Rewiring of auxin signaling under persistent shade

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Light cues from neighboring vegetation rapidly initiate plant shade-avoidance responses. Despite our detailed knowledge of the early steps of this response, the molecular events under prolonged shade are largely unclear. Here we show that persistent neighbor cues reinforce growth responses in addition to promoting auxin-responsive gene expression in *Arabidopsis* and soybean. However, while the elevation of auxin levels is well established as an early event, in *Arabidopsis*, the response to prolonged shade occurs when auxin levels have declined to the prestimulation values. Remarkably, the sustained low activity of phytochrome B under prolonged shade led to (*i*) decreased levels of PHYTOCHROME INTERACTING FACTOR 4 (PIF4) in the cotyledons (the organs that supply auxin) along with increased levels in the vascular tissues of the stem, (*ii*) elevated expression of the PIF4 targets *INDOLE-3-ACETIC ACID 19* (*IAA19*) and *IAA29*, which in turn reduced the expression of the growth-repressive *IAA17* regulator, (*iii*) reduced abundance of AUXIN RESPONSE FACTOR 6, (*iv*) reduced expression of *MIR393* and increased abundance of its targets, the auxin receptors, and (*v*) elevated auxin signaling as indicated by molecular markers. Mathematical and genetic analyses support the physiological role of this system-level rearrangement. We propose that prolonged shade rewires the connectivity between light and auxin signaling to sustain shade avoidance without enhanced auxin levels.

phytochrome B | PIF4 | Aux/IAA | auxin receptor | MIR393

Canopy shade entails a stronger reduction of red and blue than far-red and green wavelengths, due to selective absorption by photosynthetic pigments. These cues are sensed via a reduction in the activity of photosensory receptors, mainly phytochrome B (phyB) and cryptochrome 1 (cry1), consequently initiating shade-avoidance responses such as the promotion of stem and petiole elongation (1–3). Light-driven changes in canopy architecture can significantly impact yield of agricultural crops (4).

The phyB triggers the degradation and/or interferes with the specific DNA binding capacity of PHYTOCHROME INTERACTING FACTOR 3 (PIF3), PIF4, PIF5, and PIF7 (5, 6). Low red/far-red ratios reduce phyB activity and, as such, increase the activities of PIF3 (7), PIF4, PIF5 (8), and PIF7 (9). Low blue light levels reduce cry1 activity and also increase the abundance of at least PIF4 and PIF5, particularly when combined with low red/far-red ratios (10, 11). Consequently, in the presence of neighbor signals, PIFs show increased binding to the promoters of several of their targets, including the *YUCCA* (*YUC*) genes, activating their expression (9, 12, 13). Since *YUC* genes encode a rate-limiting enzyme in the synthesis of auxin, low red/far-red ratios lead to increased auxin levels in the cotyledons (14). Auxin is then transported from the cotyledons that perceive the neighbor signals to the growth-limiting epidermal tissue of the stem (15–17) and from the leaf lamina to the petioles (18, 19) to promote growth.

Auxin is perceived by the nuclear TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFBs) proteins, which effect ubiquitination and degradation of the coreceptor AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional regulators (20). Aux/IAAs are active repressors of the AUXIN RESPONSE FACTOR (ARF) transcription factors, which mediate auxin-regulated gene expression upon Aux/IAA degradation (21). *Aux/IAA* genes are, in addition, direct targets of ARFs, and their expression increases in response to auxin. The dynamic consequences of the dual action of auxin on *Aux/IAA* transcription and Aux/IAA stability are a focus of intense analysis (22–24). The microRNA *MIR393* negatively regulates TIR1, AFB2, and AFB3 transcript and protein accumulation in response to environmental stimuli (21, 25, 26).

Shade-avoidance responses can persist for weeks (27), and enhanced expression of auxin-related genes can be observed beyond the first day after the initiation of neighbor signals (14, 16, 28). However, the early activation of *YUC* genes and auxin synthesis can be severely reduced after several hours of exposure to low red/far-red ratios (29, 30), suggesting that different mechanisms might operate later on. Here we show that prolonged shade evokes a system-level commitment to shade

