Title: Integration of Phytochrome and Cryptochrome Signals Determines Plant Growth during Competition for Light.

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SUMMARY

Plants in dense vegetation perceive their neighbors primarily through changes in light quality. Initially, the ratio between red (R) and far-red (FR) light decreases due to reflection of FR by plant tissue well before shading occurs. Perception of low R:FR by the phytochrome photoreceptors induces the shade avoidance response [1], of which accelerated elongation growth of leaf-bearing organs is an important feature. Low R:FR-induced phytochrome inactivation leads to accumulation and activation of the transcription factors PHYTOCHROME INTERACTING FACTOR (PIF) 4, 5, and 7 and subsequent expression of their growth-mediating targets [2, 3]. When true shading occurs, transmitted light is especially depleted in red and blue (B) wavelengths due to absorption by chlorophyll [4]. Although reduction of blue wavelengths alone does not occur in nature, long-term exposure to low B light induces a shade avoidance-like response that is dependent on the cryptochrome photoreceptors, and the transcription factors PIF4 and PIF5 [5-7]. Here, we show in *Arabidopsis thaliana* that low B in combination with low R:FR enhances petiole elongation similar to vegetation shade, providing functional context for a low B response in plant competition. Low B potentiates the low R:FR response through PIF4, PIF5 and PIF7 and involves increased PIF5 abundance and transcriptional changes. Low B attenuates a low R:FR-induced negative feedback loop through reduced gene expression of negative regulators and reduced HFR1 levels. The enhanced response to combined phytochrome and cryptochrome inactivation shows how multiple light cues can be integrated to fine-tune the plant's response to a changing environment.

HIGHLIGHTS

- Blue light depletion combined with low R:FR mimics vegetation shade.
- Low blue light perception enhances the low R:FR response through PIFs and COP1.
- Low blue light perception counteracts a low R:FR-induced negative feedback loop.

KEYWORDS

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Reviewer #1: 'The authors have addressed my concerns and produced a much-improved manuscript. Could they please check whether, in figure 4G, the interaction between phy and COP1 is intentional in the low B condition? As far as I am aware, the involvement of COP1 in PIF degradation has only been demonstrated for PIF1.'

What we meant to depict in the low B condition in Figure 4G are in fact two separate events: 1. interaction between phytochrome and SPA proteins, which leads to inhibition of COP1 activity, and 2. interaction between phytochrome and PIF proteins, which leads to degradation of PIFs. We had not realized ourselves that from our cartoon it could be inferred that these two events are related, such that phytochrome-dependent PIF degradation would be mediated by the COP1/SPA complex. We thank the reviewer for pointing this out, and have changed this panel to avoid confusion. We have now added some space between the phytochrome symbol and the COP1/SPA complex. In fact, this makes the phytochrome-PIF interaction stand out even more, which is really at the core of our model.

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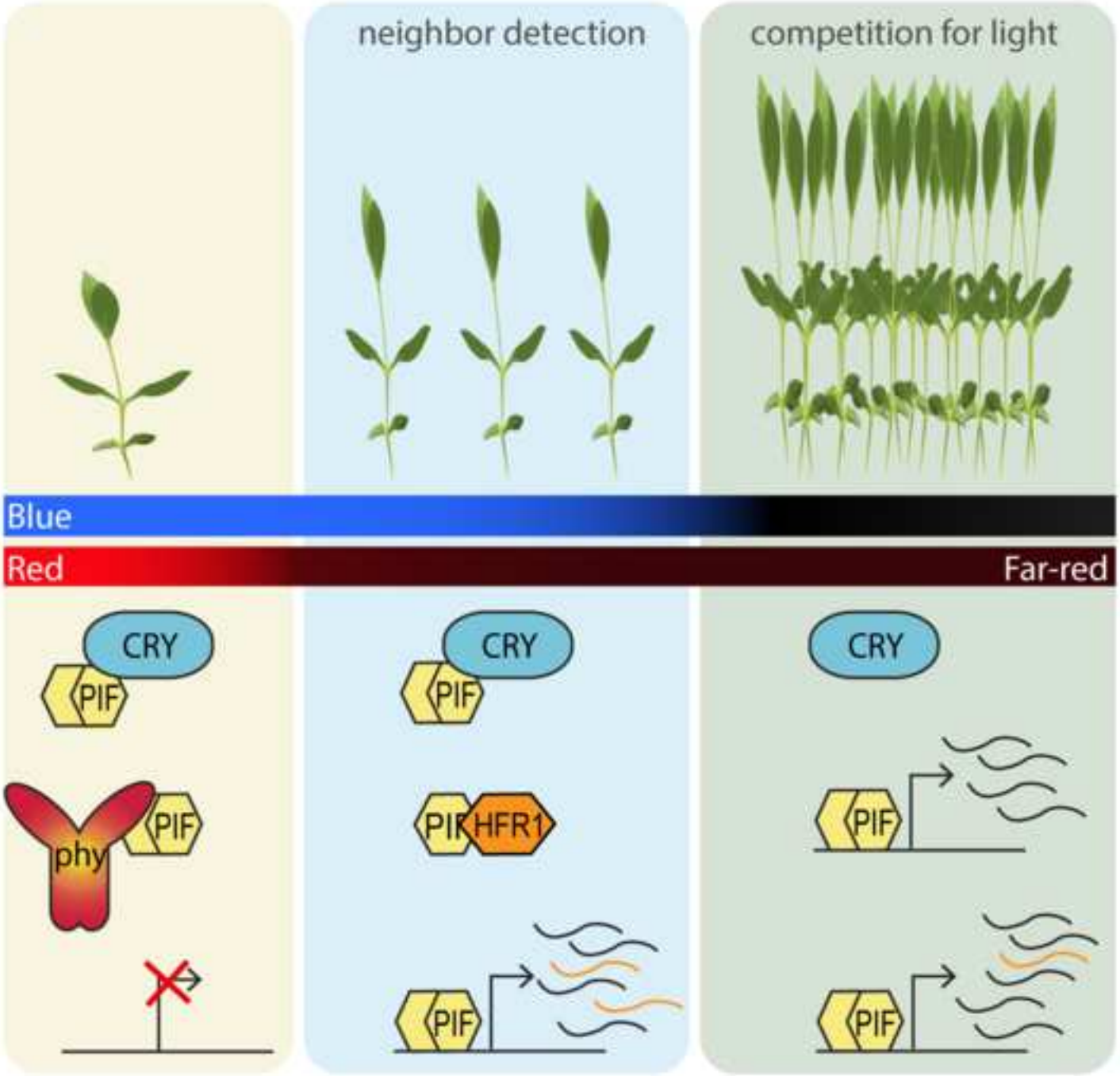
Graphical Abstract



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Plants in dense vegetation perceive their neighbors primarily through changes in light quality. Initially, the ratio between red (R) and far-red (FR) light decreases due to reflection of FR by plant tissue well before shading occurs. Perception of low R:FR by the phytochrome photoreceptors induces the shade avoidance response [1], of which accelerated elongation growth of leaf-bearing organs is an important feature. Low R:FR-induced phytochrome inactivation leads to accumulation and activation of the transcription factors PHYTOCHROME INTERACTING FACTOR (PIF) 4, 5, and 7 and subsequent expression of their growth-mediating targets [2, 3]. When true shading occurs, transmitted light is especially depleted in red and blue (B) wavelengths due to absorption by chlorophyll [4]. Although reduction of blue wavelengths alone does not occur in nature, long-term exposure to low B light induces a shade avoidance-like response that is dependent on the cryptochrome photoreceptors, and the transcription factors PIF4 and PIF5 [5-7]. Here, we show in *Arabidopsis thaliana* that low B in combination with low R:FR enhances petiole elongation similar to vegetation shade, providing functional context for a low B response in plant competition. Low B potentiates the low R:FR response through PIF4, PIF5 and PIF7 and involves increased PIF5 abundance and transcriptional changes. Low B attenuates a low R:FR-induced negative feedback loop through reduced gene expression of negative regulators and reduced HFR1 levels. The enhanced response to combined phytochrome and cryptochrome inactivation shows how multiple light cues can be integrated to fine-tune the plant's response to a changing environment.

