Genome-wide regulation of light-controlled seedling morphogenesis by three families of transcription factors

Hui Shi[a,b,1], Mohan Lyu[a,1], Yiwen Luo[a,1], Shoucheng Liu[a,1], Yue Li[a], Hang He[a], Ning Wei[c], Xing Wang Deng[a,2], and Shangwei Zhong[a,2]

[a]State Key Laboratory of Protein and Plant Gene Research, School of Advanced Agricultural Sciences and School of Life Sciences, Peking University, 100871 Beijing, China; [b]College of Life Sciences, Capital Normal University, 100048 Beijing, China; and [c]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520

Three families of transcription factors have been reported to play key roles in light control of *Arabidopsis* seedling morphogenesis. Among them, bHLH protein PIFs and plant-specific protein EIN3/ EIN3-LIKE 1 (EIN3/EIL1) accumulate in the dark to maintain skotomorphogenesis. On the other hand, HY5 and HY5 HOMOLOG (HYH), two related bZIP proteins, are stabilized in light and promote photomorphogenic development. To systemically investigate the transcriptional regulation of light-controlled seedling morphogenesis, we generated *HY5ox/pifQein3eil1*, which contained mutations of *EIN3/EIL1* and four *PIF* genes (*pifQein3eil1*) and overexpression of *HY5*. Our results show that dark-grown *HY5ox/pifQein3eil1* seedlings display a photomorphogenesis highly similar to that of wild-type seedlings grown in continuous light, with remarkably enhanced photomorphogenic phenotypes compared with the *pifQ* mutants. Consistent with the genetic evidence, transcriptome analysis indicated that PIFs, EIN3/EIL1, and HY5 are dominant transcription factors in collectively mediating a wide range of light-caused genome-wide transcriptional changes. Moreover, PIFs and EIN3/EIL1 independently control the expression of light-regulated genes such as *HLS1* to cooperatively regulate apical hook formation, hypocotyl elongation, and cotyledon opening and expansion. This study illustrates a comprehensive regulatory network of transcription activities that correspond to specific morphological aspects in seedling skotomorphogenesis and photomorphogenesis.

light signal | ethylene signal | plant photobiology | PIFs | EIN3/EIL1

L ight provides the energy source for photosynthesis and is a critical environmental regulator for plant growth. Terrestrial flowering plant seedlings grown in subterranean darkness adopt a skotomorphogenic developmental program, characterized by long hypocotyls, apical hook formation, and closed small cotyledons with etioplast (1, 2). Upon emerging from soil covering, light triggers a dramatic morphological and physiological transition from dark-grown skotomorphogenesis to light-grown photomorphogenesis, which is essential for seedling survival (2, 3). This transition involves hypocotyl elongation inhibition, apical hook unfolding, cotyledon opening and expanding, and etioplast-to-chloroplast transition (4–6).

Decades of research has identified positive and negative regulators of light-induced developmental transition. Positive regulators naturally include photoreceptors, among which the phytochromes (phyA to phyE in *Arabidopsis*) and cryptochromes are predominantly responsible for inducing photomorphogenesis in seedlings (1, 7, 8). Photoreceptors ultimately induce the light-controlled switch from skotomorphogenesis to photomorphogenesis via multiple downstream transcription factors. Two bZIP transcription factors—LONGATED HYPOCOTYL 5 (HY5) and HY5 HOMOLOG (HYH) proteins—have been genetically identified as positive regulators that promote light responses (9, 10). Loss of HY5 or HYH causes longer hypocotyl in the light (9, 10). In contrast to HY5, the bHLH transcription factors—PHYTOCHROME-INTERACTING

FACTORs (PIFs), including PIF1, PIF3, PIF4, and PIF5—accumulate in the dark and maintain skotomorphogenesis (4, 11). With light exposure, photoactivated phytochromes are translocated into the nucleus, where they directly interact with, and trigger rapid degradation of, PIF proteins (12–15). The quadruple mutant of PIFs (*pifQ*) exhibits photomorphogenic phenotype in the dark (4, 11), similar to the mutant of COP1, a central repressor of photomorphogenesis (16, 17). COP1 is an E3 ubiquitin ligase and represses light signaling predominantly through the proteasome degradation system (18–20). HY5/HYH proteins are typical transcription factors that are targeted by COP1 for degradation in the dark (10, 18).

When grown under the soil, seedlings simultaneously confront darkness and mechanical pressure from the soil covering. In response to mechanical impedance, seedlings induce ethylene production to exaggerate apical hook formation and repress cotyledon development to reduce mechanical injuries (19, 21, 22). Ethylene is a gaseous hormone and its responses are mediated by the master transcription factors ETHYLENE-INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) (23, 24). In the absence of ethylene, EIN3/ EIL1 proteins are targeted by E3 ligase SCF[EBF1/EBF2] complexes for

Significance

Seedlings of terrestrial flowering plants such as *Arabidopsis* display dramatically different morphologies depending on growing in the dark or light. Dark-grown seedlings exhibit long hypocotyls, apical hook formation, and closed small cotyledons with etioplast. In contrast, light represses hypocotyl elongation, unfolds apical hook, and promotes cotyledon development. How light-controlled seedling morphogenesis is regulated at the transcriptional level remains elusive. To date, three families of transcription factors—PIFs, EIN3/EIL1, and HY5/HYH—have been shown to mediate light responses in seedlings. This study systemically investigates their roles in regulating specific morphological aspects and provides both transcriptomic and genetic evidence that PIFs, EIN3/EIL1, and HY5 are master transcription factors for the proper establishment of seedling skotomorphogenesis and photomorphogenesis.