Significance

In agricultural crops, plants are cultivated in close proximity, exposed to mutual shading. Photosensory receptors perceive this light environment and initiate growth responses that adjust the position of plant organs within the canopy to maximize light capture. During the early hours under shade, these responses are mediated by elevated levels of the hormone auxin. Here we show that under prolonged shade, the PHYTOCHROME INTERACTING FACTOR 4 light-signaling transcription factor, selected auxin receptors, their upstream regulatory miRNA, and downstream transcriptional regulators change their abundance to enhance growth responses, while auxin levels return to those observed before shade. Thus, in the presence of persistent shade, plants elicit a system-level rearrangement of auxin signaling, which sustains growth without increased auxin.
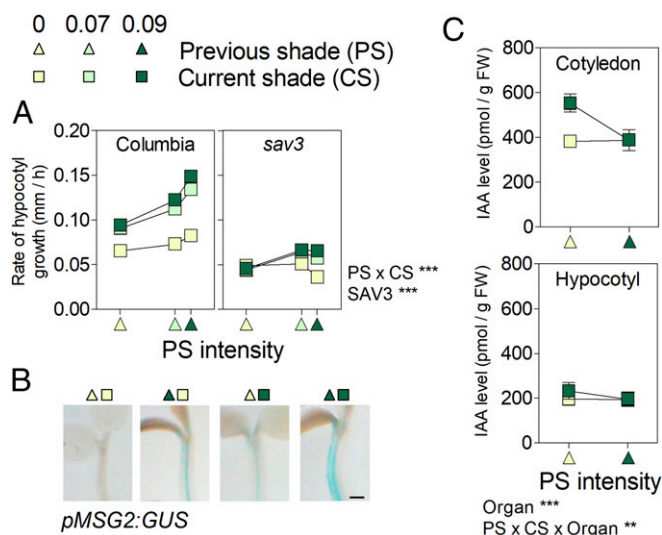
Fig. 1. Persistent shade avoidance in the absence of elevated auxin levels in *Arabidopsis*. Shade intensity (based on the inverse of photosensory receptor activity estimated from the light spectra) is 0 for simulated sunlight, 0.07 for weak shade, and 0.09 for deep shade. (*A*) Columbia wild-type and *sav3* hypocotyl growth rate during day 4 (0 h to 3 h after the beginning of the photoperiod) as affected by CS (day 4) and PS (day 3, abscissas). Means ± SE (smaller than the symbols) are from *SI Appendix*, Table S2. (*B*) Representative images of GUS stained seedlings bearing the *pMSG2/IAA19:GUS* transgene. (Scale bar = 200 μm.) (*C*) Auxin levels on day 4 (3 h after the beginning of the photoperiod). Means ± SE of four biological replicates. **$P < 0.01$; ***$P < 0.001$.

avoidance that involves changes in PIF4, *MIR393*, TIR1, AFB2, and ARF6 abundance and the relative expression of different *Aux/IAA* genes.

Results

Commitment to Shade Avoidance Under Prolonged Shade. Three-day-old *Arabidopsis* plants were transferred to simulated shade (low red/far-red, low irradiance) or left as controls. The following day, half of the plants of each group were transferred to the opposite condition, to obtain plants subjected to all possible combinations of current shade (CS) and previous shade (PS). Growth promotion was strongly reinforced when plants had been subjected to intense PS (Fig. 1*A*; note significant PS × CS interaction). The impact of PS was observed for a further 9 h, but with a decreasing magnitude (*SI Appendix*, Fig. S1). Both CS and PS treatments also led to increased GUS staining in the hypocotyl of *pMASSUGU 2* (*MSG2*)/*IAA19:GUS* reporter line (Fig. 1*B*). Therefore, prolonged shade not only maintains, it actually enhances the shade-avoidance responses. This is not a feature restricted to young (recently deetiolated) *Arabidopsis* seedlings, as stem and petiole growth and the expression of auxin-related genes such as *Aux/IAAs* (including *MSG2/IAA19*) and *SMALL AUXIN UP-REGULATED* (*SAUR*)-like were also maximal in glasshouse-grown soybean plants exposed to both PS and CS (*SI Appendix*, Figs. S2 and S3 and Table S1).

Persistent Shade Avoidance Takes Place Without Elevated Auxin Levels. Both, CS and PS hypocotyl growth responses were impaired in the *sav3* mutant (Fig. 1*A*), compromised in auxin synthesis. The slope of the relationship between growth rate under intense shade (CS = 0.09) and PS was significantly reduced ($P < 0.05$) in *sav3*, indicating that persistent response to shade is dependent on the levels of bioactive auxin. We harvested seedlings 3 h after the beginning of day 4 for auxin determination. In agreement with previous reports (14), auxin levels were higher in plants recently transferred to shade, compared with control seedlings subjected to simulated sunlight. However, in plants that had been exposed to intense PS, auxin content was similar to that

of seedlings exposed to simulated sunlight (Fig. 1*C*). These results show that auxin accumulation is only transiently induced by shade (29, 30) and demonstrate that this pattern is PS-specific and not caused by the seedling age. From these findings, we conclude that increased growth under prolonged shade depends on auxin, but it is not mediated by elevated auxin levels.

Prolonged Shade Maintains Low phyB Activity. The transient nature of the enhanced auxin levels could be due to partial recovery of phyB activity under persistent shade (31). However, *PHYB* expression (*SI Appendix*, Fig. S4A) and total cell fluorescence of *p35S:PHYB-GFP* reporter line (a proxy for phyB abundance; Fig. 2A) were not significantly affected by simulated sunlight or shade conditions. The nuclear abundance of phyB remained unaffected by prolonged shade in the cotyledons and vascular tissues of the hypocotyl and decreased with prolonged shade in epidermal cells of the hypocotyl (Fig. 2; the kinetics is shown in *SI Appendix*, Fig. S5A). Since the low red/far-red ratios of shade establish low proportions of active phyB (Pfr), and nuclear levels of phyB were either unaffected or lowered, phyB activity remains low or even decreases under prolonged shade. In the wild type, hypocotyl growth under intense shade (CS = 0.09) was accelerated by increasing PS intensity ($P < 0.0001$; note positive slope in Fig. 1*A*), but this response was absent in the *phyB* mutant (where prolonged shade inhibited rather than promoting growth, $P < 0.05$; *SI Appendix*, Table S2). This indicates that prolonged shade enhanced hypocotyl growth by maintaining low phyB activity.