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RESULTS AND DISCUSSION

Low B enhances the low R:FR-induced petiole response in a PIF-dependent manner

As reduction of specifically blue light (low B) does not naturally occur, we studied whether low B acts in concert with other shade signals. Adult plants exposed to 24 h of low B displayed only a trend towards slight petiole elongation (Figure 1A). Interestingly, combination of low B with low R:FR induced a stronger elongation response than low R:FR treatment alone, which was not further affected by reduced light intensity (green filter) (Figure 1A, light conditions in Supplemental Experimental Procedures). This suggests that low B is perceived as a signal of increasing competition in the context of shade avoidance.

The phytochrome mutant *phyB* showed an exaggerated low B response, which was not enhanced in the combination with low R:FR (Figure 1B). Cryptochrome (cry) mutants similarly showed a compromised response to the combined light treatment (Figure 1B), but retained a low R:FR response. These data indicate that the photoreceptors phyB, and both cry1 and cry2 respectively mediate the R:FR and B signaling of the interaction.

PIF4, PIF5 and PIF7 are key regulators in low R:FR signaling [2, 3] and PIF4 and PIF5 play a role in low B responses [5, 7]. Petiole elongation in the different light treatments was abolished in the *pif4pif5pif7* mutant, but not in *pif4pif5* and *pif7* (Figures S1 and 1C), indicating that the enhanced response to low R:FR + low B depends on combined action of the PIF4, PIF5 and PIF7 transcription factors. Three direct PIF target genes showed an expression pattern consistent with enhanced elongation in low R:FR + low B. Genes encoding the positive regulators ATHB2 and IAA19 were more expressed in low R:FR + low B than in low R:FR alone, while expression of the negative regulator-encoding *HFR1* was reduced in the combined light treatment (Figure 1D-F). Together, these results show that simultaneous low B perception affects both low R:FR-induced gene expression and elongation.

Wild-type plants grown at high density (canopy) experience a reduction in R:FR, B and light intensity over time and show a strong petiole elongation response ([8], Figure 2A, B). In contrast, petiole elongation was largely reduced in *pif4pif5pif7* canopies (Figure 2A, B), confirming the importance of the PIF transcription factors in plant competition. To explore the transcriptional interaction between low R:FR and low B more broadly, we studied the genome-wide transcript profile of petioles from single-grown plants subjected to the different

light treatments. We compared these with the transcript profile of canopy-grown plants of the same age. Of the light treatments, low R:FR single treatment overlapped best with low R:FR + low B treatment, both in number of differentially regulated genes (DEGs) and in direction of regulation (Figures 2C, D). In addition to light quality changes, canopy plants experienced a changed microenvironment, including reduced light intensity and mechanical stress [8]. These factors likely explain the larger number of DEGs in canopy-grown plants (Figure 2C). The combined low R:FR + low B treatment showed the best overlap in expression with the canopy profile, (Figures 2C, D and S2A, B), suggesting that integration of low R:FR and low B signals indeed occurs in competition for light.

Approximately 60% of DEGs in each treatment was previously identified as PIF4/PIF5 targets in low R:FR-treated [9] or low B-treated [7] seedlings (Figure 2E). This indicates that there was a larger and partly unique set of PIF targets among the larger number of DEGs in the low R:FR + low B and canopy treatments (Figure 2E). R-activated phyB mediates PIF4 and PIF5 degradation and PIF7 inactivation [2, 3], and B-activated cry1 binds to PIF4 and PIF5 and inhibits PIF4 activity [7, 10]. Combined cry and phy inactivation may thus relieve inhibition of PIF abundance and activity, leading to increased regulation of PIF targets in shade.

Auxin and brassinosteroid positively regulate elongation in low R:FR + low B

GO terms for the plant hormones auxin and brassinosteroid (BR) were particularly enriched in the low R:FR + low B and canopy transcriptomes (Table S1). Auxin regulates both low R:FR and low B responses, and several auxin-related genes are direct PIF targets [3, 6, 9, 11]. BR, together with auxin, is also implicated in low R:FR and low B responses [5, 6, 12, 13, 14]. The transcription factors ARF6 (auxin-related), BZR1 (BR-related), and PIF4 directly interact and cooperatively induce genes involved in hypocotyl elongation [15], suggesting auxin and BR together can stimulate PIF-dependent growth. To study whether auxin and BR mediate enhanced elongation in low R:FR + low B, we used a seedling hypocotyl assay to accelerate the experimental cycle and facilitate pharmacological manipulation. Although hypocotyls strongly responded to low B, they elongated more in combined R:FR and low B similar to petioles (Figure S3A). This response was dependent on PIF4, PIF5 and PIF7, with a more prominent role for PIF7 in low R:FR-induced elongation in hypocotyls than in petioles (Figure

S3A, Figure S1B). Simultaneous impairment of auxin and BR pathways was achieved by combining mutants with chemical inhibitors. Inhibition of both hormone pathways reduced the elongation response to low R:FR + low B more than inhibition of a single pathway, but did not completely suppress it (Figure 3A). This suggests that although auxin and BR indeed promote enhanced elongation in low R:FR + low B, further modes of regulation may exist, such as for example gibberellin [16].

Low B enhances low R:FR response through a COP1-dependent mechanism

To study whether the increased number of PIF-targets expressed in low R:FR + low B reflects increased PIF abundance, we studied PIF5 protein levels in *PIF5:PIF5-HA* seedlings. Indeed, PIF5 accumulated more in low R:FR + low B than in low R:FR after 1h, although PIF5 abundance did not significantly increase in low B alone (Fig 3B). PIF-dependent transcription could further be enhanced by counteracting low R:FR-induced negative feedback loops. Several negative regulators of the shade avoidance response are induced by low R:FR, such as *LONG HYPOCOTYL IN FAR-RED 1 (HFR1), PHYTOCHROME RAPIDLY REGULATED 1 (PAR1*) and *PAR2*, *LONG HYPOCOTYL 5 (HY5)* and *HOMOLOG OF HY5 (HYH)* [17-19]. These negative regulators may prevent exaggerated elongation in low R:FR, and present a putative target for cross-talk. We therefore measured hypocotyl elongation in mutants of known negative regulators of shade avoidance. Although *35S:PAR1-GFP (PAR1-G)* hypocotyls elongated less in all light treatments, both the *PAR1-RNAi* line (mildly reduced levels of *PAR1* and *PAR2* [18]) and the *par2-1* mutant maintained a wild type-like low R:FR + low B response (Figure 3C,D), indicating that the *PARs* do not play a major role. In contrast, the *hfr1* and *hy5hyh* mutants showed enhanced hypocotyl elongation. Whereas *hy5hyh* elongated more than wild type in all light treatments, *hfr1* only did so in low R:FR and low R:FR + low B, suggesting a more specific interaction (Figure 3C). A line overexpressing a truncated stable version of HFR1 (G-BH-03, [20]) was impaired in all light treatments (Figure 3C), confirming that HFR1 can be a potent inhibitor of light quality-induced hypocotyl elongation [17, 21]. We therefore studied HFR1 protein levels in *HFR1:HFR1-HA* seedlings. After 1h, HFR1 abundance had increased in low R:FR, decreased in low B and was similar to white light in low R:FR + low B (Figure 3E). This attenuated HFR1 accumulation in low R:FR

+ low B was also observed at later time points (Figure S3) and is consistent with HFR1 protein destabilization during prolonged shade [22]. HFR1 forms non-DNA-binding heterodimers with PIF4 and PIF5, thereby inhibiting their transcriptional activity [21]. By reducing HFR1 abundance, low B signaling may thus increase availability of PIFs for transcription.

