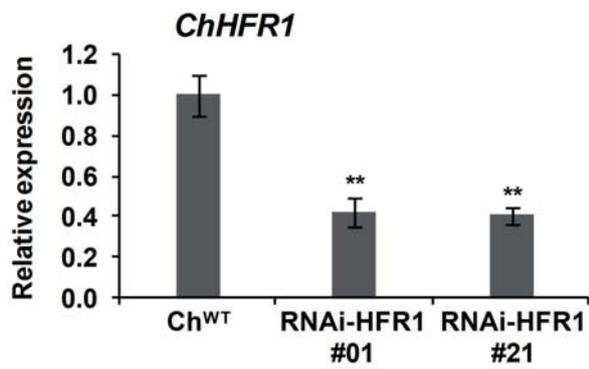
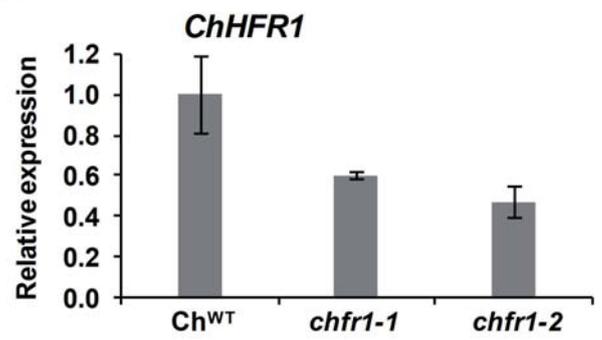
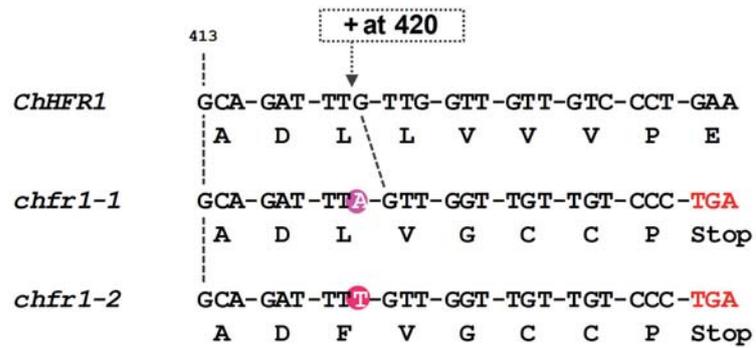
$HFR1 \approx PIFs$



C. hirsuta

$HFR1 \gg PIFs$



A**B****C**

A

SPO88 **SPO89**
AtHFR1 TCA **CCAGCTTCTTCTCCTCA**GTCTCTT CACGGT TTGGTT CCT TAT TTC CCAAGT TTC TTG **GATTTTCTTCC**
ChHFR1 TCG **CCAGCTTCTTCTCCTCA**A TGTCTT TACGGT TTGGTT CCT TGT TTC CCAAGT TTC TTT **GATTTTCTTCC**
 ** . ***** . * **** . ***** . ***** . ***** . ***** . ***** . *****

AtHFR1 **CATGCGATG**AGA
ChHFR1 **CATGCGATG**GGA
 ***** . **

SPO108
AtPIF4 GAT **CCAATACCCTCCAGATGAAGAC**CCATT CGAACC CGACGACTT CTC CTC CCACTT CTT CTC AACCAT GGA
ChPIF4 GAT **CCAATACCCTCCAGATGAAGAC**CCATT TGA TGC CGACGACTT CTC CTC CCACTT CTT CTC AACCGT TAA
 ***** . ** ***** . ***** . ***** . ***** . ***** . ***** . ***** . *****

SPO109
AtPIF4 TCCCTT **CCAGAGACCAACCTCAGAGAC**CGG
ChPIF4 TCCCTT **CCAGAGACCAACCTCAGAGAT**G
 ***** . **

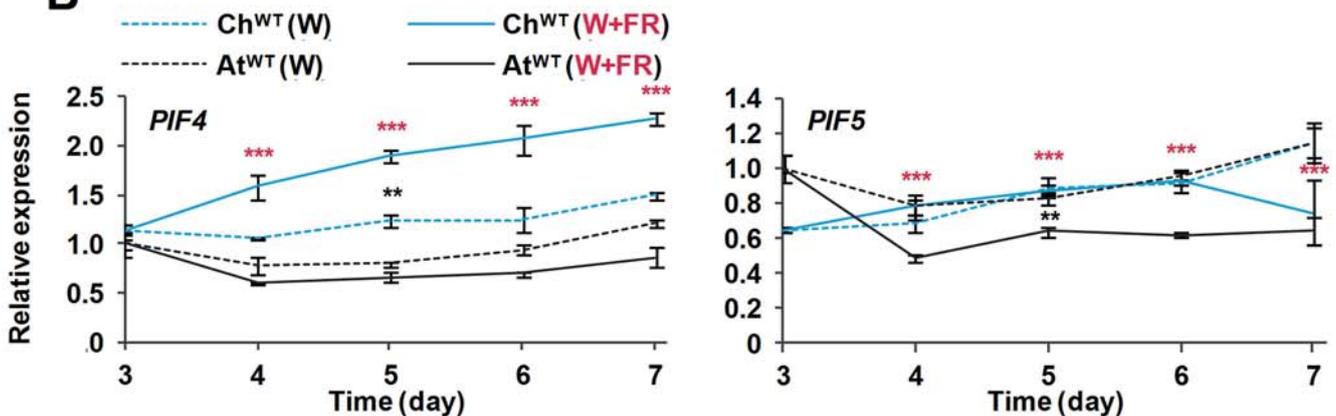
SPO110
AtPIF5 ATA **CATTAATCAGATGGCTATGCA**AAGTCAGAT GCAATT GTC TCAATT CCC GGT TAT GAACCGGTC CGCTCC
ChPIF5 GTT **CATTAATCAGATGGCTATGCA**GAA TCAGAT GCAATT GCC TCAATT TCC GGT TAT GAACCGGTC CAGTGC
 . * ***** . * . ***** . ***** . ***** . ***** . ***** . ***** . * *