degradation (25–27). Ethylene represses the action of EBF1/EBF2 to stabilize EIN3 and activate ethylene responses (28, 29). EIN3 proteins are highly accumulated in buried seedlings through both mechanical stress-evoked ethylene production and darkness (19, 21, 22). Previous studies suggest that EIN3 and PIFs regulate the expressions of their common target genes in either an additive or interdependent way (30). Recently, EIN3/EIL1 have been shown to promote seedling soil emergence by exaggerating apical hook formation (22), inhibiting cotyledon opening and expansion (19), and repressing chloroplast development (6).

Here, we present genetic and transcriptomic evidence to demonstrate that PIFs, EIN3/EIL1, and HY5 are master transcription factors for the proper establishment of seedling skoto- and photomorphogenesis. In addition, PIFs and EIN3/EIL1 are further shown to act independently in regulating the main processes of light-controlled seedling morphological development, including apical hook formation, hypocotyl elongation, and cotyledon opening and expansion.

Results

PIFs, EIN3/EIL1, and HY5 Are Essential for Establishment of Light-Controlled Seedling Morphogenesis.
It is known that the proteins of four PIFs (PIF1, PIF3, PIF4, and PIF5) accumulate and act redundantly in maintaining skotomorphogenesis in the dark (4, 11), whereas HY5 protein is elevated in light to promote photomorphogenesis (9, 18). EIN3/EIL1 have been recently identified as a new class of regulators that suppress light-induced seedling developmental transition (5, 19). In 10-d-old dark-grown *ein3eil1* mutant seedlings, apical hooks were unfolded and cotyledons were partially open, similar to those of *pif1pif3* mutants (*SI Appendix*, Fig. S1), while the *pif1pif3ein3eil1* quadruple mutants displayed an additive effect in cotyledon opening (*SI Appendix*, Fig. S1). As previously documented, the 4-d-old dark-grown *pif1pif3pif4pif5* (*pifQ*) mutant showed partially constitutive photomorphogenesis, manifested by reduced hypocotyl elongation, unfolded apical hook, and largely opened and expanded cotyledons (Fig. 1A). To determine the relationship of EIN3/EIL1 and PIFs in repressing photomorphogenesis, we crossed *pifQ* and *ein3eil1* mutants to generate the *pifQein3eil1* (*pifQee*) sextuple mutant. The cotyledons of 4-d-old dark-grown *pifQee* seedlings were fully opened and expanded, similar to those of 4-d-old dark-grown *cop1-4* mutant or continuous red light (R4)-grown wild-type (WT) seedlings (Fig. 1). Quantitative cotyledon areas and open angles indicate that PIFs and EIN3/EIL1 additively repressed cotyledon development in the dark (Fig. 1 B and D and *SI Appendix*, Fig. S2).

Notably, mutations of *EIN3/EIL1* did not further alter the hypocotyl lengths of *pifQ*, and the hypocotyls of *pifQee* were still much longer than those of light-grown WT (Fig. 1 A and C). HY5 has been shown to accumulate in light to repress hypocotyl elongation (9, 18). We therefore examined the protein levels of HY5 in the seedlings and found that HY5 proteins were stabilized in light-grown WT, but mutation of *PIFs* and *EIN3/EIL1* in the dark did not change its abundance (*SI Appendix*, Fig. S3). To investigate whether HY5 regulates the hypocotyl lengths of *pifQee*, we constitutively overexpressed HY5 in dark-grown *pifQee* to reach a comparable level with light-grown WT (Fig. 1A). Although the developmental pattern of cotyledons was not altered by overexpressing *HY5* (Fig. 1 A and B), the hypocotyl lengths of *HY5*ox/*pifQee* were further shortened (Fig. 1 A and C). Remarkably, the dark-grown *HY5*ox/*pifQee* showed photomorphogenic phenotypes most similar to those of dark-grown *cop1-4* or light-grown WT (Fig. 1A). These pieces of genetic evidence indicate that PIFs, EIN3/EIL1, and HY5 are central transcription factors which function additively in establishing seedling morphogenesis responding to light.
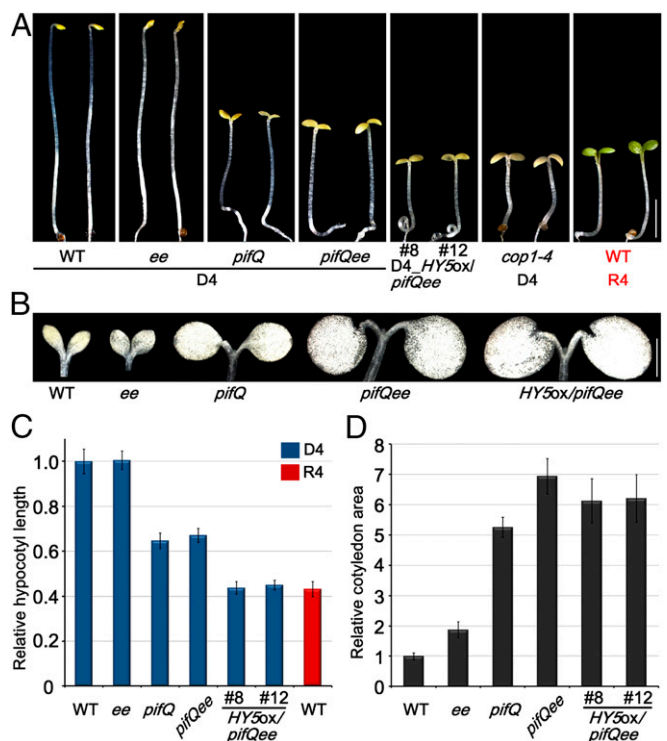