Regulation of protein abundance may occur at the transcript level, as is suggested by partially reduced *HFR1* expression in petioles in the combined light treatment (Figure 1F). Transcriptome analysis suggested that *HY5* expression was similarly reduced in low R:FR + low B, and QPCR analysis confirmed reduced expression of *HFR1, HY5* and *HYH* in petioles of plants treated with low R:FR + low B compared to low R:FR (Figure 4A-C). How transcript levels of these genes might selectively be reduced by addition of a low B signal is currently not understood.

As *HFR1* transcript levels are elevated in low R:FR + low B compared to white light while protein levels are similar, protein stability may also be regulated in the combined light treatment. HFR1, HY5 and HYH are targets of the COP1 / SUPPRESSOR OF PHYTOCHROME (SPA) E3 ubiquitin ligase complex, which labels them for degradation [23]. COP1 is indeed involved in shade-induced elongation and accumulates in the nucleus both in low R:FR and low B [22, 24, 25]. Moreover, crys and phys are associated with the COP1/SPA complex and their light-activation inhibits COP1/SPA activity [26-29]. In low R:FR + low B, COP1 nuclear localization and relieved inhibition through de-activation of crys may thus provide more favorable conditions for degradation of COP1 targets than low R:FR alone. Accordingly, the *cop1-4* mutant did not show enhanced petiole elongation in combined low R:FR + low B (Figure 4D). This shows that low B stimulation of the low R:FR response is COP1 dependent, and suggests that degradation of low R:FR-induced negative regulators is indeed required for enhanced elongation in low R:FR + low B.

Despite the COP1-dependency of the petiole response, the reduced gene expression of COP1 targets in petioles, and the obvious growth-inhibiting roles of HFR1 and HY5/HYH in hypocotyls, petiole elongation was not enhanced in *hfr1* adult plants subjected to light treatment or canopy growth (Figure 4E, F). Similarly, *hy5* responses to low R:FR and combined low R:FR + low B were not significantly different from wild type ($p > 0.05$, Student's

t-test) (Figure 4E, F). This suggests that in adult plants other or a combination of COP1 targets inhibit elongation, and that negative regulators of shade avoidance may depend on developmental stage.

A model for phy and cry signaling integration in plant competition

Combination of low B with low R:FR is a specific signature of plant competition, posing a serious threat to light capture. Low B stimulation of the low R:FR response suggests that blue and red light signals are integrated to respond adequately to the transition from neighbor detection to real competition. We propose that low B potentiates the low R:FR pathway through enhanced PIF action: In addition to increased abundance, PIF activity is likely enhanced directly through cry inactivation and indirectly through relieved inhibition of COP1, which increases degradation of negative regulators of PIF-mediated transcription such as HFR1 (Figure 4G). As PIFs are thought to be signaling hubs [30], these may be common mechanisms through which plants adapt their growth to changing environmental conditions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table and Experimental Procedures.

AUTHOR CONTRIBUTIONS

Conceptualization, M.dW. and R.P.; Investigation, M.dW., D.H.K., F.J.B., C.M.M.G., E.R., and C-M.-C.; Resources, P.H. and C.F.; Funding Acquisition, M.dW., R.P. and C.F.; Writing, M.dW., R.P. and D.H.K..; Supervision, R.P.

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FIGURE LEGENDS

Figure 1. Low B enhances the low R:FR response. (A-C) Elongation of approximately 5-mm-long petioles of 29-day-old plants over 24h of light treatment (Supplemental Experimental Procedures) (n=10). Green filter combines low R:FR, low B and low light intensity. (D-F) Expression relative to t=0 of PIF-dependent genes over time in petioles of light-treated plants. Data represent means ± SE (n=4). Different letters indicate significant difference ($p<0.05$) within genotype. ns, not significant.

Figure 2. Genome-wide transcript analysis of competition for light. (A) Plants grown at low (single) or high (canopy) density. (B) Length of 3rd youngest petiole of 37-day-old single and canopy-grown plants. Data represent means ± SE (n=5). Different letters indicate significant difference ($p<0.05$). (C) Venn diagram of genes expressed differentially to control in petioles of 29-day-old light-treated (24h) single plants and canopy-grown plants. Microarray analysis using a cut-off of $p < 0.05$ and $|\log2FC| > 1$, (n=3). (D) Heat maps of log2FC of

significantly regulated genes in canopy and at least one of the light treatments. (E) Number of differentially regulated genes in each of the treatments that are putative PIF4 and/or PIF5 targets. Different colors indicate targets shared with at least one other treatment, and unique targets not expressed in the other treatments.

Figure 3. Effect of auxin, BR and negative regulators in hypocotyl elongation. (A,C,D) Hypocotyl length of light-treated (A) auxin mutant *wei8-1* and BR mutant *bri1-1* and (C,D) negative regulator mutants (n > 16). Chemical inhibitors of auxin perception (50 μM α-(phenylethyl-2-one)-IAA, PEO-IAA) and brassinosteroid biosynthesis (0.5 μM Brassinazole, Brz) were added to medium right before light treatments started. (B,E) Protein accumulation in *PIF5:PIF5-HA* (B) and *HFR1:HFR1-HA* (E) seedlings detected with anti-HA antibody from total protein extract after 1h of light treatment, quantified and normalized to DET3 signal (n=3). Bands of representative blot correspond with bars in graph above. Data represent means ± SE. Asterisks indicate significant difference between light treatment and its respective chemical-treated control, different letters indicate significant difference within genotype ($p<0.05$,). w, white light; FR, low R:FR; LB, low B; FR+LB, low R:FR + low B.

Figure 4 Interaction between low R:FR and low B is COP1-dependent. (A-C) Gene expression relative to white light in petioles of four-week-old light-treated (4h) plants (n=6). (D,E) Petiole elongation of three-week-old (*cop1-4* – flowers early*)* or four-week-old (*hfr1-5*, *hy5-215)* mutants involved in the COP1 signaling pathway over 24h of light treatment (n=10). (F) Length of 3rd youngest petiole in 35-day-old plants grown at low (single) or high (canopy) density (n=5). Data represent means ± SE. Different letters indicate significant difference within genotype ($p<0.05$).

(G) Model of phytochrome (phy) and cryptochrome (cry) signaling integration during competition for light. In low R:FR, phy is inactivated and resides in the cytosol [31]. This allows PIF accumulation in the nucleus and subsequent transcription of positive, but also negative (red mRNA) regulators of shade avoidance [2,17], such as HFR1 that forms non-DNA-binding heterodimers with PIFs [21]. In low B, PIFs may accumulate [7] and cryptochrome inactivation relieves its direct inhibition of PIF-mediated transcription [10]. In

combined low R:FR and low B, PIF-mediated transcription is thus facilitated likely through both enhanced PIF abundance (this paper) and activity. Additionally, low R:FR + low B leads to reduced accumulation of negative regulators of shade avoidance such as HFR1 (this paper), many of which are targets of the E3 ubiquitin ligase COP1. Low R:FR and low B both induce nuclear translocation of COP1 [25], while both cry and phy inactivation relieves their repression on the COP1/SPA complex [26-29]. This allows for enhanced degradation of COP1 targets. Furthermore, transcription of low R:FR-induced negative regulators is reduced in the combination with low B (this paper) through an unknown mechanism.