Prolonged Shade Modifies the PIF4 Profile. Among phyB targets, PIF4 has a large effect on shade avoidance. *PIF4* expression was not significantly affected by shade (*SI Appendix*, Fig. S4 *B* and *C*). To describe the dynamics of the PIF4 protein, we generated lines bearing the *pPIF4:PIF4-GFP* transgene. Confocal microscopy analyses revealed that nuclear abundance of the PIF4 protein decreased in mesophyll cells in response to prolonged shade, with much lower nuclear GFP levels observed in cotyledons of plants subjected to prolonged shade than in those exposed to a first shade event (Fig. 3 *A* and *B*). Since *YUC8* and *YUC9* are direct targets of PIF4 (12), this reduction in PIF4 protein abundance could account for the decrease in auxin levels observed under prolonged shade (Fig. 1*C*). By contrast, under prolonged shade, PIF4 levels remained high in epidermal/subepidermal cells of the hypocotyl (Fig. 3 *A* and *B*; kinetics are shown in *SI Appendix*, Fig. S5B) and specifically overaccumulated in the vascular
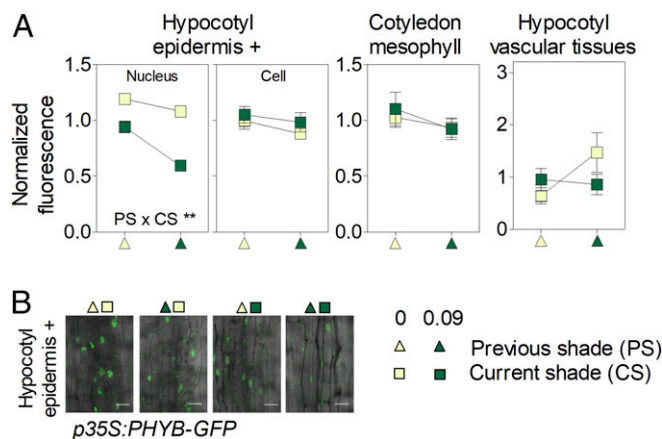

Fig. 2. Dynamics of phyB under persistent shade. Shown are cell and nuclear fluorescence driven by the *p35S:PHYB-GFP* transgene in mesophyll cells of the cotyledons, the epidermal and subepidermal cells of the hypocotyl (epidermis +), and the vascular tissues of the hypocotyl as affected by CS and PS. (*A*) Means ± SE (whenever larger than the symbols) of at least 24 seedlings. **$P < 0.01$. (*B*) Representative confocal images of the epidermal and subepidermal cells of the hypocotyl. (Scale bar = 25 μm.)

tissues of the hypocotyl (Fig. 3 *A* and *B*). In protein blots of entire seedlings, PIF4-HA increased additively in response to CS and PS (Fig. 3 *C* and *D*), suggesting that PIF4 in the stem vascular tissues makes a significant contribution to the overall pool of the protein, consistent with the pattern of *PIF4* gene expression in seedlings (*SI Appendix*, Fig. S4C). Transcriptional activity of PIF4 also depends on the abundance of negative regulators such as HFR1 (32) and ELF3 (33, 34), but stability of these proteins was not affected by PS (*SI Appendix*, Fig. S6), despite the strong response of HFR1 to CS (35).

Under prolonged shade, the abundance of PIF4 remained elevated in the epidermis of hypocotyl tissues, continued to increase (i.e., it was overelevated) in vascular tissues, and decreased in the cotyledons. We therefore investigated which one of these three temporal patterns shows the best correlation with growth. The model based on a transient increase in PIF activity showed poor goodness of fit compared with the model based on elevated or overelevated PIF activity under persistent shade (Fig. 3 *E* and *F*). This result suggests that the commitment to shade avoidance requires persistent PIF activity. To test this prediction, we compared the effects of PS intensity in the wild type and the *pif4 pif5 pif7* mutant exposed to intense CS. The significantly lower slope of the mutant confirms the prediction of the model (Fig. 3*G*). The model based on overelevated PIF activity outperformed that based on stably elevated activity (Fig. 3*F*), suggesting that the overaccumulated PIF4 observed in vascular tissues could be important. To test this hypothesis independently, we analyzed whether overaccumulation of PIF4 in vascular tissues promotes growth. Expression of PIF4 in phloem companion cells, by using the *SUC2* promoter, was, per se, sufficient to promote hypocotyl growth in transgenic seedlings (Fig. 3*H*), confirming the prediction of the model.