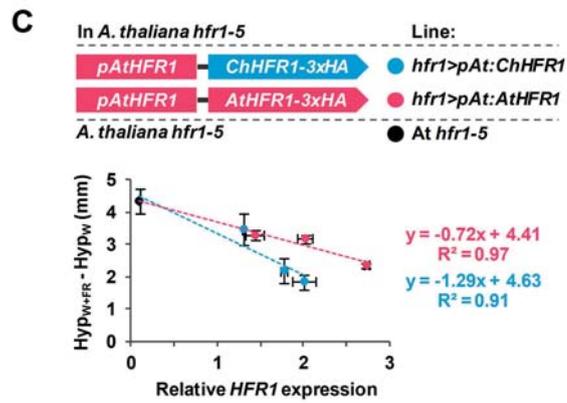
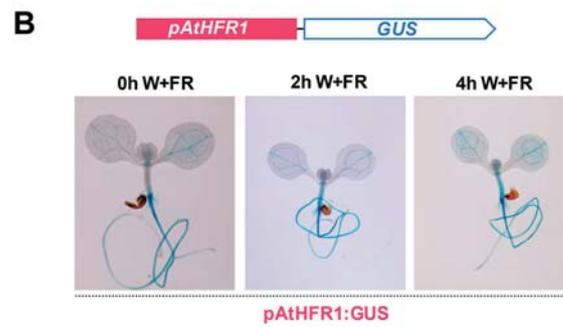
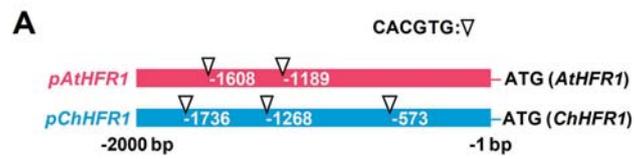
SPO111
AtPIF5 GCAGAACCA TCC CGGTTT AGT A **TGTCAAAA CCCGGTACAGTT** GCA
ChPIF5 ACAAAA TCA TCC CGGTTT AGT A **TGTCAAAA CCCGGTACAGTT** CCA
 . ** . ** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . **

SPO112
AtPIF7 ATT **TCCGCTCTGATCGGAAACTC**AAGATACTGAAGGAGATGAAC AAGAGACAA GAGGAGAAGCAGGTAGAT
ChPIF7 ATA **TCCGCTCTGATCGGAAACTC**AAGATACTGAAGGAGATGAAC AAGAGACAA GAGGAGAAGGTGGAGAT
 ** ***** . ***** . ***** . ***** . ***** . ***** . ***** . ***** . *****