Figure 1

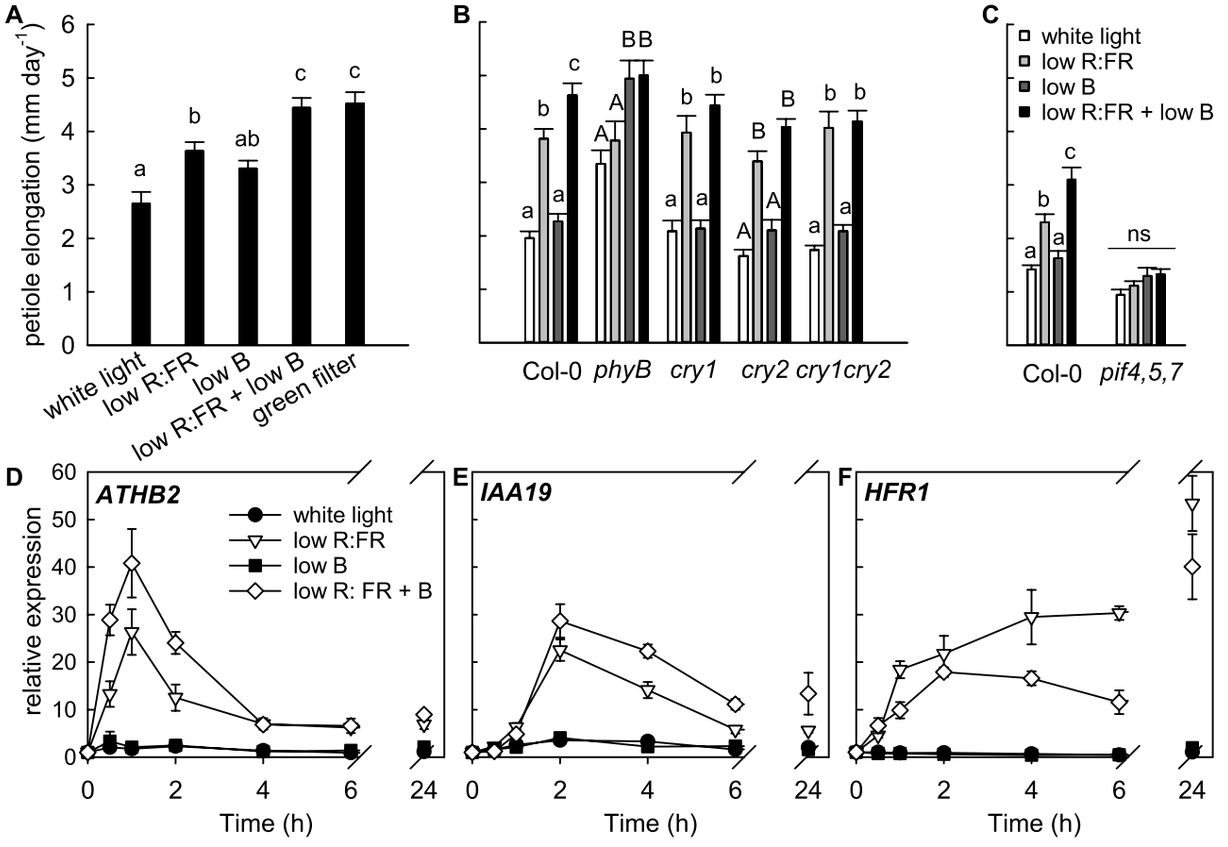

A
petiole elongation (mm day-1)
white light
low R:FR
low B
low R:FR + low B
green filter
B
Col-0
phyB
cry1
cry2
cry1cry2
C
white light
low R:FR
low B
low R:FR + low B
Col-0
pif4,5,7
ns
D
ATHB2
relative expression
white light
low R:FR
low B
low R: FR + B
Time (h)
E
IAA19
Time (h)
F
HFR1
Time (h)

Figure 2

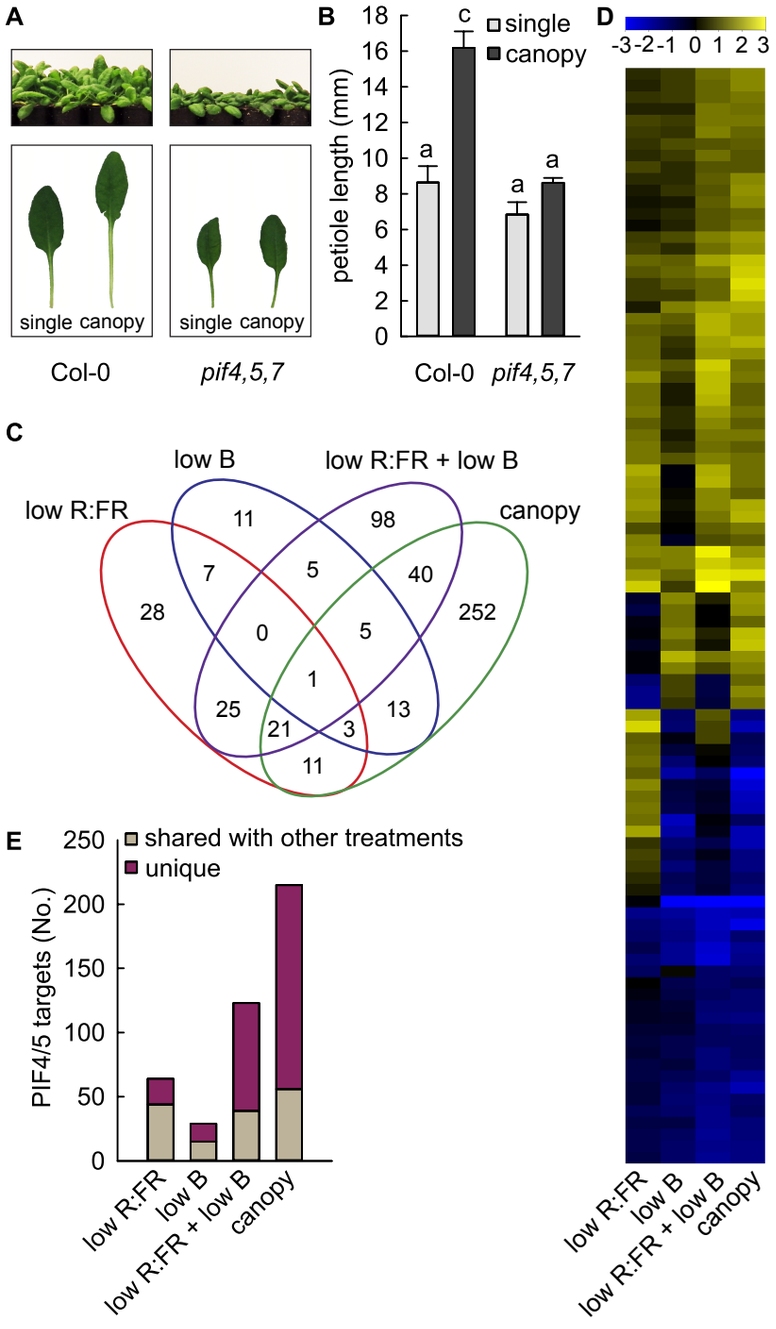

A
single canopy
single canopy
Col-0
pif4,5,7
B
petiole length (mm)
single
canopy
a
c
a
a
Col-0
pif4,5,7
C
low B
low R:FR + low B
low R:FR
canopy
11
98
7
5
40
28
0
5
252
1
25
13
21
3
11
D
-3 -2 -1 0 1 2 3
low R:FR
low B
low R:FR + low B
canopy
E
PIF4/5 targets (No.)
shared with other treatments
unique
low R:FR
low B
low R:FR + low B
canopy