Fig. 1. PIFs, EIN3/EIL1, and HY5 are the major transcription factors in directing light-controlled seedling morphogenesis. (*A*) Images of 4-d-old Col-0 (WT), *ein3eil1* (ee), *pifQ*, *pifQee*, *HY5*ox/*pifQee*, and *cop1-4* seedlings grown in the dark (D4) or in continuous red light (R4). (Scale bar, 2 mm). (*B*) Cotyledon photographs of 10-d-old dark-grown seedlings. (Scale bar, 0.5 mm.) (*C*) Hypocotyl lengths of 4-d-old seedlings grown in the dark (D4) or in R4. The WT value was set as 1, and the relative hypocotyl lengths were calculated. Error bars represent SD (n = 20). (*D*) Cotyledon areas of 10-d-old dark-grown etiolated seedlings. The WT value was set as 1, and the relative cotyledon areas were calculated. Error bars represent SD (n = 20).

Light-Directed Seedling Transcriptome Changes Are Largely Mediated by PIFs, EIN3/EIL1, and HY5.
To understand how PIFs, EIN3/EIL1, and HY5 control light-responsive seedling morphogenesis, we further examined the transcriptomes in *pifQ*, *pifQee*, and *HY5*ox/*pifQee* in comparison with the light-grown profile by mRNA deep-sequencing analysis. Seedlings were grown in the dark (D4) or under R4 for 4 d and harvested for the transcriptomic analysis. Our results showed that about one-fourth (7,015 genes) of the whole genome in WT was statistically significantly twofold (SSTF) changed by red light (R4-WT versus D4-WT) (Fig. 2A). We then compared the gene expression profiles of WT with those of dark-grown *pifQ*, *pifQee*, and *HY5*ox/*pifQee* and identified 2,629 SSTF genes in *pifQ*; 3,620 SSTF genes in *pifQee*; and 3,558 SSTF genes in *HY5*ox/*pifQee* (Fig. 2A and *SI Appendix*, Fig. S4A). Moreover, 4,772 genes were shown to be significantly regulated at least in one mutant background and were identified as the PIFs/EIN3/HY5-regulated genes (Fig. 2A and *SI Appendix*, Fig. S4A). Among them, 71% (3,384 genes) were commonly regulated at least in two mutant backgrounds (Fig. 2A and *SI Appendix*, Fig. S4A). By comparing the R4- and PIFs/EIN3/HY5-regulated genes, we found that PIFs/EIN3/HY5-regulated genes in the dark were estimated to account for half of the light-directed transcriptome changes (Fig. 2A and *SI Appendix*, Fig. S4B). Together, the genome-wide gene expression analysis supports our genetic data demonstrating the essential roles of PIFs, EIN3/EIL1, and HY5 in regulating light-controlled seedling morphogenesis.
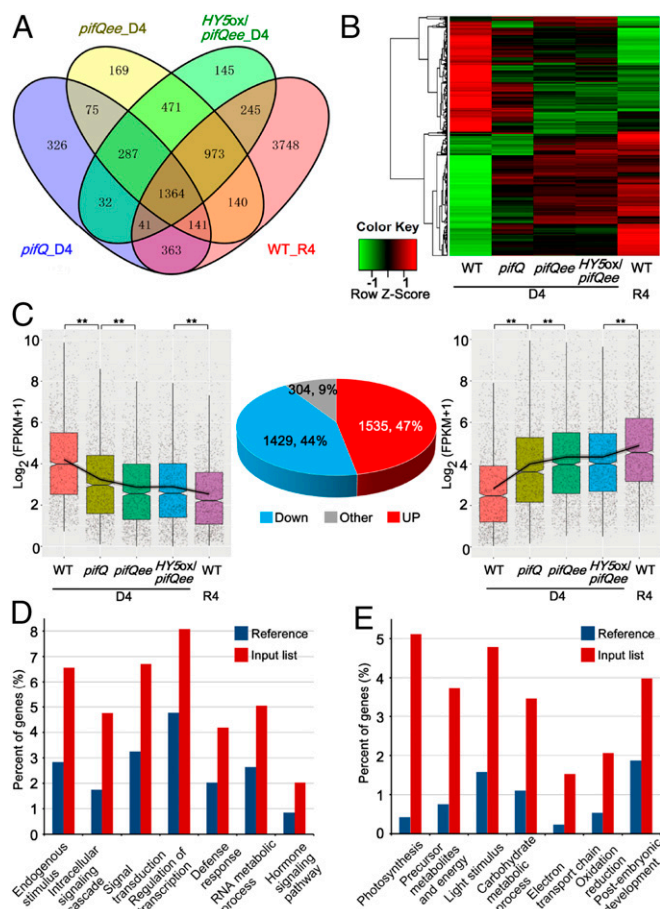
Fig. 2. Light-induced genome-wide transcriptional changes are largely mediated by PIFs, EIN3/EIL1, and HY5. (*A*) Venn diagram showing overlaps among genes regulated by light, *pifQ*, *pifQee*, and *HY5ox/pifQee*. The seedlings were grown for 4 d in the dark (D4) or in R4. (*B*) Expression levels of the PIFs/EIN3/HY5- and light-coregulated 3,267 genes in different genotypes. The FPKM values of these genes were normalized based on the Z-score method. (*C*) Box plot representation of the PIFs/EIN3/HY5-mediated light down-regulated (*Left*) or light up-regulated (*Right*) gene FPKM values in different genotypes. **$P < 0.01$, Wilcoxon test. (*D* and *E*) Gene ontology analysis of the light down-regulated (*D*), or light up-regulated (*E*) genes mediated by the PIFs/EIN3/HY5 pathway.