Prolonged Shade Reshapes Auxin Perception and Signaling. Since growth under prolonged shade depends on auxin but is not mediated by enhanced auxin levels, we investigated the levels of auxin perception and signaling components. The *MSG2/IAA19* and *IAA29* genes are direct targets of PIF4 (12) expressed in vascular tissues of the hypocotyl (36, 37), and prolonged shade enhanced their expression in a PIF4-dependent manner (Fig. 4*A*, see also Fig. 1*B*). Prolonged shade also enhanced the abundance of the AFB2 and TIR1 auxin receptor proteins in the hypocotyl (Fig. 4 *B* and *C* and *SI Appendix*, Fig. S8). This effect correlated with a reduction in the expression of *MIR393* (Fig. 4 *D* and *E* and *SI Appendix*, Fig. S8), which is a negative regulator of auxin receptor genes (25, 26). The GUS signal, driven by a mutant *pAFB2:AFB2m-GUS* transgene resistant to *MIR393*, showed distorted responses (Fig. 4 *B* and *C*). Finally, CS and PS both slightly induced *ARF6* expression (*SI Appendix*, Fig. S7 and Table S1), although ARF6 protein levels were smaller under PS, probably due to an effect of PS on ARF6 stability (Fig. 4 *F* and *G*).

Changes in Auxin Perception/Signaling Sustain Growth Promotion. The slope of the growth response to PS in seedlings exposed to intense CS was significantly reduced in the *tir1 afb2* mutant compared with the wild type (growth rate/PS, 1.07 ± 0.08 and 1.69 ± 0.04, respectively; $P < 0.0001$), indicating that the commitment to shade avoidance requires auxin perception. To assess the physiological significance of the temporal dynamics of auxin
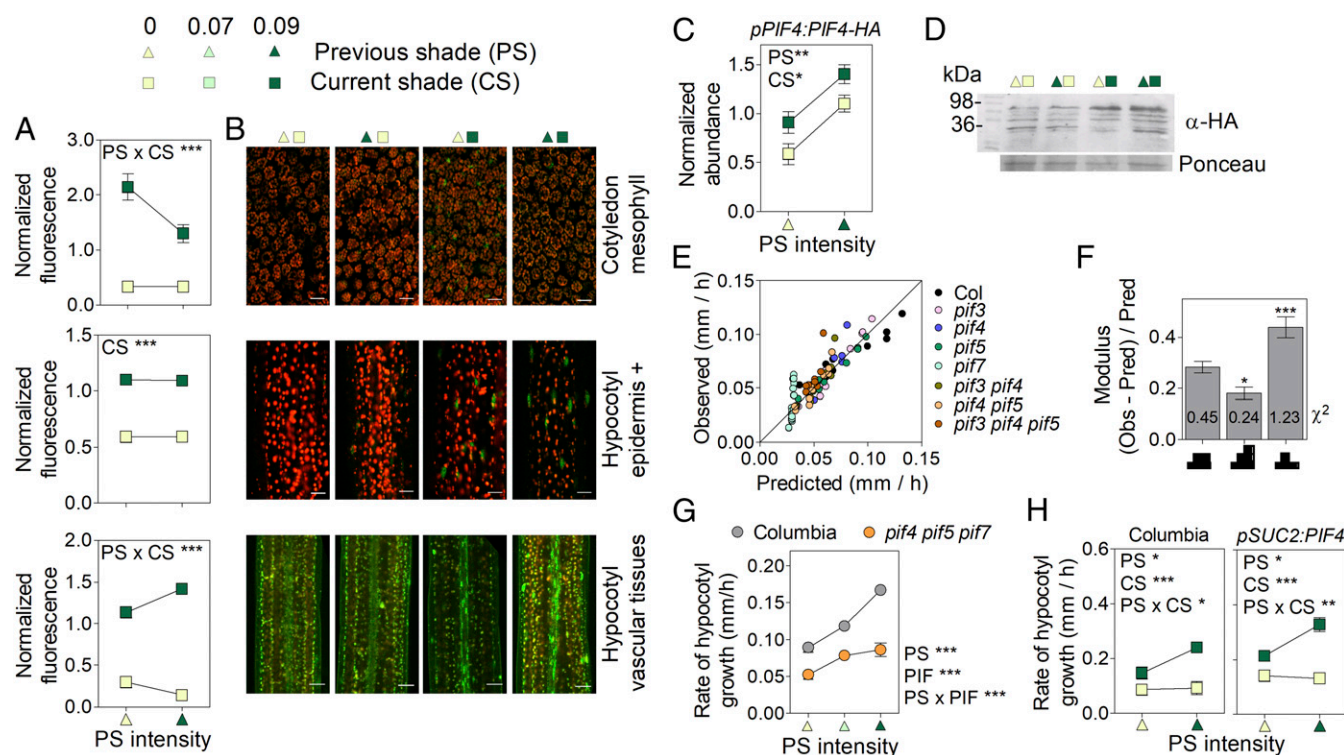