Figure 3

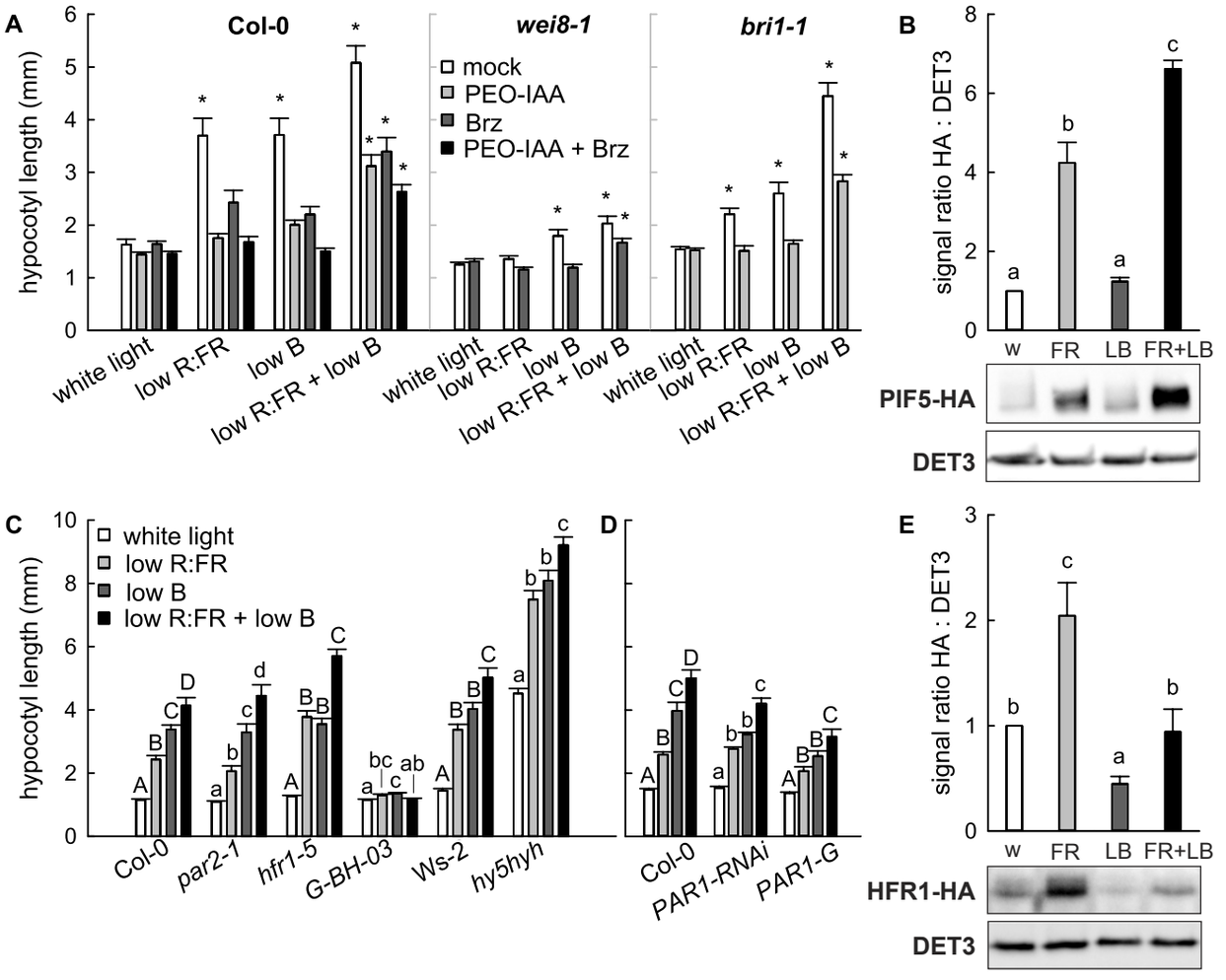
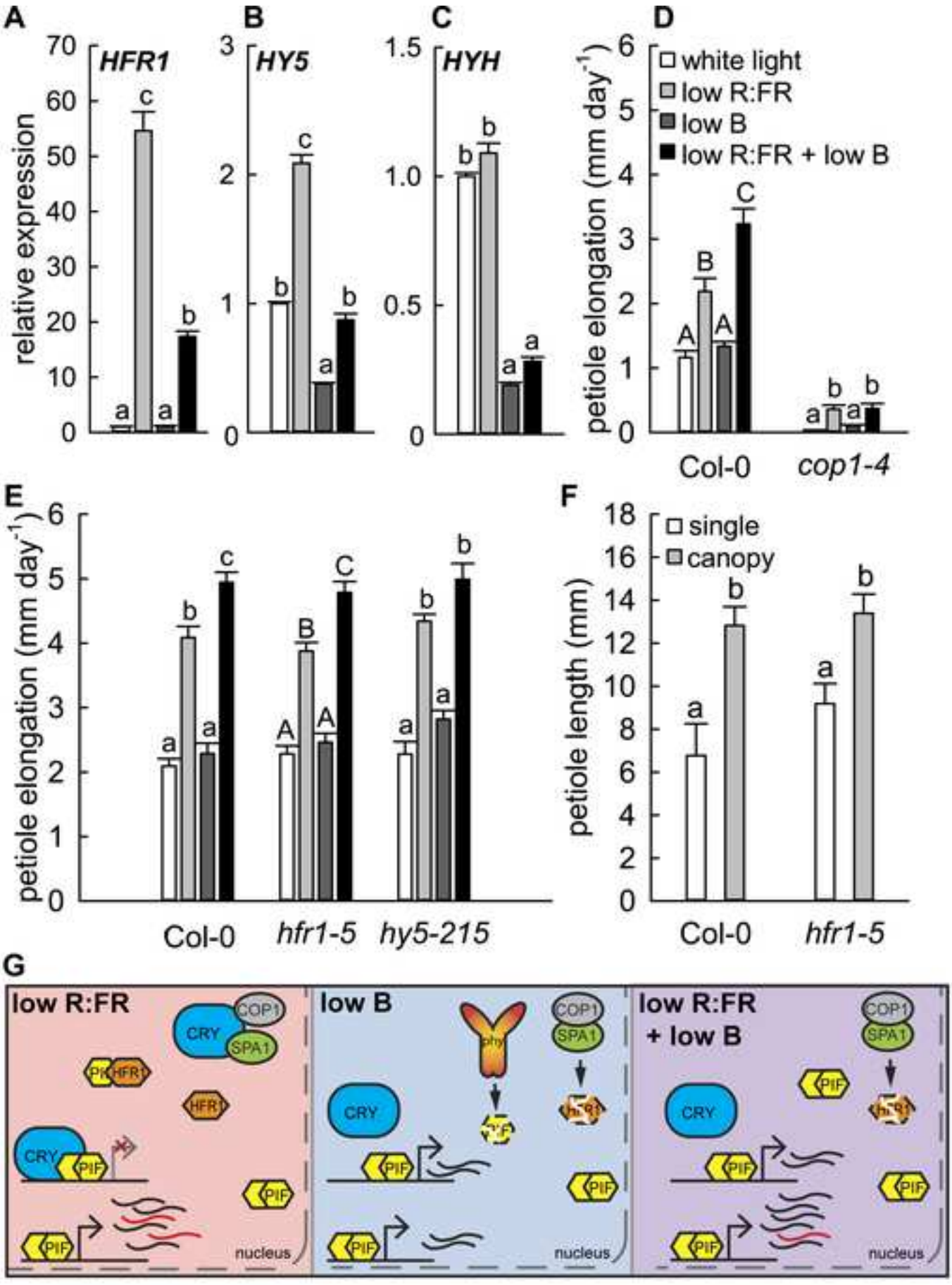

A
Col-0
wei8-1
bri1-1
mock
PEO-IAA
Brz
PEO-IAA + Brz
hypocotyl length (mm)
white light
low R:FR
low B
low R:FR + low B
B
signal ratio HA : DET3
w
FR
LB
FR+LB
PIF5-HA
DET3
C
white light
low R:FR
low B
low R:FR + low B
hypocotyl length (mm)
Col-0
par2-1
hfr1-5
G-BH-03
Ws-2
hy5hyh
D
Col-0
PAR1-RNAi
PAR1-G
E
signal ratio HA : DET3
HFR1-HA
DET3

Figure 4

Click here to download Figure Fig4.tif



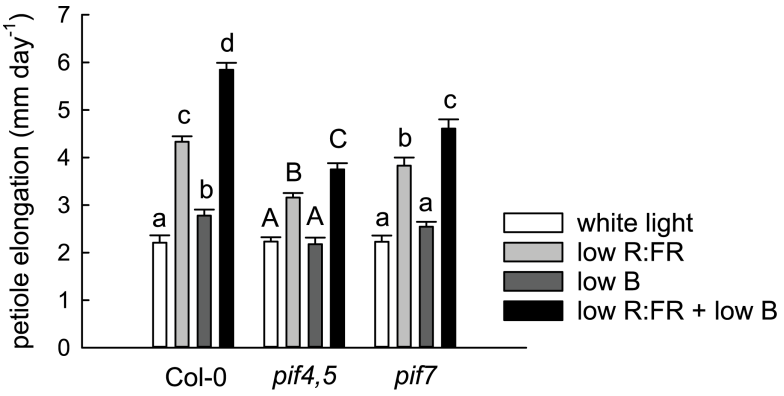


Figure S1. Related to Figure 1.
Petiole elongation in *pif* mutants. Petiole elongation of *pif4pif5* and *pif7* mutants over 24h of light treatment. Data represent means ± SE. Different letters indicate significant difference ($p<0.05$) within genotype.