PIFs, EIN3/EIL1, and HY5 Collectively Direct a Wide Range of Light-Regulated Biological Processes. By comparing the SSTF genes regulated by PIFs/EIN3/HY5 and light, we identified 3,267 overlapped genes (*SI Appendix*, Fig. S4*B* and Dataset S1). After normalizing the expression levels [FPKM (fragments per kilobase of exon per million fragments mapped)] of the 3,267 genes in different samples based on the Z-score method, we found that about half of the 3,267 genes in D4-*pifQ* were redirected to the expressing patterns of R4-WT (Fig. 2*B*). Mutation of *EIN3/EIL1* in *pifQ* remarkably exaggerated the expressing pattern changes (Fig. 2*B*), and most of the 3,267 genes in D4-*HY5ox/pifQee* were regulated in the same direction as those of R4-WT (Fig. 2*B*). We further analyzed the regulation pattern of these 3,267 genes and found that 91% (2,964 genes) were modulated in the same way by light and by PIFs/EIN3/HY5 (Fig. 2*C*). We referred to these 2,964 genes as members of the PIFs/EIN3/HY5-mediated light signaling pathway. The box plots of average FPKM values similarly showed that the expression of light-repressed genes was gradually inhibited, while the levels of light-activated genes were progressively increased in the seedlings of *pifQ* and *pifQee* (Fig. 2*C*). These analyses

suggest that PIFs, EIN3/EIL1, and HY5 act collectively in directing light-induced transcriptome changes.

We then performed a gene ontology (GO) enrichment analysis to investigate the biological processes that PIFs/EIN3/HY5 mediate in light responses (Dataset S2). Our results revealed that among the light-repressed genes, PIFs/EIN3/HY5 most significantly regulated the following GO categories: response to endogenous stimulus ($P < 10^{-11}$), intracellular signaling cascade ($P < 10^{-11}$), signal transduction ($P < 10^{-9}$), regulation of transcription ($P < 10^{-6}$), defense response ($P < 10^{-6}$), RNA metabolic process ($P < 10^{-6}$), and hormone signaling pathway ($P < 10^{-4}$) (Fig. 2*D*). In contrast, among the light-activated genes, the top enriched GO categories mediated by PIFs/EIN3/HY5 are photosynthesis ($P < 10^{-48}$), precursor metabolites and energy ($P < 10^{-19}$), response to light stimulus ($P < 10^{-14}$), carbohydrate metabolic process ($P < 10^{-11}$), electron transport chain ($P < 10^{-10}$), oxidation reduction ($P < 10^{-8}$), and postembryonic development ($P < 10^{-6}$) (Fig. 2*E*). Thus, PIFs, EIN3/EIL1, and HY5 integrate both internal and external effects to mediate a wide range of biological processes in light responses.

PIFs and EIN3/EIL1 Independently Regulate the Expression of Common Downstream Genes. Given the essential roles of PIFs and EIN3/EIL1 in sustaining skotomorphogenesis, we next investigated how PIFs and EIN3/EIL1 regulate the expression of common downstream genes. We adopted a 5X*EBS:GUS* transgenic line in which the glucuronidase (*GUS*) reporter gene was driven by five tandem repeats of EIN3 binding sequence (*EBS*) to monitor the action of EIN3/EIL1 (31). GUS staining analysis showed that EIN3/EIL1 were active in the tissues of cotyledons, apical hook, and the upper hypocotyl, which was notably enhanced by ethylene treatment (Fig. 3*A*). Then we used the *PIFs*-promoter:*GUS* reporter lines in which the GUS gene was driven by each of the *PIF* promoters (32), and assayed the distribution of PIFs' expression in etiolated seedlings. We found that all four PIF genes were highly expressed in the same tissues where EIN3/EIL1 function (Fig. 3*A*). These results suggest that EIN3/EIL1 and PIFs probably act in a similar spatial pattern.

Next, we expressed PIFs and EIN3 genes individually into the *pifQee* sextuple mutant and examined the expression levels of three *pifQee* down-regulated genes by qRT-PCR in these transgenic lines. Intriguingly, the transcriptions of all three highly repressed genes (*PIL2*, *BEE1*, and *EDF3*) in *pifQee* were remarkably activated by single *PIF*s or *EIN3* to the comparable levels as seen in WT (Fig. 3*B*). Conversely, we also examined the expression of four *pifQee* up-regulated genes (*EXPA1*, *AT5G61290*, *BRS1*, and *RCA*), and found that the high expression of all four genes in *pifQee* was dramatically suppressed by introducing single *PIF*s or *EIN3* (Fig. 3*C*). Thus, PIFs and EIN3/EIL1 could regulate downstream gene expressions in an independent and redundant manner.