Fig. 3. Persistent shade increases PIF4 levels to promote growth. Shown are effects of CS and PS on (*A* and *B*) nuclear fluorescence driven by the *pPIF4P:PIF4-GFP* transgene in mesophyll cells of the cotyledons, the epidermal and subepidermal cells of the hypocotyl (epidermis +), and the vascular tissues of the hypocotyl and on (*C* and *D*) PIF4 abundance in protein blots. (In *B*, scale bar = 25 μm. For representative confocal images, the GFP signal is green, while red represents autofluorescence of chlorophyll. In *D*, a representative protein blot is shown.) (*E*) Observed hypocotyl growth (*SI Appendix*, Table S2) vs. values predicted by a model based on PIF4 levels increased by CS and PS (i.e., overincreasing under persistent shade). (*F*) Goodness-of-fit of the model [as denoted by low observed (Obs)/predicted (Pred) Obs ratios and the χ^2 values (numbers on bars)] when considering either stably increased, overincreasing, or decreasing PIF activity (represented in abscissa). (*G*) The *pif4 pif5 pif7* mutant has significantly reduced response to PS. (*H*) Expression of PIF4 in vascular tissues promotes hypocotyl growth in *Arabidopsis* (both lines in the Columbia wild-type background). Means ± SE (whenever larger than the symbols) of (*A* and *G*) 20 or (*H*) 15 seedlings or (*C*) 3 biological replicates. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
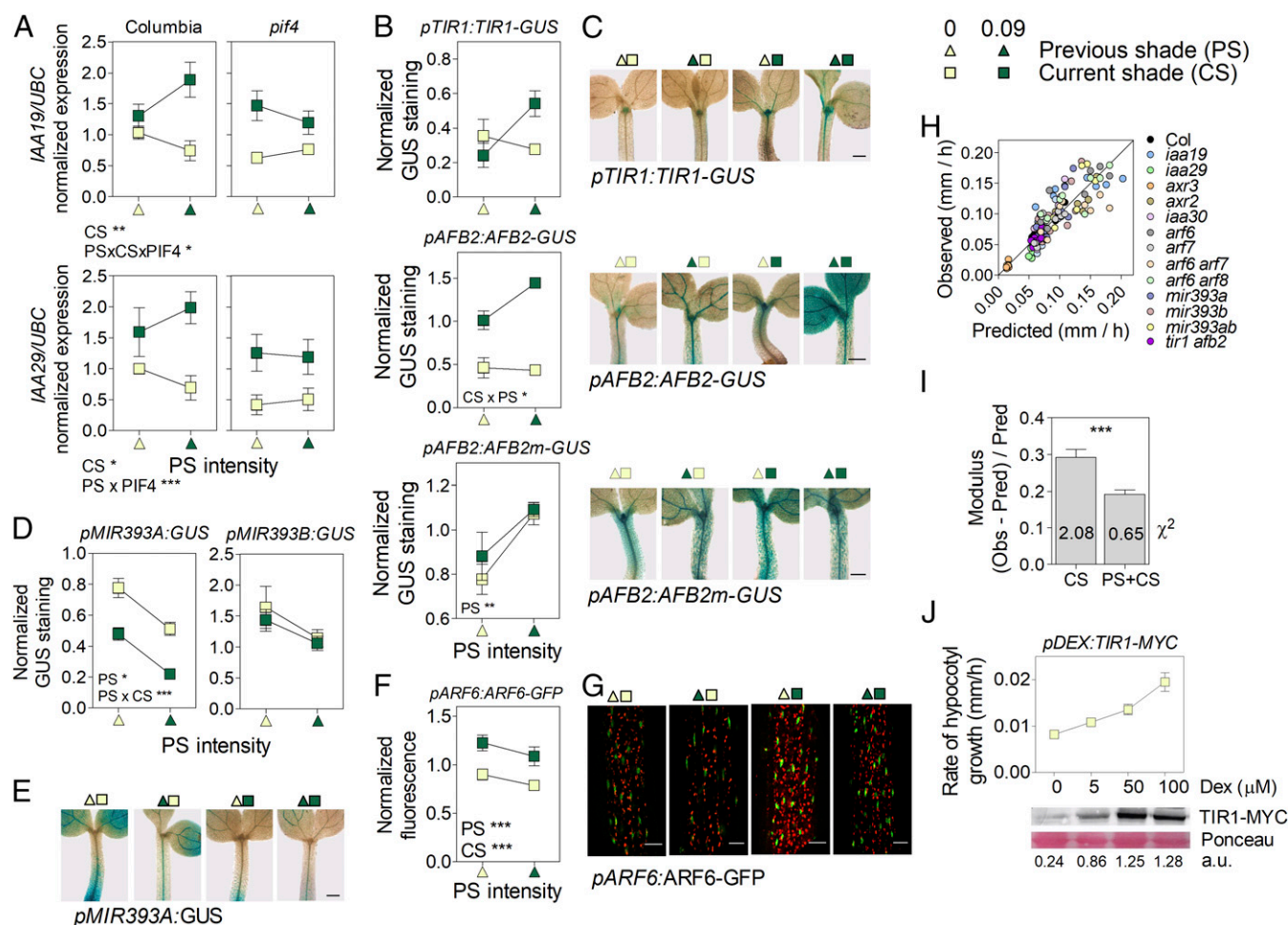
Fig. 4. Persistent shade modifies auxin perception and signaling to promote growth. Effects of (*A*) CS and PS on *MSG2/IAA19* and *IAA29* expression in Columbia wild type and *pif4*, (*B* and *C*) GUS staining driven by the *pAFB2:AFB2-GUS, pTIR1:TIR1-GUS*, or *pAFB2:AFB2m-GUS* transgenes, (*D* and *E*) GUS staining driven by the *pMIR393a:GUS* transgene, and (*F* and *G*) fluorescence driven by the *pARF6:ARF6-GFP* transgene. (In *C* and *E*, scale bar = 200 μm. In *G*, scale bar = 35 μm.) (*H*) Observed hypocotyl growth (*SI Appendix*, Table S2) vs. values predicted by a model based on *MSG2/IAA19, IAA29, ARF6*, AFB2, and TIR1 levels as affected by CS and PS. (*I*) Goodness-of-fit of the models [as denoted by low Obs-pred/Obs ratios and the χ^2 values (numbers on bars)] when considering either CS alone or CS and PS. (*J*) Enhanced expression of TIR1 by Dexamethasone induction is enough to promote hypocotyl growth. Means ± SE of (*A, D*, and *F*) five and (*B*) seven biological replicates or (*J*) 15 seedlings, and (*C, E*, and *G*) representative images. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