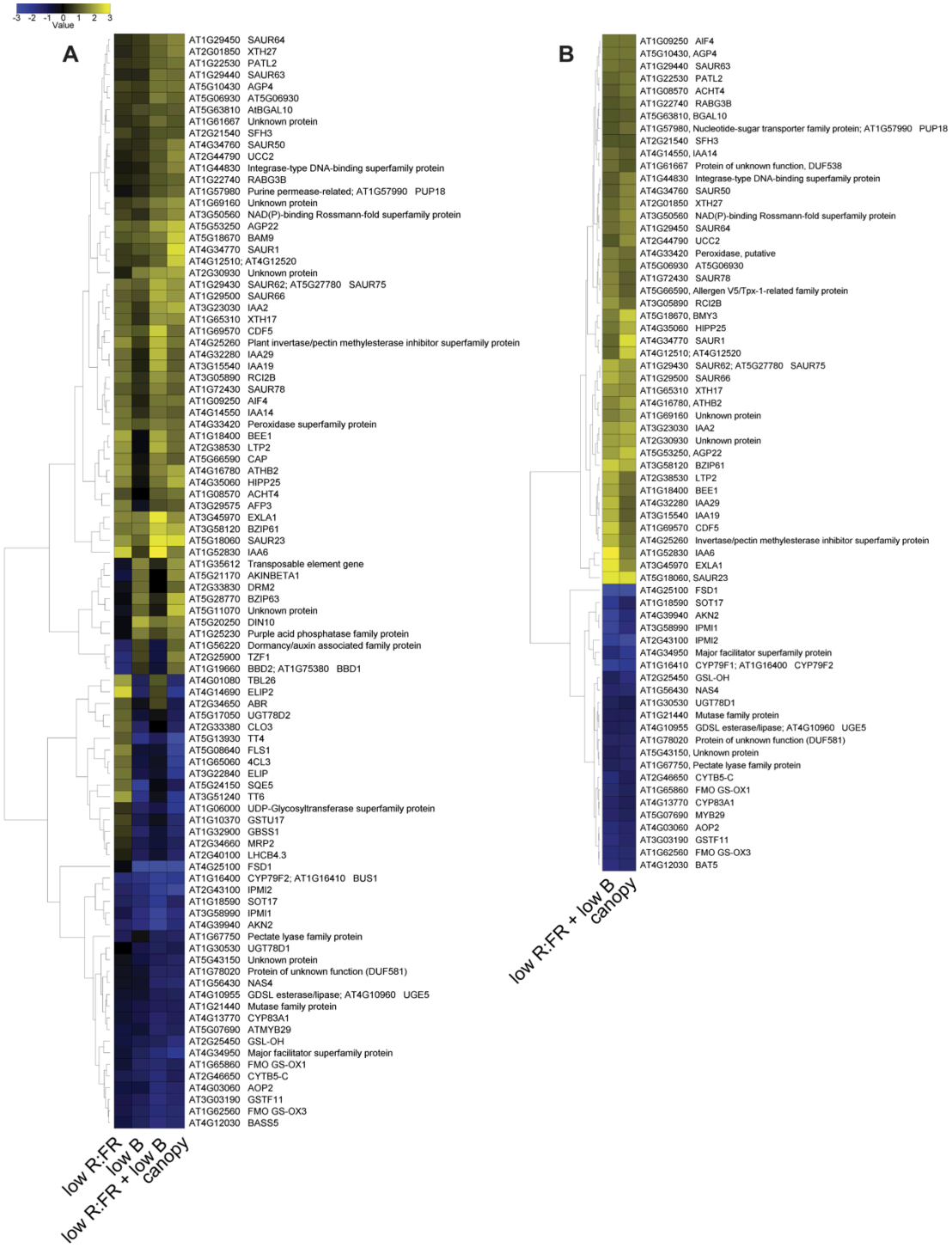


Figure S2. Related to Figure 2.

Overlap in expressed genes between treatments. Heatmaps of ^{2}log fold changes of differentially expressed genes significantly regulated in (A) canopy and at least one of the light treatments, (B) canopy and low R:FR + low B.

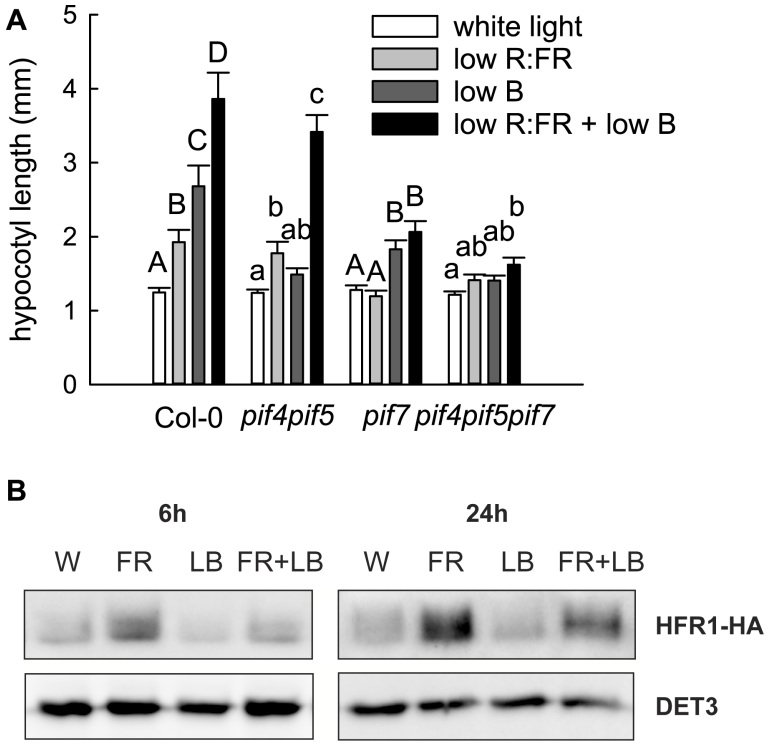


Figure S3. Related to Figures 3 and 4.

Light treatments affect hypocotyl elongation and HFR1 protein abundance. (A) Hypocotyl elongation of *pif* mutants in light treatments. Data represent means ± SE. Different letters indicate significant difference ($p<0.05$) within genotype. (B) HFR1 protein accumulation in *HFR1:HFR1-HA* seedlings from total protein extracts after 6h or 24h of light treatment. Protein was detected with anti-HA or anti-DET3 antibody. W, white light; FR, low R:FR; LB, low B; FR+LB, low R:FR + low B.