PIFs and EIN3/EIL1 Additively Activate Transcription of *HLS1* to Promote Apical Hook Formation in the Dark. Apical hook formation is a characteristic feature of etiolated seedlings at the apex region. Because all four PIFs and EIN3 were expressed in apical hook (Fig. 3*A*), we wondered how PIFs and EIN3 may synergistically regulate this morphological trait. Previous studies have reported that, in response to ethylene, EIN3 activates *HOOK-LESS1* (*HLS1*) gene expression to promote apical hook formation (22, 33, 34). Agreeably, the angle of the apical hook was visibly reduced in *ee* (Fig. 4*A*). Treatment with aminocyclopropane carboxylic acid (ACC), the ethylene biosynthetic precursor, greatly enhanced the apical hook angles in WT, *pifQ*, and *EIN3/pifQee*, but not in lines that lacked *EIN3/EIL1* (Fig. 4*A*). Mutation of *PIFs* showed dramatically reduced hook formation, and the apical hooks of *pifQee* seedlings were fully unfolded (Fig. 4*A*). We further expressed individual *PIFs* into *pifQee* and found that expression of each single *PIF* was sufficient to restore the hook formation to various degrees (Fig. 4*B* and *SI Appendix*, Fig. S5).
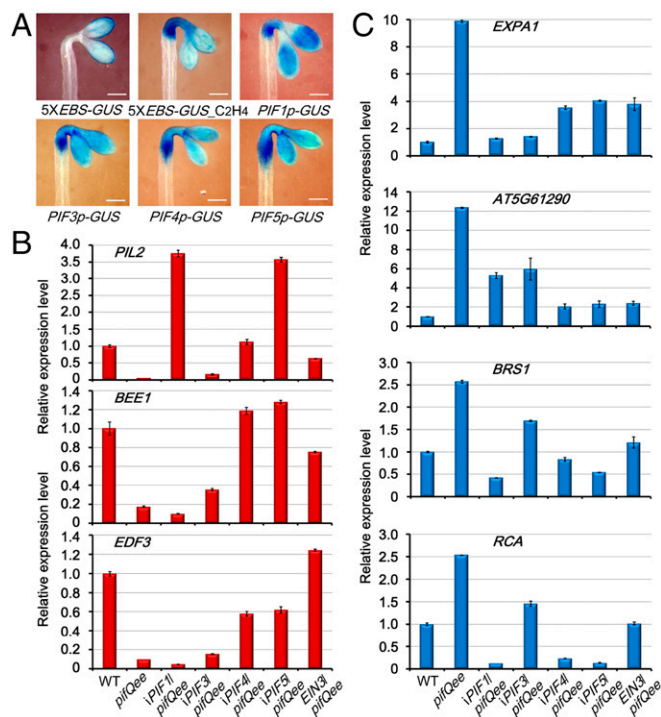
Fig. 3. PIFs and EIN3/EIL1 independently regulate the expression of light-responsive genes. (*A*) Representative images of GUS staining in 4-d-old dark-grown seedlings. 5X*EBS-GUS* transgenic lines harbor five tandem repeats of *EBS* fused with the *GUS* reporter gene. C_2H_4 indicates that the seedlings were treated with C_2H_4 gas for 4 h before staining. *PIF1p*- to *PIF5p*-GUS indicates that the *GUS* reporter gene is driven, respectively, by the promoter of *PIF1* to *PIF5* genes. (Scale bar, 0.2 mm.) (*B* and *C*) qRT-PCR analysis of the expression levels of light-repressed (*B*) or light-activated (*C*) genes in 4-d-old etiolated seedlings grown on half-strength Murashige and Skoog (1/2 MS) medium supplemented with 10 μM β-estradiol. i*PIF1/pifQee* to i*PIF5/pifQee* are the inducible transgenic plants in which the respective *PIF1* to *PIF5* genes driven by β-estradiol–induced *pER8* promoters are transformed into *pifQee* background. *EIN3/pifQee* presents the transgenic plants with *EIN3* native promoter-driven *EIN3* gene in the *pifQee* background. The gene expression levels were normalized to that of two internal control genes (*PP2A* and *SAND*) and relative to the WT sample. Error bars indicate the SD of three technical repeats. All experiments were repeated independently three times.

This genetic evidence indicates that EIN3 or each of the PIFs alone is capable of promoting hook formation.

Since HLS1 is the main regulator of hook formation, with *hls1* exhibiting fully unfolded apical hook (33), we next analyzed the regulation of *HLS1* gene expression by EIN3 and PIFs. qRT-PCR results showed that mutation of either *EIN3/EIL1* or *PIF*s notably reduced *HLS1*'s transcription, which was further declined in *pifQee*, suggesting that EIN3/EIL1 and PIFs function additively in activating the expression of *HLS1* (Fig. 4*C*). In agreement with the hook formation phenotypes, the expression levels of *HLS1* were partially restored by introducing *EIN3* back into *pifQee* and were fully rescued to WT levels in ACC-treated *pifQ* or *EIN3/pifQee* (Fig. 4*C*). Furthermore, the highly inhibited *HLS1* transcription in *pifQee* was reactivated by introducing single *PIF*s into it (Fig. 4*D*). Collectively, these data demonstrate that EIN3 and PIFs promote apical hook formation by activating *HLS1* gene expression additively and independently of each other.