SPO64
AtPIF7 CTA **ATGGACGACGGGACGAGCA**
ChPIF7 CTA **ATGGACGACGGGACGAGCA**GCT

B

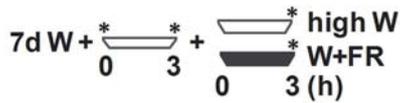




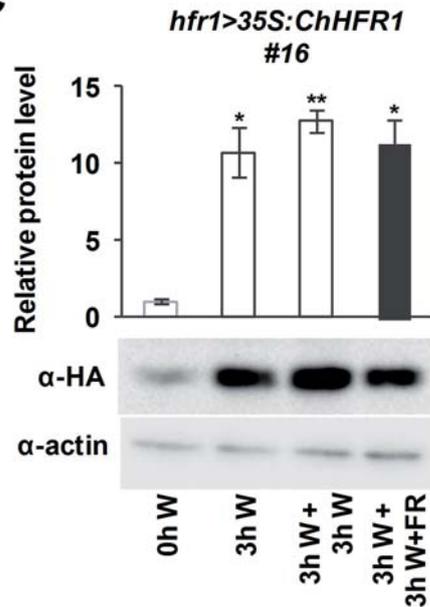
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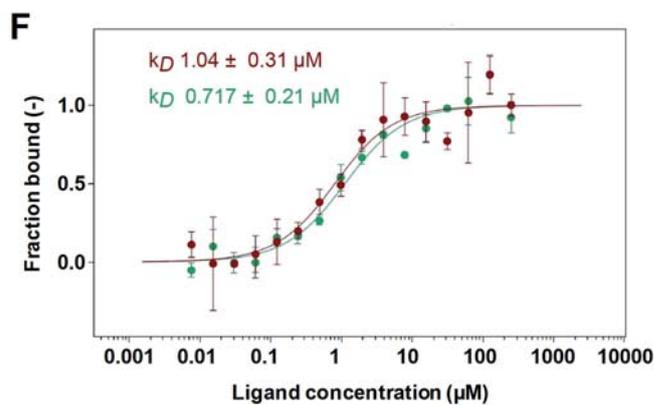
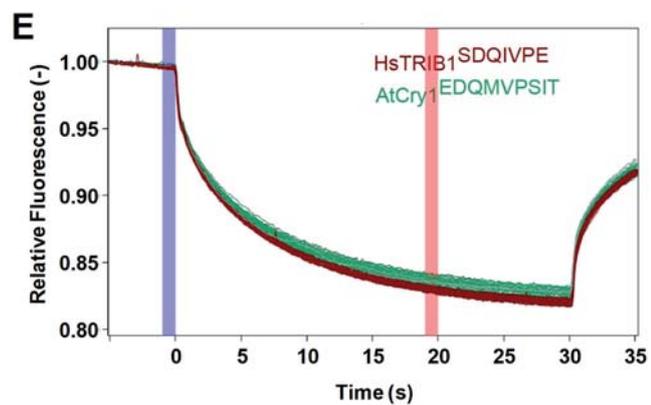
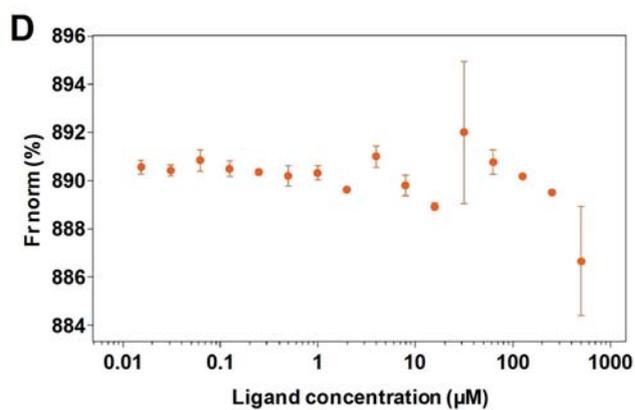
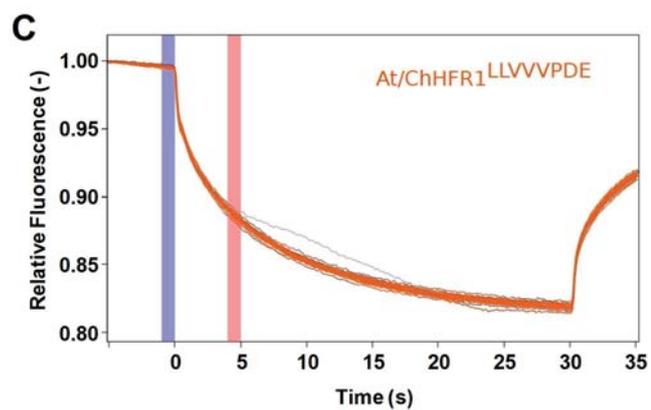
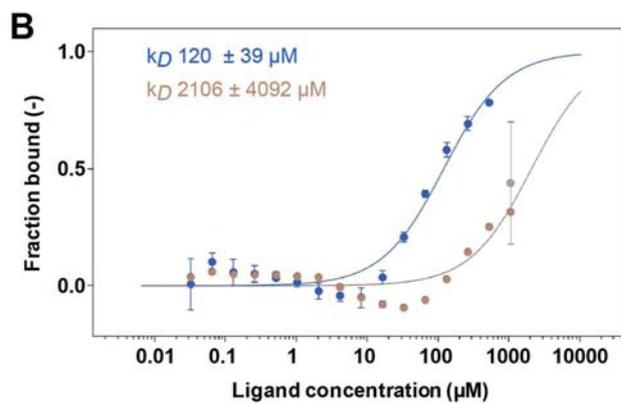
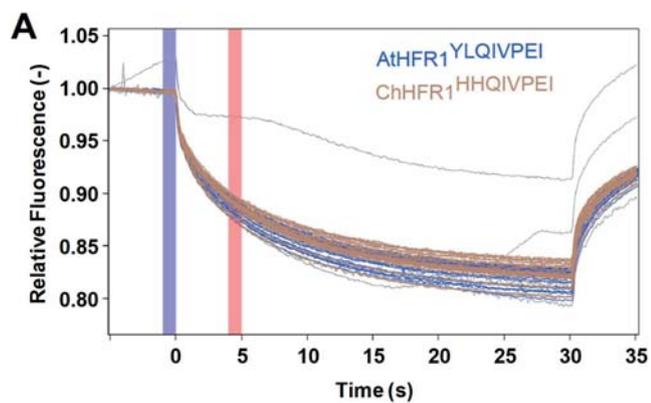
AtHFR1	-----	-----	-----	MSNNQAFMEL	GWRNDVGS LA	VKDQGMMSER	ARSD EDRLIN
ChHFR1	MGF PFSRTNL	KSPK KNSFLK	FSVPDFSLVN	MFSNQDFMEL	GWRNEVE SLA	LKDHG-ITDI	ARSD EDRLIN
				* . ** ****	*****: * **	: **: * :::	*****
AtHFR1	GLK WGYGYFD	HDQT DN-YLQ	IVPEI HKEVE	NAK-EDLLVV	VPDEHSE TDD	HH--HIKDFS	ERSDHRFYLR
ChHFR1	GLK WSYGYFG	HDQT HNDHHQ	IVPEI QKEER	LLKTADLLVV	VPDEHSE TGD	YHHDHIDDYS	DSSDNL CYLR
	*** . **** .	**** . * : *	*****: ** .	* *****	***** ** . *	: * ** . *: *	: * *: ** *
AtHFR1	NKHENPKRRR	IQVLS SDDDES	EEFTREVP SV	TRKGS-KRRR	RDEKMSN KMR	KLQQLVP NCH	KTDKVS VLDK
ChHFR1	NKHENPKRRR	VQIW-SDEES	YGFTREVP SL	TRKGSKRRR	RDDELSN KMS	TLQELLP NCH	KADTVS VLDN
	*****: **	: *: ** : **	*****: *	***** ** *	* *: : ** *	. *: *: ** *	*: * . *****:
AtHFR1	TIEYMKNLQL	QLQMMSTVGV	NPYFLPATLG	FGMHNH-MLT	AMASAHGLNP	ANHMMPSPLI	PALNWPLPPF
ChHFR1	AIEYMKNLQL	QLQVMSAMGM	NPYFP PATLD	FGMSNH YMLT	AMALAHIQNP	AYQKTSSPLI	PASNWPLLPF
	: *****	***: ** : *	*****: *	*** ** ** *	*** ** ** *	* : . *****	** ***** *
AtHFR1	TNISFP HSSS	QSLFLTT SSP	ASSPQSLHGL	VPYFPSFLDF	SSHAMRRL		
ChHFR1	TN-----	-PLFLTTASP	ASSPQCLYGL	VPCFPSFFDF	SSHAMGRL		
	**	. *****: **	*****: * : **	** *****: **	***** **		

B



C





1 **APPENDIX PDF**

2

3 **Adaptation to plant shade relies on rebalancing the transcriptional activity of**
4 **the PIF7-HFR1 regulatory module.**

5

6 Sandi Paulišić, Wenting Qin, Harshul Arora Verasztó, Christiane Then, Benjamin
7 Alary, Fabien Nogue, Miltos Tsiantis, Michael Hothorn, Jaime F. Martínez-García

8

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13 4. Appendix Table S1.