perception and signaling components, we modeled growth as a function of the levels of AFB2 plus TIR1, MSG2/IAA19, IAA29, and ARF6 as established by CS (i.e., the values established by CS with PS = 0) or the combination of CS and PS, including several auxin-related mutants in the analysis. The model where dynamics of these components was modified according to CS and PS showed better goodness of fit (Fig. 4 *H* and *I*), suggesting that the effects of PS on auxin perception/signaling are important for the commitment to shade avoidance. If the growth promotion induced by prolonged shade is at least partially mediated by enhanced levels of auxin receptors, increasing the levels of TIR1 should, per se, promote hypocotyl growth. The enhanced growth observed in a *TIR1* inducible line in the presence of inductor and the absence of shade (CS = 0, PS = 0) confirms this prediction of the model (Fig. 4*J*).

Persistent Shade Enhances Sensitivity to Auxin. The model also predicts that persistent shade should enhance the sensitivity to auxin, and we tested this possibility with molecular markers. We observed reduced DII fluorescence in the epidermal cells of the hypocotyl (Fig. 5*A*) and increased GUS staining driven by DR5: GUS (Fig. 5*B*) in the hypocotyl in seedlings exposed to persistent shade compared with the light controls. The seedlings transferred from simulated shade to light showed relatively high DII

and low DR5:GUS activities, indicating that the patterns observed under persistent shade are not the confounding consequence of lack of time to revert the effects of the high auxin levels experienced earlier under shade. Since light controls and seedlings exposed to persistent shade have similar levels of endogenous auxin (Fig. 1*C*), the differences in the activity of the DII and DR5:GUS markers can be attributed to enhanced sensitivity to auxin. Conversely, the reduced DII and high DR5: GUS staining observed in seedlings recently transferred to shade can be attributed to high auxin levels (Fig. 1*C*). In the *mir393ab* background, DR5:GUS levels failed to decrease normally upon transfer from simulated shade to sunlight (Fig. 5 *B* and *C*), confirming the importance of *MIR393* in the control of sensitivity to auxin under fluctuating shade/light conditions.

MSG2/IAA19 and IAA29 Can Act to Promote Auxin Signaling. The mutant analysis revealed that *MSG2/IAA19, IAA29*, and *AXR2/IAA7* promoted and *AXR3/IAA17* inhibited hypocotyl growth (*SI Appendix*, Table S2). Given that hypocotyl growth under PS depends on auxin and Aux/IAA proteins repress auxin responses (20, 21), we propose that some Aux/IAA could indirectly promote growth, by suppressing auxin-induced expression of a subset of *Aux/IAA* genes with stronger growth inhibitory function. In favor of this interpretation, *MSG2/IAA19* and *IAA29*
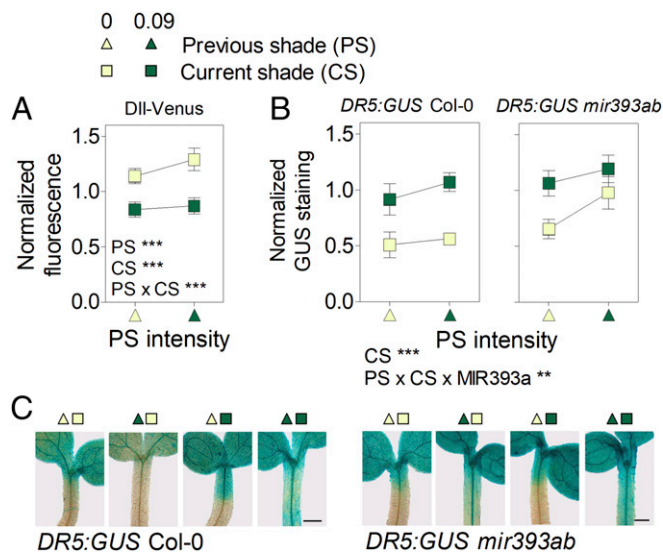
Fig. 5. Persistent shade increases auxin signaling despite the reduced auxin levels. (*A*) DII fluorescence in epidermal and subepidermal tissues of the hypocotyl. (*B* and *C*) GUS staining driven by *DR5:GUS* in the hypocotyl of seedlings of the Columbia wild type and of the *mir393ab* mutant background. (In *C*, scale bar = 200 μm.) Means ± SE of (*A*) 40 or (*B*) 8 biological replicates, and (*C*) representative images. **P < 0.01; ***P < 0.001.