Table S1. GO analysis of differentially expressed genes falling in the category 'Biological Process'. Corrected p-values are depicted for up- and downregulated genes for each treatment separately.

				low R:FR		low B		low R:FR+LB		canopy	
				corr. p-value		corr. p-value		corr. p-value		corr. p-value	
		Description	GO-ID	up	down	up	down	up	down	up	down
regulation of biological process		regulation of G2/M transition of mitotic cell cycle	10389	4.79E-02							
		positive regulation of anthocyanin metabolic process	31539	3.58E-02							
		regulation of transcription	45449	1.88E-02							
		regulation of potassium ion transport	43266		4.00E-02				8.35E-03		
		regulation of glucosinolate biosynthetic process	10439						3.14E-13		2.95E-05
		regulation of anthocyanin biosynthetic process	31540								9.31E-03
		photosystem I stabilization	42550								3.90E-02
		monovalent inorganic cation homeostasis	55067								9.31E-03
response to stimulus	light	red or far-red light signaling pathway	10017	3.98E-06							
		response to far red light	10218	1.13E-07			5.43E-03	4.70E-03			
		response to low fluence red light stimulus	10202	2.25E-02							
		response to red light	10114				7.04E-03				
		response to UV-B	10224	1.24E-04			5.28E-03				9.10E-04
		phototropism	9638	6.74E-03						3.97E-02	
		shade avoidance	9641					1.83E-02			
		response to blue light	9637					3.07E-02			
	hormones	response to auxin stimulus	9733	2.92E-07				1.00E-19		2.88E-10	
		response to jasmonic acid stimulus	9753	4.71E-02					8.42E-03		3.67E-05
		response to salicylic acid stimulus	9751								2.95E-02
		response to gibberellin stimulus	9739						4.18E-02		
		response to cytokinin stimulus	9735							2.02E-02	
		response to abscisic acid stimulus	9737							7.57E-03	
		response to brassinosteroid stimulus	9741					3.48E-02			
	mineral nutrients	cellular response to sulfate starvation	9970				3.72E-02				
		cellular response to sulfur starvation	10438						5.00E-04		
		cellular response to nitrogen starvation	6995				2.49E-02				
		cellular response to potassium ion starvation	51365						1.47E-02		
		cellular response to potassium ion starvation	51365		4.00E-02						
		cellular response to phosphate starvation	16036								1.19E-11
		response to zinc ion	10043								4.30E-02
	other	gravitropism	9630	2.81E-03							
		response to cold	9409	2.96E-02			1.25E-03			1.73E-02	1.64E-02
		cold acclimation	9631				4.93E-02				
		response to freezing	50826				3.06E-02				
		transpiration	10148	2.25E-02							
		response to fungus	9620				2.88E-02				1.33E-02
		phospholipid transfer to membrane	6649	2.25E-02							
		response to water deprivation	9414								2.30E-02
		hyperosmotic salinity response	42538								1.23E-02
		response to mannitol stimulus	10555		4.39E-02				3.61E-02		
		cellular response to sucrose starvation	43617		4.00E-02						
		sucrose mediated signaling	9745								3.90E-02
		response to carbon dioxide	10037				1.80E-02				
		response to desiccation	9269				4.45E-02				
		removal of superoxide radicals	19430						3.97E-02		5.37E-03
Cellular process		G2/M transition of mitotic cell cycle	86	3.58E-02							
		circadian regulation of calcium ion oscillation	10617	4.79E-02							
		cell growth	16049					2.62E-02			
		plant-type cell wall modification	9827					2.67E-02		5.42E-03	
		plant-type cell wall modification involved in multidimensional cell growth	9831								3.54E-02
		plant-type cell wall loosening	9828							2.80E-03	
		cuticle development	42335					4.11E-02			
		unidimensional cell growth	9826							8.43E-04	
		de-etiolation	9704							1.42E-02	
Localization		tryptophan transport	15827								2.30E-02
		aspartate transport	15810								2.30E-02
		sodium ion transport	6814								2.51E-02
		sulfate transport	8272								1.78E-02
		phosphate transport	6817								7.36E-04
Metabolic process		phosphatidylglycerol metabolic process	46471	4.79E-02							
		sterol biosynthetic process	16126	1.88E-02							
		oxylipin metabolic process	31407	2.25E-02							4.05E-02
		flavonoid biosynthetic process	9813	4.40E-02							
		methionine catabolic process via 2-oxobutanoate	19458	2.25E-02							
		ubiquinone biosynthetic process	6744				1.48E-02				
		sulfate assimilation	103				3.72E-02				
		glucosinolate biosynthetic process	19761				3.63E-03				
		regulation of glucosinolate biosynthetic process	10439				1.80E-02				
		regulation of anthocyanin biosynthetic process	31540				2.88E-02				
		chalcone biosynthetic process	9715				5.94E-03				2.30E-02
		flavonol biosynthetic process	51555				2.49E-02				6.79E-03
		chlorophyll catabolic process	15996				2.66E-02				
		PSII associated light-harvesting complex II catabolic process	10304				2.49E-02				
		cellular glucan metabolic process	6073					6.75E-05			
		wax biosynthetic process	10025					3.48E-02			
		toxin catabolic process	9407						1.18E-02		
		glucosinolate biosynthetic process from homomethionine	33506						8.35E-03		2.30E-02
		leucine biosynthetic process	9098						8.29E-04		7.99E-03
		nicotianamine biosynthetic process	30418						2.56E-02		
		brassinosteroid biosynthetic process	16132							2.49E-02	
		starch catabolic process	5983								1.78E-02
		galactolipid biosynthetic process	19375								1.07E-06
		L-ascorbic acid biosynthetic process	19853								9.31E-03
		glycerol metabolic process	6071								2.72E-02
		polyamine catabolic process	6598								3.90E-02
Reproduction		male gamete generation	48232				4.27E-02				

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plant growth and measurements

The *hy5hyh* double mutant was in the Wassilewskija (Ws-2) background [1]. All other mutants were in Columbia (Col-0) background: *phyb-9* [2], *cry1-304* [3], *cry2-1* [4], *pif4pif5* [5], *pif7-1* [6], *pif4pif5pif7* [7], *wei8-1* [8], *bri1-1* [9], *PAR-RNAi, 35S:PAR1-GFP* and *par2-1* [10], *hfr1-5* [11], *p35S:G-BH-03* [12], *cop1-4* [13], *hy5-215* [14].

Plants were grown on fertilized 1:2 potting soil:perlite substrate in individual pots of 70 ml as previously described [15]. Canopy plants were grown in individual pots of 19 ml in a checkerboard design of 7x7 plants (2066 plants m^{-2}). The single plants used for the microarray analysis of different light treatments were grown in similar pots of 19 ml soil to keep all conditions similar to canopy-grown plants except for the presence of close neighbors. Plants were watered daily and grown in a nine-hour light period of 180 µmol m^{-2} s^{-1} Photosynthetically Active Radiation (PAR) and a R:FR of 1.8 (Philips HPI-T Plus, 400W) at 20°C and 70% RH.

Adult plants were used for experiments when they were four weeks old, except for the *cop1-4* mutant which flowers early and was measured after 21d. One petiole of approximately 5 mm was measured per plant with a digital calliper. Elongation was calculated as difference in length per 24h. Petioles of the same developmental age (29 d old) were used in the microarray assay. Petiole lengths of canopy-grown plants were measured 35d (*hfr1-5)* or 37d (*pif4pif5pif7*) after germination.

For hypocotyl experiments seeds were surface-sterilized and sown on MS plates containing 8 g l^{-1} agar and 0.22 g l^{-1} Murashige and Skoog (Duchefa Haarlem, the Netherlands). After 4d of stratification (dark, 4˚C) seeds were germinated under long-day light regime (16h light, 8h dark) in PAR of 80 µmol m^{-2} s^{-1}, thus matching the PAR conditions of further treatments (see below). The seedlings were placed in different light treatments when cotyledons were completely unfolded (approximately 24h after germination). Hypocotyl lengths were measured from scans taken after 4d of light treatment using the open-source software package ImageJ [16].

Light and pharmacological treatments

Light treatments on adult plants were conducted under short day (9h light / 15h dark) regime, hypocotyls were kept in long days (16h light 7 8h dark). A low R:FR of 0.3 was obtained through supplemental far-red LEDs (730 nm; Philips Green Power, the Netherlands). A reduced B light environment was obtained by filtering the background white light through a layer of Lee Medium Yellow 010 filter (Lee Hampshire, United Kingdom). The combined treatment of low R:FR and low B consisted of a combination of a layer of Lee yellow filter and FR LEDs. Low R:FR, low B and low PAR treatment was achieved using Lee 122 Fern Green filter (Lee Hampshire, United Kingdom). Reduction of control light treatment to equal PAR of the different light treatments (80 µmol m^{-2} s^{-1}) and low PAR were obtained through shading with a spectrally neutral cloth. Final light conditions are given in the table below.