14 5. Appendix Table S2.

15 6. Appendix Table S3.

16 7. Appendix References.

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1 **1. APPENDIX FIGURE S1.**

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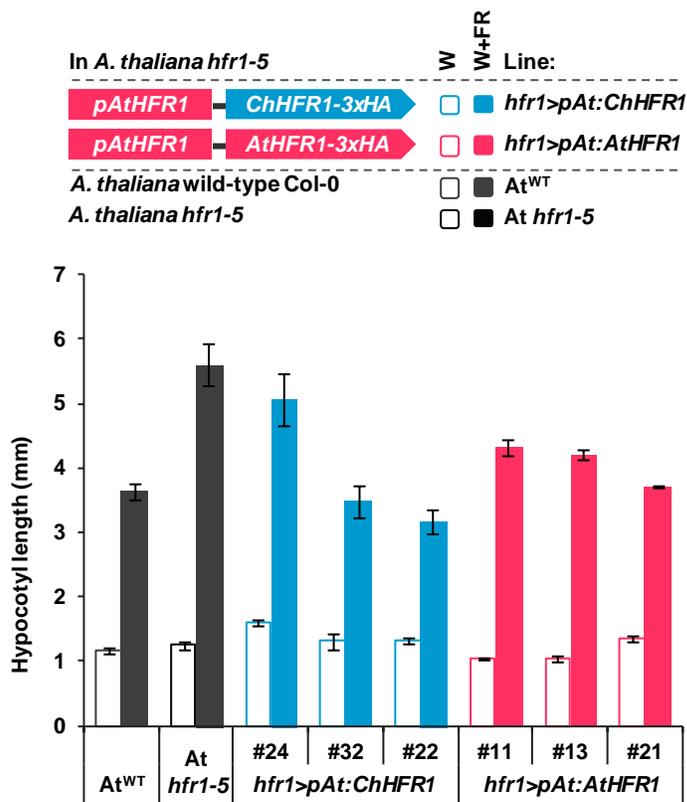
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14 **Appendix Figure S1. ChHFR1 and AtHFR1 complement the *A. thaliana* *hfr1-5***

15 **mutant long hypocotyl phenotype.** Hypocotyl length of the shown lines grown as

16 indicated in Fig 3C. Values were used to generate data on Fig 3C.

17

1 **2. APPENDIX FIGURE S2.**

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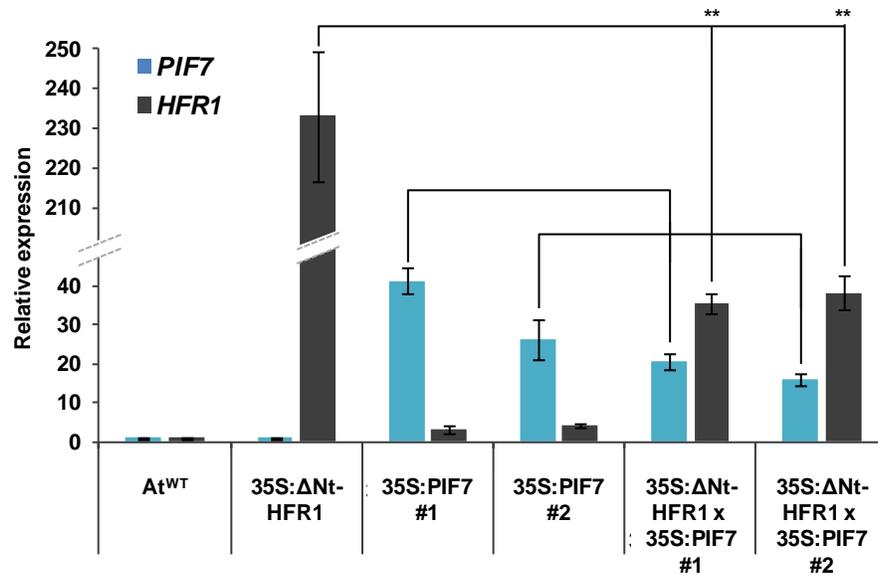
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13 **Appendix Figure S2. Relative expression levels of *AtHFR1* and *AtPIF7* genes**

14 **in transgenic lines overexpressing *GFP-ΔNt-HFR1* and/or *PIF7-CFP*.** Relative

15 expression, normalized to *UBQ10*, was estimated in seedlings grown for 7 days in

16 W. Expression values are the mean ± SE of three independent biological replicates

17 relative to At^{WT}. Asterisks mark significant differences in expression (Student *t*-test:

18 ** p-value <0.01; * p-value <0.05) relative to 35S:*GFP-ΔNt-HFR1-GFP* or

19 35S:*PIF7-CFP* values.

20

21

3. APPENDIX SUPPLEMENTARY METHODS.

Generation of RNAi-HFR1 plants of *C. hirsuta*

To generate an RNAi construct for silencing of the endogenous *ChHFR1*, a fragment of 222 bp was PCR amplified using primers CTO35 + CTO36 (Appendix Table S2) and cDNA of 7-day old *C. hirsuta* seedlings grown 1 h under W+FR. This partial fragment of *ChHFR1* (ptChHFR1) was cloned into pCRII-TOPO (Invitrogen, www.thermofisher.com) to generate pCT17, which was confirmed by sequencing. An *EcoRI* fragment of pCT17 was subcloned into pENTR3C vector (Invitrogen), to create the Gateway entry clone pCT19 (to have ptChHFR1 flanked with attL1 and attL2, attL1<ptChHFR1<attL2). Recombination of pCT19 with the destination vector pB7GWIWG2(I), which contains attR1 and attR2 sites, using Gateway LR Clonase II (Invitrogen), gave pCT33 (35S:attB1<RNAi-ChHFR1<attB2). This plasmid is a binary vector conferring resistance to the herbicide phosphinothricin (PPT) in plants and the antibiotic spectinomycin in bacteria. *Agrobacterium tumefaciens* strain C₅₈C₁ (pGV2260) was transformed with pCT33 by electroporation and colonies were selected on solid YEB medium with rifampicin (100 µg/mL), kanamycin (25 µg/mL) and spectinomycin (100 µg/mL). Wild-type *C. hirsuta* (Ox, Ch^{WT}) plants were transformed by floral dipping and transgenic seedlings were selected on 0.5xGM- medium (Roig-Villanova *et al*, 2006) containing 50 µg/mL PPT. Transgene in seedlings of T1 generation was verified by PCR genotyping using specific primers. Plants homozygous for the transgene were finally used for experiments.