PIFs and EIN3/EIL1 Bind to the Promoter of *HLS1* in an Independent Manner. To further understand how PIFs and EIN3 regulate the gene expression of *HLS1*, we analyzed the genomic occupancy of PIFs and EIN3 based on previously reported chromatin immunoprecipitation sequencing (ChIP-seq) data (32, 35). The ChIP-seq results revealed the specific associations of EIN3 and PIFs with the promoter of *HLS1* (Fig. 4*E*). Moreover, the binding peaks of EIN3 and PIFs in the *HLS1* promoter region largely overlapped (Fig. 4*E*), suggesting that EIN3 and PIFs coassociated with the same regions. To investigate the interrelationship of PIFs and EIN3 in binding to the *HLS1* promoter regions, we performed ChIP-qPCR assays using transgenic lines in which the individual *PIF*s or *EIN3* was expressed in the *pifQee* background. Compared with the *PP2A* promoter region, the DNA fragments of the *HLS1* promoter region were evidently enriched in immunoprecipitation by single PIFs or by EIN3 (Fig. 4*F*). In addition, the enrichment of *HLS1* promoter fragments immunoprecipitated by EIN3 in the *pifQee* background was strongly enhanced by ACC treatment (Fig. 4*F*). Moreover, the relative enrichment of *HLS1p* by individual PIFs or EIN3 correlated with the expression levels of *HLS1* and with the restoration of the apical hook in the corresponding transgenic lines (Fig. 4 *A–D*). Thus, these results imply that PIFs and EIN3 are each capable of binding to the promoter of *HLS1* to activate *HLS1*'s transcription.

PIFs and EIN3 Cooperatively Regulate Hypocotyl Elongation and Cotyledon Development. Decreasing rate of hypocotyl elongation is usually quantitatively correlated to the level of light responses in a plant seedling (18). We found that the shortened hypocotyl of *pifQee* could be reversed by expressing individual PIFs (PIF3, PIF4, or PIF5) in *pifQee* in the dark (Fig. 5 *A* and *B*). However, expressing EIN3 could not restore the hypocotyl elongation of *pifQee* (*SI Appendix*, Fig. S6). Instead, the hypocotyl lengths of
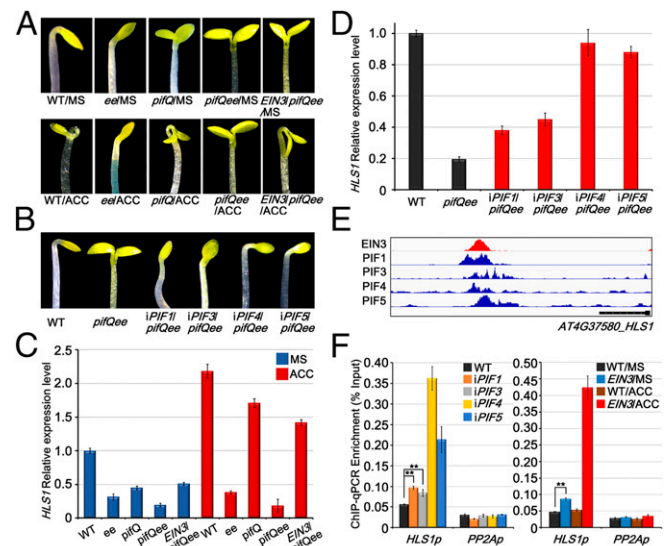