inhibited the expression of *AXR3/IAA17* (Fig. 6*A*; based on gain- and loss-of-function mutants for *IAA19* and *IAA29*, respectively). We also performed transactivation experiments by transiently coexpressing the *pIAA17:LUC* reporter together with a β-estra-diol inducible *IAA19* effector cassette in *Nicotiana benthamiana* leaves. The β-estradiol induction of the *IAA19* effector decreased *pIAA17* activity in this system, providing independent support to the idea that IAA19 can repress the expression of *IAA17* (Fig. 6 *B* and *C*). Noteworthy is that PIF4 enhances the expression of *IAA19* and *IAA29* (Fig. 4*A*), and β-estradiol induction of the PIF4 effector also reduced *pIAA17* activity (Fig. 6*B*). A meta-analysis showed that *MSG2/IAA19* and *IAA29* expression is more significantly induced by both shade and auxin in *Arabidopsis* than the expression of *AXR3/IAA17* (Fig. 6*D*). Similarly, in soybean, four genes with similarity to *MSG2/IAA19* showed increased expression in response to CS and PS (cluster 1) whereas *AXR3/IAA17* (cluster 3) only showed a modest response (*SI Appendix*, Table S1). The weak promotion of *AXR3/IAA17* by shade or auxin is consistent with the strong induction of *IAA19* and *IAA29* by these stimuli.

Discussion

The promotion of stem growth can be induced by neighbor sig-nals within minutes (38–40) and persist for weeks (27). Analyses of plants of the same age subjected to different PS conditions exposed a further temporal dimension of the shade-avoidance syndrome, where prolonged shade reinforces the growth re-sponse and its auxin-related gene expression signature, both in *Arabidopsis* (Figs. 1*B* and 4*A*) and soybean (*SI Appendix*, Fig. S3 and Table S1).

The commitment to shade avoidance under prolonged shade requires auxin but is not mediated by sustained auxin accumu-lation (Fig. 1 *A* and *C*). We cannot rule out that prolonged shade has localized effects on auxin abundance that escaped our detec-tion, but it is clear that the early auxin burst observed in re-sponse to shade does not accompany the response to prolonged shade. Previous studies based on pharmacology or expression of auxin-related genes suggested enhanced auxin sensitivity in re-sponse to neighbor signals (29, 30, 41, 42). Here we show that prolonged shade (*i*) increases the sensitivity to auxin as revealed by low DII fluorescence and high DR5:GUS staining (Fig. 5 *B*

and *C*) in the absence of high auxin levels (Fig. 1*C*) and (*ii*) modifies auxin perception/signaling at multiple levels, by en-hancing the abundance of AFB2 and TIR1, inducing *MSG2/IAA19* and *IAA29* expression, and reducing ARF6 abundance (Fig. 4 *A–F*). Mathematical modeling suggests a contribution of increased AFB2, TIR1, *MSG2/IAA19*, and *IAA29* and reduced ARF6 to the promotion of growth under prolonged shade (Fig. 4 *H* and *I* and *SI Appendix*, Table S2). The role of TIR1 and AFB2 in the commitment to shade avoidance was confirmed by the reduced response to PS in *tir1 afb2* and the growth promotion caused in the absence of shade by increased *TIR1* expression in an inducible line (Fig. 4*J*).

We propose that the growth promotion by MSG2/IAA19 and IAA29 observed here (*SI Appendix*, Table S2) is indirect, via an incoherent feed-forward loop where they inhibit *AXR3/IAA17* expression (Fig. 6*E*). In fact, *AXR3/IAA17* is a strong growth inhibitor (*SI Appendix*, Table S2), and its expression is inhibited by *MSG2/IAA19* and *IAA29* (Fig. 6 *A–C*). Based on gene ex-pression patterns, *MSG2/IAA19* and *IAA29* had been proposed to promote growth early in the morning (43); yet, these IAAs can antagonize auxin action in other contexts (18, 44). The observed growth inhibition by ARF6 (*SI Appendix*, Table S2) provides experimental support to the predictions of mathematical mod-eling indicating that increased ARF levels can reduce auxin re-sponses in some contexts (23).