1 **Isolation of *HFR1* mutants of *C. hirsuta***

2 To obtain loss-of-function mutants of *ChHFR1* in *C. hirsuta* (named as *chfr1*)
3 we employed the CRISPR-Cas9 gene editing system (Morineau *et al*, 2017). The
4 guide RNA targeting *ChHFR1* (gRNA_{ChHFR1}, 5'-GTT-GAA-GAC-TGC-AGA-TTT-GT-
5 3') was synthesized to be under the control of the *A. thaliana* *U6* promoter (pU6)
6 sequence and flanked by the Gateway attB1 and attB2 recombination sites (IDT,
7 <https://eu.idtdna.com/pages>) (attB1<pU6:gRNA_{ChHFR1}<attB2). This sequence was
8 recombined with the vector pDONR207 using Gateway BP Clonase II (Invitrogen)
9 to generate the entry vector pSP101 (attL1<pU6:gRNA_{ChHFR1}<attL2). In a
10 recombination reaction of pSP101 with pDE-Cas9 (Fauser *et al*, 2014) using
11 Gateway LR Clonase II, a binary vector pSP102 was created
12 (attB1<pU6:gRNA_{ChHFR1}<attB2, Cas9). This vector, that contains the information to
13 target *ChHFR1*, confers resistance to PPT in plants and spectinomycin in bacteria.
14 *A. tumefaciens* strain C₅₈C₁ (pGV2260) was transformed with pSP102 by
15 electroporation and colonies were selected on solid YEB medium with antibiotics,
16 as indicated before for pCT33. Wild-type *C. hirsuta* (Ox, Ch^{WT}) plants were
17 transformed by floral dipping and resistant transgenic seedlings were selected on
18 0.5xGM- medium containing PPT (30 µg/mL). These T1 seedlings were PCR
19 genotyped using primers MJO27 and MJO28 (Appendix Table S2) to detect the
20 presence of the transgene. In the following T2 generation, a total of six seedlings
21 with a *sis* phenotype from 1 independent transgenic line were selected and grown
22 to maturity. An *HFR1* fragment of 664 bp around the gRNA_{ChHFR1} target sequence
23 was amplified by PCR from gDNA of each plant using primers CTO29 + CTO36
24 (Appendix Table S2). Sequencing of these fragments indicated the presence of

1 mutations in the *ChHFR1* gene. Descendants of these plants (T3 generation) were
2 reselected in shade and sequenced to confirm the unambiguous presence of the
3 mutated *chfr1* alleles. In the T4 generation, seedlings sensitive to PPT (indicating
4 the loss of T-DNA insertion) were selected, which resulted in the isolation of the
5 *chfr1-1* and *chfr1-2* mutant allele lines (Fig EV1). The wild-type and these mutant
6 alleles were genotyped by PCR using primers SPO104 + SPO107 (for *ChPIF7*),
7 SPO105 + SPO107 (for *chfr1-1*) and SPO106 + SPO107 (for *chfr1-2*) (Appendix
8 Table S2).

9

10 **Generation of *A. thaliana hfr1-5* transgenic lines expressing *AtHFR1* or** 11 ***ChHFR1* under the control of different promoters**

12 We amplified a 2 kbp fragment of *AtHFR1* promoter starting immediately
13 before the ATG of *AtHFR1* gene using gDNA of *A. thaliana* wild-type Col-0 (*At*^{WT})
14 as a template and primers SPO26 + SPO27 (Appendix Table S2). This fragment
15 was subcloned into pCRII-TOPO to generate pSP51. From the different clones
16 analyzed, the best one was pSP51.10, with three 1 bp-deletions in the amplified
17 region, none affecting the G-boxes, known to be necessary for PIF binding.

18 *AtHFR1* coding sequence was amplified from pJB30 (Galstyan *et al*, 2011)
19 using primers RO25 + SPO30 (Appendix Table S2), which removed the stop codon
20 and introduced a *XhoI* site at the N-terminal site. After subcloning this fragment into
21 pCRII-TOPO, which gave pSP54 (*AtHFR1*), the insert was sequenced to confirm
22 its identity. The 3xHA fragment was amplified from plasmid pEN-R2-3xHA-L3
23 (Karimi *et al*, 2007) and primers SPO31 (which added a *SalI* site) + SPO32 (which
24 added a *XhoI* site, Appendix Table S2). This fragment was subcloned into pCRII-

1 TOPO to generate pSP55 (3xHA), whose insert was sequenced to confirm its
2 identity. A *Bam*HI-*Xho*I fragment of pSP54 was subcloned into pSP55 digested
3 with *Bam*HI and *Sal*I to generate pSP57 (*AtHFR1*-3xHA). A *Bam*HI-*Xho*I fragment
4 of pSP57 was subcloned into the same sites of pENTR3C vector which gave
5 pSP59. This plasmid contained *AtHFR1*-3xHA, with an extra *Xba*I site in the C-
6 terminus end, flanked with attL1 and attL2 sites (attL1<*AtHFR1*-3xHA^{*Xba*I}<attL2).
7 *Xba*I restriction site in pSP59 was removed by filling the site with Klenow enzyme
8 after digestion, and religation to generate pSP84 (attL1<*AtHFR1*-3xHA<attL2).
9 Recombination of pSP84 with the binary vector pIR101 (attR1<*ccdB*<attR2)
10 (Molina-Contreras *et al*, 2019) (using Gateway LR Clonase II) resulted in pSP88
11 (attB1<*AtHFR1*-3xHA<attB2). An *Xba*I fragment of pSP51 was subcloned into the
12 same site of pSP88 which gave pSP90 (*pAtHFR1*:attB1<*AtHFR1*-3xHA<attB2).
13 This binary vector confers resistance to spectinomycin in bacteria and PPT in
14 plants.