Fig. 4. Apical hook formation is promoted by PIFs and EIN3/EIL1 through directly activating *HLS1* transcription. (*A* and *B*) Apical hook images of 4-d-old etiolated seedlings grown on half-strength Murashige and Skoog (1/2 MS) medium without (MS) or with (ACC) 10 μM ACC supplementation (*A*) or on 1/2 MS medium supplemented with 10 μM β-estradiol (*B*). (*C* and *D*) qRT-PCR analysis of *HLS1* gene expression levels in 4-d-old etiolated seedlings grown on 1/2 MS medium supplemented without or with 10 μM ACC (*C*) or on 1/2 MS medium supplemented with 10 μM β-estradiol (*D*). (*E*) Visualization of EIN3 and PIFs ChIP-seq data in the genomic regions encompassing the promoter region of *HLS1* gene. (*F*) ChIP-qPCR analyzing the associations of PIFs and EIN3 with HLS1 promoter in 4-d-old etiolated seedlings grown on 1/2 MS medium supplemented with 10 μM β-estradiol (*Left*) and without or with 10 μM ACC (*Right*). iPIF1 to iPIF5 are the respective iPIF1/*pifQee* to iPIF5/*pifQee* transgenic plants, and *EIN3* presents the *EIN3/pifQee* transgenic plants. WT and *PP2A* promoter (*PP2Ap*) were used as controls. **$P < 0.01$, Student's *t* test. Error bars indicate the SD of three technical repeats. All experiments were repeated independently three times.
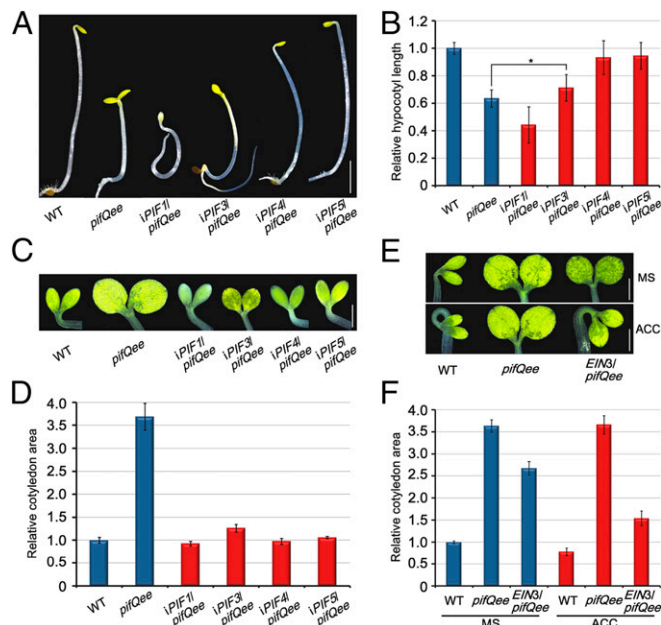
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Fig. 5. PIFs and EIN3/EIL1 cooperatively regulate hypocotyl elongation, cotyledon opening, and cotyledon expansion. (*A* and *B*) Representative images (*A*) and hypocotyl lengths (*B*) of 4-d-old etiolated seedlings grown on half-strength Murashige and Skoog (1/2 MS) medium supplemented with 10 μM β-estradiol. (Scale bar, 2 mm.) The WT value was set as 1, and the relative hypocotyl lengths were calculated. Error bars represent SD (*n* = 20). *P < 0.05, Student's *t* test. (*C*–*F*) Representative cotyledon images (*C* and *E*) and cotyledon areas (*D* and *F*) of 4-d-old etiolated seedlings grown on 1/2 MS medium supplemented with 10 μM β-estradiol (*C* and *D*) and without (MS) or with (ACC) 10 μM ACC (*E* and *F*). (Scale bar, 0.5 mm.) The WT value was set as 1, and the relative cotyledon areas were calculated. Error bars represent SD (*n* = 20).

EIN3/*pifQee* were further shortened by ACC application (*SI Appendix*, Fig. S6). In previous studies, we revealed that EIN3 concomitantly activates a PIF3-dependent promoting pathway and an ERF1-mediated repressing pathway, which antagonistically regulated hypocotyl elongation (36). Thus, it is likely that EIN3 activates the ERF1 pathway in *pifQee* to repress hypocotyl elongation.

Before emerging from the soil, cotyledon development of buried seedlings is strictly inhibited to reduce soil mechanical resistance. We investigated how PIFs and EIN3/EIL1 act in repressing cotyledon development. Remarkably, the results showed that individual PIFs alone could fully restore the etiolated cotyledon morphology, in contrast to the opened and expanded cotyledons of *pifQee* (Fig. 5 *C* and *D*). Expressing *EIN3* in *pifQee* partially reduced the area of cotyledons, and ACC treatment further decreased the cotyledon expansion of *EIN3*/*pifQee* to a comparable level with WT (Fig. 5 *E* and *F*). Similar to the regulation of cotyledon expansion, a single *PIF* fully rescued the cotyledon-opening phenotype of *pifQee* (Fig. 4*B* and *SI Appendix*, Fig. S7). With ACC application, the cotyledons of *EIN3*/*pifQee* were also largely closed (Fig. 4*A* and *SI Appendix*, Fig. S7). Together, these genetic results indicate that PIFs and EIN3 repress cotyledon development in an independent way.

Discussion

Most higher plants, such as *Arabidopsis*, exhibit distinct morphogenesis depending on the light environment. How light controls genomic gene expression to direct seedling morphogenesis has been extensively studied in the past decades (37). PIFs are well documented to accumulate and sustain seedlings' skotomorphogenic state in darkness (4, 11, 38), whereas HY5 is accumulated in light and promotes light responses (9, 18, 39). EIN3/EIL1 have previously been reported to be indispensable for ethylene signaling

transduction pathway (23, 24). In this study, we propose that the three families of transcription factors—PIFs, EIN3/EIL1, and HY5/HYH—function collectively in directing the transcriptional network underlying light-induced developmental transition (Fig. 6*A*). When grown in subterranean darkness, phyB is inactive and COP1 is activated to degrade HY5 and EBF1/EBF2. PIFs and EIN3/EIL1 proteins are highly accumulated and maintain skotomorphogenesis. Upon light exposure, photoactivated phyB directly binds to, and induces rapid degradation of, PIFs and EIN3/EIL1, thus removing the repression of PIFs and EIN3/EIL1 on light responses. At the same time, COP1 is repressed and HY5 proteins are stabilized to further promote light-induced photomorphogenesis (Fig. 6*B*).