The phyB-PIF4 module and *MIR393* linked the prolonged-shade cue to growth via changes in auxin sensitivity. The activ-ity of phyB did not recover under prolonged shade (Fig. 2 *A* and *B* and *SI Appendix*, Figs. S4*A* and S5*A*). Persistent shade acted mainly by establishing and maintaining low levels of phyB activity (the response was fully distorted in the *phyB* mutant). In all
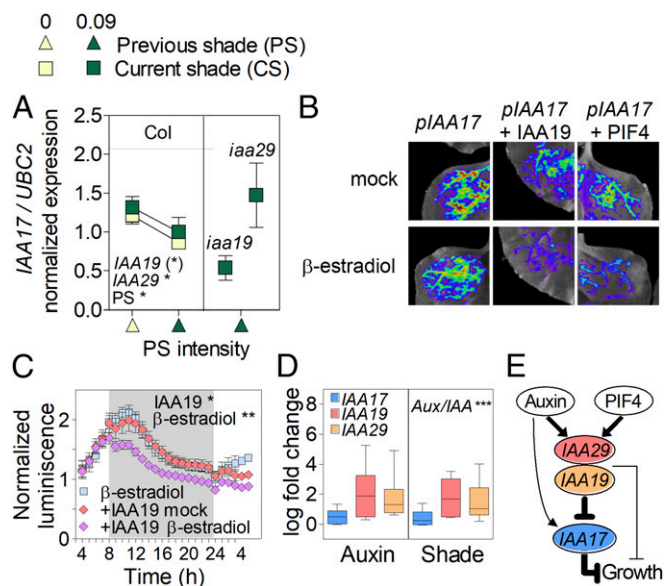

Fig. 6. *MSG2/IAA19* and *IAA29* negatively regulate the strong growth in-hibitor *AXR3/IAA17* under prolonged shade. (*A*) Expression of *IAA17* in the *iaa19/msg2* gain of function and the *iaa29* loss of function mutants com-pared with the Columbia wild type. Means ± SE of three biological repli-cates. (*) P < 0.06; *P < 0.05. (*B* and *C*) The β-estradiol induction of *IAA19* or PIF4 effectors inhibits the activity of *pIAA17:LUC* reporter when transiently expressed in *Nicotiana benthamiana* leaves: (*B*) in vivo imaging and (*C*) time course of *pIAA17:LUC* activity in response to the β-estradiol–induced *IAA19* effector. Median ± SE of 12 biological replicates. *P < 0.05; **P < 0.01. (*D*) Metaanalysis showing that neighbor signals and auxin induce *MSG2/IAA19* and *IAA29* expression to higher levels than expression of *IAA17*. Shown is fold change relative to controls without auxin or shade. The significance of the differences among *Aux/IAA* genes is indicated. ***P < 0.001. (*E*) Incoherent feed-forward loop involved in the promotion of growth by *IAA19* and *IAA29*. Thicker lines indicate stronger positive (→) or negative (⊣) regulations.

tissues examined, we observed a rapid increment of the fluorescence driven by the *pPIF4:PIF4-GFP* transgene in response to shade (8). Prolonged shade maintained elevated levels of PIF4 in the epidermal and subepidermal tissues of the hypocotyl, and enhanced the accumulation of PIF4 in the hypocotyl vascular tissues (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S5*B*). These high PIF4 levels would directly activate the expression of *MSG2/IAA19* and *IAA29* in the hypocotyl (Figs. 1*B* and 4*A*). However, prolonged shade led to a subsequent decrease in nuclear PIF4 levels in the cotyledon mesophyll cells, which can be linked to the decay in auxin levels. The reduction in *MIR393* expression with prolonged shade (Fig. 4 *D* and *E*) could account for the increased accumulation of TIR1 and AFB2 (25, 26) (Fig. 4 *B* and *C*).

We conclude that sustained shade avoidance under prolonged cues from neighboring vegetation proceeds in two distinct temporal phases. During an initial triggering phase, PIFs bind to the promoters of auxin synthesis genes and generate a burst of auxin that promotes growth. This phase appears important to break the growth inertia. Later, the commitment phase is characterized by a system-level shift of the growth-controlling network which leads to a stronger physiological response, without the elevated levels of auxin observed during the initiation state. The initial phase triggered at the cotyledons gives place to a phase apparently dominated by hypocotyl events. Reduced levels of nuclear phyB, *MIR393*, *IAA17/AXR3*, and ARF6, together with the accumulation of PIF4, AFB2, TIR1, *MSG2/IAA19*, and *IAA29*, are characteristic of the second phase.

Materials and Methods

Plant materials, generation of transgenic plants, and shade intensities are described in *SI Appendix, SI Materials and Methods*. The details and procedures for the analyses of gene expression (RNA-seq, qRT-PCR, metaanalysis), growth rates, confocal microscopy, IAA abundance, protein blots, GUS activity (staining and fluorometric), dexamethasone induction, agroinfiltration, and transient activation assays are provided in *SI Appendix, SI Materials and Methods*.

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