15 *ChHFR1* CDS was amplified using cDNA from wild-type *C. hirsuta* (Ox,
16 Ch^{WT}) seedlings and primers SPO28 + SPO29 (Appendix Table S2), which
17 removed the stop codon and introduced a *Xho*I site. This PCR product was
18 subcloned into pCRII-TOPO to generate pSP53 (*ChHFR1*). Selected colonies were
19 sequenced to confirm their identity. A *Bam*HI-*Xho*I fragment of pSP53 was
20 subcloned into pSP55 digested with *Bam*HI-*Sal*I to generate pSP56 (*ChHFR1*-
21 3xHA). A *Bam*HI-*Xho*I fragment of pSP56 was subcloned into the same site of
22 pENTR3C vector, which gave pSP58. This plasmid contained *ChHFR1*-3xHA, with
23 an *Xba*I site in the C-terminus end, flanked with attL1 and attL2 sites
24 (attL1<*ChHFR1*-3xHA^{*Xba*I}<attL2). *Xba*I restriction site in pSP58 was removed by

1 filling the site with Klenow enzyme after digestion, and religation to generate
2 pSP83 (attL1<*ChHFR1*-3xHA<attL2). Recombination of pSP83 with the binary
3 vector pIR101 using Gateway LR Clonase II resulted in pSP87 (attB1<*ChHFR1*-
4 3xHA<attB2). An *Xba*I fragment of pSP51 was subcloned into the same site of
5 pSP87 which gave pSP89 (*pAtHFR1*:attB1<*ChHFR1*-3xHA<attB2). This binary
6 vector confers resistance to spectinomycin in bacteria and PPT in plants.

7 To overexpress *ChHFR1*, a *Bam*HI-*Xho*I fragment of pSP58 was subcloned
8 into the *Bam*HI-*Sal*I digested pCAMBIA1300 based pCS14 (Sorin *et al*, 2009) to
9 generate pSP81 (35S:*ChHFR1*-3xHA). This binary vector confers resistance to
10 kanamycin in bacteria and hygromycin in plants.

11 *A. thaliana hfr1-5* plants were transformed with pSP81, pSP89 and pSP90,
12 as previously described. Transgenic seedlings were selected on 0.5xGM- medium
13 with PPT (15 µg/mL) or hygromycin (30 µg/mL), verified by PCR genotyping using
14 specific primers. Homozygous transgenic plants with 1 T-DNA insertion were finally
15 used for experiments.

16

17 **Generation of constructs for transient expression in *N. benthamiana* leaves**

18 To overexpress *ChHFR1* and *AtHFR1* in *N. benthamiana*, a Gateway vector
19 was created using pCAMBIA1302 (35S:*mGFP5*) as a backbone. An *Nsi*I-*Hind*III
20 fragment of pEarlyGate 100 (35S:attR1<*ccdB*<attR2) (Earley *et al*, 2006) was
21 subcloned into pCAMBIA1302 digested with *Pst*I-*Hind*III, which gave pSP135
22 (35S:attR1<*ccdB*<attR2, 35S:*mGFP5*). Recombination of pSP58 and pSP59 (both
23 linearized with *Nhe*I) with the binary vector pSP135 using Gateway LR Clonase II
24 gave pSP141 (35S:attB1<*ChHFR1*-3xHA<attB2, 35S:*mGFP5*) and pSP142

1 (35S:attB1<AtHFR1-3xHA<attB2, 35S:mGFP5), respectively. These two binary
2 vectors also overexpress mGFP5 and confer resistance to kanamycin in bacteria.

3 To generate constructs overexpressing *ChHFR1* and *AtHFR1* with the
4 COP1 binding domains exchanged (Fig 5C), we employed a PCR-based
5 mutagenesis. Using pSP90 as a template, a fragment of 205 bp was amplified with
6 RO25 and SPO126 primers, and a larger fragment of 821 bp was amplified using
7 SPO127 and SPO32 primers. Both PCR fragments were used to amplify *AtHFR1*
8 with the COP1 binding domain from *ChHFR1* (named in here as *AtHFR1**). The
9 resulting fragment was subcloned into pCR8/GW/TOPO (Invitrogen) to generate
10 pSP130, which was confirmed by sequencing. Using pSP89 as a template, a
11 fragment of 291 bp was amplified with SPO28 and SPO128 primers, and a
12 fragment of 800 bp was amplified using SPO129 and SPO32. Both PCR fragments
13 were used to amplify *ChHFR1* with the COP1 binding domain from *AtHFR1*
14 (named in here as *ChHFR1**). The resulting fragment was subcloned into
15 pCR8/GW/TOPO to generate pSP131, which was confirmed by sequencing.
16 Recombination of pSP130 and pSP131 with the binary vector pSP135 using
17 Gateway LR Clonase II gave pSP132 (35S:attB1<*AtHFR1**-3xHA<attB2,
18 35S:mGFP5) and pSP133 (35S:attB1<*ChHFR1**-3xHA<attB2, 35S:mGFP5),
19 respectively. Both vectors also overexpress mGFP5 and confer resistance to
20 kanamycin in bacteria.

21 *N. benthamiana* plants were agroinfiltrated with the *A. tumefaciens* (strain
22 GV3101) transformed with pSP141, pSP142, pSP132 or pSP133, and the same
23 strain expressing the HcPro protein (Vilela *et al*, 2013) and kept in the greenhouse
24 under long-day photoperiods. Samples were taken 3 days after agroinfiltration.