Skotomorphogenesis is a dark-adaptive strategy adopted by seedlings grown in subterranean darkness. The findings presented here indicate that maintenance of skotomorphogenic development requires the concerted action of PIFs and EIN3/EIL1. Remarkably, we demonstrate that overexpressing single *PIF*s or *EIN3* alone can reverse the constitutive photomorphogenic phenotype of *pifQee*, including apical hook formation, hypocotyl elongation,
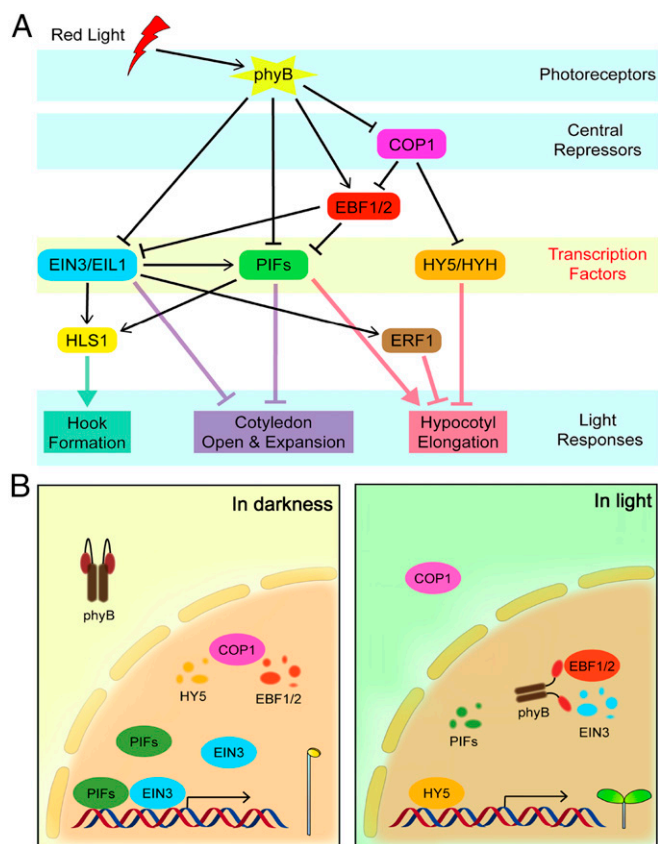

Fig. 6. Model of the transcriptional regulation of light-controlled seedling morphogenesis by PIFs, EIN3/EIL1, and HY5. (*A*) PIFs, EIN3/EIL1, and HY5 direct the transcriptional network underlying light responses in seedlings. PIFs and EIN3/EIL1 cooperatively repress light-induced morphological transition, including apical hook unfolding, cotyledon opening and expansion, and inhibition of hypocotyl elongation, whereas HY5 inhibits hypocotyl elongation to promote light responses. (*B*) Light oppositely regulates the protein levels of PIFs, EIN3/EIL1, and HY5 to initiate seedling morphological transition. When grown in the dark, photoreceptor phyB is inactive, and the central repressor COP1 is activated to control the protein abundance of downstream factors. PIFs and EIN3/EIL1 accumulate and function collectively in sustaining skotomorphogenesis. Upon light activation, phyB directly induces rapid degradation of PIFs and EIN3/EIL1, and indirectly stabilizes HY5 by repressing COP1's action to promote the photomorphogenic development.

and cotyledon development. The independent and redundant nature of PIFs and EIN3/EIL1 provides a molecular mechanism by which the skotomorphogenesis could be sustained when either darkness or mechanical stress exists alone. This idea may provide insight into how seedlings deal with the complex situations during soil emergence. For example, buried seedlings could grow in darkness with minimum mechanical stress in hollow soil or under dense canopy upon emergence. Skotomorphogenic pattern would be maintained by PIFs alone to allow seedlings to grow vigorously toward light. Upon breaking out, seedlings could be exposed to light, with soil block or stone retaining on the top. Mechanical stress-elevated EIN3 slows down apical hook unfolding and cotyledon development, enabling seedlings to push aside obstructions and fully emerge. With regard to the physiology, we have recently shown that EIN3 and PIF3 form an interdependent module in repressing chloroplast development (6). It is likely that seedlings have to rapidly initiate photoautotrophic growth upon light regardless of mechanical stress, due to the limited reserves in seeds. This hypothesis is consistent with the observations that dark-grown *EIN3*ox turned green normally under initial light irradiation, with apical hook folded and cotyledons closed and unexpanded (5).

Plant morphogenesis is profoundly influenced by signals from environmental factors as well as endogenous hormones. Collective studies have shown that PIFs, EIN3/EIL1, and HY5 serve as central integrators of environmental and hormonal signals in controlling seedling morphological development. Gibberellins (GAs) induce rapid degradation of DELLA proteins, which directly sequester the transcriptional activity of PIF3 and PIF4 (40, 41). Brassinosteroid-activated transcription factor BZR1 is found to interact and cooperatively promote hypocotyl cell elongation with PIF4 (42, 43), while GA and cytokinin have been reported to regulate the protein levels of HY5 (44, 45). During seedling soil emergence, EIN3/EIL1 are key transcription factors that precisely adjust seedling morphogenesis by converging both light and mechanical pressure signals (5, 19, 21). Thus, our studies provide genetic and molecular evidence and resources for elucidating the integration of environmental and hormonal signals.

Materials and Methods

The details and procedures of plant materials and growth conditions, immunoblot assays, ChIP analysis, RNA extraction, qRT-PCR analysis, transcriptome assays, and histochemical GUS staining assays are provided in *SI Appendix, Materials and Methods*.

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