Canopies were kept at a short day regime at 180 µmol m^{-2} s^{-1}. In canopies of 29 d old, PAR had decreased to 33 µmol m^{-2} s^{-1}, with a B component of 1.4 µmol m^{-2} s^{-1} and a low R:FR that was reduced to 0.7. The R:FR in further developed canopies of 36 d old had decreased to 0.3.

Light qualities were verified with a Licor1800 spectroradiometer.

Light conditions in experimental set-up

Treatment / Signal	white light	low R:FR	low B	low R:FR + low B	green filter
PAR (400-700 nm, µmol m^{-2} s^{-1})	80	80	80	80	20
R:FR (R, 654-664; FR, 724-734 nm)	2.1	0.3	2.1	0.3	0.1
B (400-500 nm, µmol m^{-2} s^{-1})	17	17	1	1	2.5

50 µM α-(phenylethyl-2-one)-IAA (PEO-IAA; [17]) was used to block auxin perception and 0.5 µM Brassinazole (Brz; TCI Europe Tokyo, Japan) was used to block brassinosteroid biosynthesis. A concentrated, sterile solution was administered to the agar and allowed to diffuse through the medium right before the start of the light treatments.

Transcript profiling and Real Time RT-PCR

Three biological replicates each consisting of four pooled petioles from different plants were harvested at ZT2 for each treatment. Total RNA was extracted from homogenized material using the RNeasy plant mini kit with on-column DNA digestion (Qiagen) according to the manufacturer's instructions. cDNA synthesis, cRNA synthesis and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were executed by Service XS Leiden, The Netherlands (authorized service provider Affymetrix). Microarray data were normalized with the RMA algorithm[18] and differential expression was assessed using the empirical Bayes method [19] and Benjamini and Hochberg multiple testing correction [20] in the Bioconductor packages in R (www.bioconductor.org). Genes were considered differentially expressed if $p < 0.05$ and $-1 < FC > 1$. Heatmaps were created using the heatmap.2 function in the R package gplots [21]. Complete linkage hierarchical clustering using a Euclidian distance measure was performed using the *hclustfun* and *distfun* arguments. GO analysis was done with the Bingo plug-in of Cytoscape [22]. For PIF5 target identification, differentially expressed genes were compared with Table S1 provided in Hornitschek et al., 2012 [23] and Table S4 provided in Pedmale et al., [24].
Data are available at the NCBI gene expression and hybridization array data repository, Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo; accession no. GSE87770.

For Real Time RT-PCR time course four to six biological replicates were used, each containing six petioles pooled from three different plants. RNA was reverse transcribed using Reverse Transcriptase SSIII (Invitrogen) with RNAse inhibitors and random primers. Quantitative RT-PCR was performed in a 5 µl reaction with SybrGreen Supermix on a ViiA 7 Real Time PCR system (384 wells). The average CT of *UBQ5* (time-course) or *TUBULIN, APT1* and *AT1G13320* were used as a reference. Oligo sequences are given in table below.

Oligo sequences (5'-3') used for Real Time RT-PCR

gene	forward	reverse
ATHB2	GAGGTAGACTGCGAGTTCTTAC	GCATGTAGAACTGAGGAGAGA
IAA19	TAAGCTCTTCGGTTTCCGTG	ACATCCCCCAAGGTACATCA
HFR1	ACGTCGTATCCAGGTCTTAAGT	GAGAACCGAAACCTTGTCCG
HY5	ATGAGGAGATACGGCGAGTG	TCCCTCGCTTCCTTTGACTT
HYH	TCCCTCGCTTCCTTTGACTT	ACACATGTTGATCCAGCTGC
UBQ5	CCAAGCCGAAGAAGATCAAG	ACTCCTTCCTCAAACGCTGA
TUB-6	ATAGCTCCCCGAGGTCTCTC	TCCATCTCGTCCATTCCTTC
APT1	AATGGCGACTGAAGATGTGC	TCAGTGTCGAGAAGAAGCGT
AT1G13320	GTAGGACCGGAGCCAACTAG	ACAGGGAAGAATGTGCTGGA

HA-tagged lines

pSL113 (PIF5pro:PIF5-3xHA) was constructed by introducing the *PIF5-3HA* coding sequence into *pGemT* to generate pSL88 (*PIF5-3HA*) after amplification by PCR from the plasmid pCF404 [5] using the primers **5'-** ATCGCTAGCATGGAACAAGTGTTTGCTGA**-3'** and 5'TACCTCGAGCCAAATGTTTGAACGATCTG-3'. 2,5 kB 5' of the *PIF5* ATG was amplified with primers 5'-ATC**GCTAGC*GGATCC***CGCCACCGCCGCCTGAATGTT-3' and 5'-ATCGGATCCGCTAGCGTCAGATCTGTAAAGACACT-3' using BAC F17J16 as a template. The PCR product was digested by *NheI* and cloned into the *NheI*-digested pSL88 (*PIF5pro:PIF5-3HA* in *pGemT*). After sequencing, a *BamH1*-digested fragment containing *PIF5* promoter and a part of the *PIF5* CDS were introduced into *BamH1*-digested pAM04. pAM04 is a binary vector containing the *PIF5-3HA* coding sequence under the control of a shorter version of *PIF5* promoter. It was generated by a three-way ligation between *BamHI-NheI*-digested PIF5 promoter sequence, *NheI-XhoI*-digested PIF5-3HA coding sequence and the *BamHI-SalI* digested pCF300 binary vector. This construct was transformed into *pif5-1* and lines with a single insertion site that complement the *pif5* phenotype were selected.

pPH73 (HFR1pro:HFR1-3xHA) was constructed by amplifying 2.1kb 5' of the *HFR1* ATG using primers 5'-tgactctagaggtaccggcgatcgctacgaaaagaagaag-3' and 5'-gtcaggatccttagttaaagagatatcggagatga-3', *HFR1* cDNA with a triple HA tag at the C-terminus was amplified from vector pCF396 described in [25]. HFR1 promoter and cDNA were ligated into pPZP211 including an *RBCS* terminator sequence 3' of the *HFR1* gene. This construct was transformed into *hfr1-101* and lines with a single insertion site that complement the *hfr1* phenotype were selected.

Protein analysis

For protein extraction, 30 *HFR1::HFR1-HA* seedlings were pooled, ground in extraction buffer (125 mM Tris pH6.8, 4% SDS, 20% glycerol, 0.02% bromophenolblue, 10% mercaptoethanol) heated at 95°C for five minutes. Samples were centrifuged for five minutes at maximum speed and supernatant was transferred to a clean tube, frozen in liquid nitrogen and stored at -20 °C until further handling. Samples were re-heated for two minutes at 95°C before loading on gel. After separation on SDS-PAGE gel and transfer to nitrocellulose membrane, proteins were detected with anti-HA conjugated with peroxidase (Roche) or polyclonal antibodies directed to DET3. HFR1 blots were stripped (2% SDS, 0.7% 2-ME in 1x PBS) before probing with DET3 antibodies. Data was repeated in three biological experiments. For protein quantification bands were normalised to the control light sample using the ImageQuant TL software (GE Healthcare Life Sciences) after which the ratio between HFR1 and DET3 protein signal was calculated. Means were calculated from three technical replicates from one experiment.

Statistical analysis

Hypocotyl and petiole data were analyzed by analysis of variance (ANOVA) and Tukey's HSD post-hoc test separately for each genotype to determine significant difference between means. In figures, lower case letters and capital letters are alternated to show data was analyzed per genotype. Difference in hypocotyl length between light treatments in the pharmacological experiment (Figure 3A) was analyzed with unpaired Student's *t*-test comparing to white light of the same chemical treatment and genotype. Analyses were done in the R statistical environment (R Development Core Team 2015). Microarray data were analyzed as mentioned above.

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