1

2 **Generation of constructs for the Yeast 2 Hybrid (Y2H) assays**

3 *AtPIF7* CDS was amplified using cDNA of *A. thaliana* wild-type Col-0 (*At*^{WT})
4 seedlings and primers JO414 + JO415 (Appendix Table S2), which removed the
5 STOP codon and introduced a *Xho*I site. This PCR product was subcloned into
6 pCRII-TOPO to generate pRA1 (*AtPIF7*). The insert was sequenced to confirm its
7 identity. A *Xho*I fragment of pRA1 was subcloned into pSP55 digested with *Sal*I to
8 generate pRA2 (*AtPIF7-3xHA*). An *Eco*RI fragment of pRA2 was subcloned into
9 the same site of pENTR3C entry vector which gave pRA3 (*attL1<AtPIF7-*
10 *3xHA<attL2*). This PIF7-3xHA had a stop codon immediately before the ATG,
11 which prevented from cloning it in frame with the yeast derived proteins. Therefore,
12 the *PIF7-3xHA* gene was PCR amplified using pRA3 as a DNA template and
13 primers BAO4 + BAO5 (Appendix Table S2) to add *attB1* and *attB2* sequences
14 (*attB1<AtPIF7-3xHA<attB2*). This fragment was recombined with pDONR207 using
15 Gateway BP Clonase II to obtain pBA7 (*attL1<AtPIF7-3xHA<attL2*). The insert was
16 sequenced to confirm its identity. In a recombination reaction of pBA7 and
17 pGBKT7-GW (Chini *et al*, 2009) which contained the Gal4 DNA-binding domain
18 (BD, *attR1<ccdB<attL2*; it confers Trp auxotrophy), and pBA7 and pGADT7-GW
19 (Chini *et al*, 2009) which contained the Gal4 activation domain (AD,
20 *attR1<ccdB<attL2*; it confers Leu auxotrophy), using Gateway LR Clonase II, pBA10
21 (BD-*attB1<AtPIF7-3xHA<attB2*) and pBA11 (AD-*attB1<AtPIF7-3xHA<attB2*) were
22 obtained. These plasmids allowed expressing the fusion BD-PIF7-3xHA or AD-
23 PIF7-3xHA proteins under the *ADH1* promoter in yeast, respectively.

24

1 **Protein expression and purification for the MST experiments**

2 Sf9 cells were cultured in HyClone SFX-Insect Cell Culture Media. The
3 codon optimized COP1 gene (residues 349-765 corresponding to the WD40
4 domain) for expression in Sf9 cells, was PCR amplified and cloned into a modified
5 pFastBac (Geneva Biotech) insect cell expression vector using Gibson assembly
6 (Gibson *et al*, 2009). The final construct contained a tandem N-terminal His10-
7 Twin-Strep-tags, a TEV (tobacco etch virus protease) cleavage site prior to COP1
8 WD40 coding sequence in the pFastBac vector. This construct was transformed
9 into DH10MultiBac cells (Geneva Biotech). White colonies, implying successful
10 recombination, were selected and bacmids were purified by the alkaline lysis
11 method. Sf9 cells were transfected with the bacmid using Profectin (AB Vector).
12 eYFP-positive cells (P0) were observed after 1 week and subjected to two rounds
13 of viral amplification. Sf9 cells at a density of $1-2 \times 10^6$ cells·ml⁻¹ were infected with
14 amplified P2 virus at a Multiplicity of infection (MOI) between 2 to 3. Infected Sf9
15 cells were grown for 72 h at 28°C and 110 rpm. The cell pellet was then harvested
16 by centrifugation at 2000 x g for 15 min, pellets were flash frozen and stored at –
17 20°C.

18 Pellets from one liter of Sf9 cell culture were dissolved in 25 ml of buffer A
19 (20 mM HEPES pH 7.5, 300 mM NaCl, 2 mM β-ME), supplemented with 10% [v/v]
20 glycerol, a pinch of DNase, and 1 Roche cOmplete™ protease inhibitor tablet.
21 Dissolved pellets were lysed by sonication and centrifuged at 60,000 x g for 45
22 minutes at 4°C. The supernatant was consecutively filtered through 2-µm 1-µm and
23 0.45-µm filters prior to loading onto Ni²⁺-affinity column (HisTrap excel, GE
24 Healthcare). After the loading, Ni²⁺-affinity column was washed with buffer A and

1 eluted directly onto a coupled Strep-Tactin Superflow XT column (IBA) using buffer
2 B (20 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 2 mM β -ME). The
3 Strep-Tactin column was washed with buffer A and COP1 was eluted with 1x
4 Buffer BXT (IBA) supplemented with 2 mM β -ME. It was cleaved overnight at 4°C
5 with TEV protease and subsequently purified from the protease and affinity tag by
6 a second Ni²⁺ affinity column. COP1 WD40 was concentrated to 10 μ M and was
7 labeled immediately.

8

9 **GUS lines**

10 Transgenic lines expressing GUS were based on a modified pIR101 plasmid
11 (Molina-Contreras *et al*, 2019) which contains the reporter *GUS* gene in a
12 promoterless context (attB1<*GUS*<attB2). *Xba*I fragment of pSP51 was subcloned
13 into the same site of modified pIR101 to give pSP86 (*pAtHFR1*:attB1<*GUS*<attB2).
14 This binary vector confers resistance to spectinomycin in bacteria and PPT in
15 plants. *A. thaliana* wild-type Col-0 (At^{WT}) plants were transformed with this
16 construct as described previously.

17

18 **GUS staining**

19 Histochemical GUS assays were done as described (Roig-Villanova *et al*,
20 2006), incubating seedlings at 37°C without ferricyanide/ferrocyanide.

21

22

23

1 **4. APPENDIX TABLE S1. Primers used for gene expression analyses.** Primers
2 BO40 and BO41 for amplifying *UBQ10* (Sorin *et al*, 2009), SPO102 and SPO103
3 (*AtEF1 α* and *ChEF1 α*), SPO113 and SPO114 (*AtSPC25* and *ChSPC25*), and
4 SPO115 and SPO116 (*AtYLS8* and *ChYLS8*) have been described before (Molina-
5 Contreras *et al*, 2019).

6

Gene	Primer name	Sequence (5' – 3')
<i>ChEF1α</i>	CTO9 (F)	GGCCGATTGTGCTGTCCTTA
	CTO10 (R)	TCACGGGTCTGACCATCCTTA
<i>ChHFR1</i>	CTO13 (F)	CGGCGTCGTGTCCAGATC
	CTO14 (R)	TGAACCTTTTCGCGTCAGTG
<i>ChPIL1</i>	CTO17 (F)	GAAGACCCCAAACAACGGTT
	CTO18 (R)	CCCTCATCGTACTCGGTCTCA
<i>ChYUC8</i>	CTO51 (F)	TTACGCCGGGAAAAAAGTTCT
	CTO52 (R)	GCGAAATGGTTGGCTAGGTC
<i>ChXTR7</i>	CTO69 (F)	TGGTGTTCTTTCCCAAAAAA
	CTO70 (R)	CCACCTCTCGTAGCCCAATC
<i>AtHFR1, ChHFR1</i>	SPO88 (F)	CCAGCTTCTTCTCCTCA
	SPO89 (R)	CATCGCATGGGAAGAAAAATC
<i>AtPIF4, ChPIF4</i>	SPO108 (F)	CCAATACCCTCCAGATGAAGAC
	SPO109 (R)	TCTCTGAGGTTGGTCTCTGG
<i>AtPIF5, ChPIF5</i>	SPO110 (F)	CATTAATCAGATGGCTATGCA
	SPO111 (R)	AACTGTACCGGGTTTTGACA
<i>AtPIF7, ChPIF7</i>	SPO112 (F)	TCCGCTCTGGATCGGAAACTC
	SPO64 (R)	TGCTCGTCCCCGTCGTCCAT
	SPO142 (R)	TCTCATCCTCTGGTTTATCC

7

8

1 **5. APPENDIX TABLE S2. Primers used for cloning or/and genotyping.** Primer

2 RO25 (Roig-Villanova *et al*, 2007) has been described before.

3

Gene	Primer name	Sequence (5' – 3')
<i>ChHFR1</i> WT	SPO104 (F)	CTGTTGAAGACTGCAGATTTG
	SPO107 (R)	CCTAAGGCAAGATTCTTTGAA
<i>chfr1-1</i> <i>chfr1-2</i>	SPO105 (F)	CTGTTGAAGACTGCAGATTA
	SPO106 (F)	CTGTTGAAGACTGCAGATTTT
attB1 attB2	MJO27 (F)	GGGACAAGTTTGTACAAAAAAGCAGGCT
	MJO28 (R)	GGGACCACTTTGTACAAGAAAGCTGGGT
<i>pAtHFR1</i>	SPO26 (F)	GCTCTAGAGTAAAGATAACGTTCT
	SPO27 (R)	GCTCTAGAGTTAGTTAAAGAGATA
<i>ChHFR1</i>	SPO28 (F)	CCATGGGTTTTCCATTTTCTCG
	SPO29 (R)	GGCTCGAGGAGTCTTCCCATCGCA
<i>ChHFR1</i>	CTO29 (F)	ATGATCATCATCAAATTGTTCT
<i>AtHFR1</i>	RO25 (F)	AACATGTCGAATAATCAAGCTTTTCATG
	SPO30 (R)	GGCTCGAGTAGTCTTCTCATCGCA
3xHA	SPO31 (F)	CCGTCGACGGTGGAGGCGGTTTCAG
	SPO32 (R)	GGCTCGAGTCAAGCGTAATCTGGA
RNAi- <i>ChHFR1</i>	CTO35 (F)	CAAACACATAATGATCATCATC
	CTO36 (R)	ATCACTCCAGATCTGGACACGA
<i>ChHFR1</i> *	SPO128 (R)	CTTCTTTATGAATCTCTGGAACAATCTGAAGA TAATTATCTGTTTGATCATGACCAAAA
	SPO129 (F)	GTTCCAGAGATTCATAAAGAAGTAGAAAATGC GAAGGAGGATTTGTTGGTTGTTGTC
<i>AtHFR1</i> *	SPO126 (R)	CTTTCTGAATCTCTGGAACAATTTGATGATGA TCATTATGAGTTTGATCATGATCAAAG
	SPO127 (F)	GTTCCAGAGATTCAGAAAGAAGAACGACTGTT GAAGACTGCAGATTTATTGGTTGTTGTC
<i>AtPIF7</i>	JO414 (F)	TAACACATGTCGAATTATGGAG
	JO415 (R)	GGCTCGAGATCTCTTTTCTCATGATTC
<i>AtPIF7</i> + attB1	BAO4 (F)	GGGACAAGTTTGTACAAAAAAGCAGGCTAC ATGTCGAATTATGGAGTTAAAG
<i>AtPIF7</i> + attB2	BAO5 (R)	GGGACCACTTTGTACAAGAAAGCTGGGTGT CAAGCGTAATCTGGAACGTC

4

5

1 **6. APPENDIX TABLE S3. Synthetic peptides used for microscale**
2 **thermophoresis (MST) experiments.** The peptides were acetylated (Ac) at the N-
3 terminal and aminated (-NH₂) at the C-terminal. The C-terminal tyrosine (Y)
4 residue was added to quantify peptide concentrations via absorbance at 280 nm.

5

Name	Sequence	Company
AtHFR1 VP	Ac-YLQIVPEI-NH ₂	Genescript
ChHFR1 VP	Ac-HHQIVPEIY-NH ₂	Genescript
At/ChHFR1 VP	Ac-LLVVVPDEY-NH ₂	Genescript
AtCRY1	Ac-EDQMVPSITY-NH ₂	Peptide Synthesis Laboratory
HsTRIB1	Ac-SDQIVPEY-NH ₂	Peptide Synthesis Laboratory

6

7

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