Phytochromes inhibit hypocotyl negative gravitropism by regulating the development of endodermal amyloplasts through phytochrome-interacting factors

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Phytochromes are red and far-red light photoreceptors that regulate various aspects of plant development. One of the less-understood roles of phytochromes is the inhibition of hypocotyl negative gravitropism, which refers to the loss of hypocotyl gravitropism and resulting random growth direction in red or far-red light. This light response allows seedlings to curve toward blue light after emergence from the soil and enhances seedling establishment in the presence of mulch. Phytochromes inhibit hypocotyl negative gravitropism by inhibiting four phytochrome-interacting factors (PIF1, PIF3, PIF4, PIF5), as shown by hypocotyl agravitropism of dark-grown *pif1 pif3 pif4 pif5* quadruple mutants. We show that phytochromes inhibit negative gravitropism by converting starch-filled gravity-sensing endodermal amyloplasts to other plastids with chloroplastic or etioplastic features in red or far-red light, whereas PIFs promote negative gravitropism by inhibiting the conversion of endodermal amyloplasts to etioplasts in the dark. By analyzing transgenic plants expressing *PIF1* with an endodermis-specific *SCARECROW* promoter, we further show that endodermal *PIF1* is sufficient to inhibit the conversion of endodermal amyloplasts to etioplasts and hypocotyl negative gravitropism of the *pif* quadruple mutant in the dark. Although the functions of phytochromes in gravitropism and chloroplast development are normally considered distinct, our results indicate that these two functions are closely related.

Phytochromes are red and far-red light plant photoreceptors that regulate various light responses, including seed germination, seedling photomorphogenesis, and shade avoidance. Phytochromes regulate light responses partly by inhibiting phytochrome-interacting factors (PIFs), a set of bHLH transcription factors that negatively regulate various light responses (1, 2). A series of experiments has demonstrated that phytochromes promote light responses partly by activating degradation of these PIFs (3). Consistent with these findings, *pif1 pif3 pif4 pif5* quadruple mutants (*pifQ*) display constitutive photomorphogenic phenotypes and ectopic expression of chloroplast-related genes in the dark, and *pif4 pif5* double mutants show suppressed shade avoidance responses and associated repression of shade-avoidance marker genes in a low-red/far-red light (4–8). At the molecular level, PIFs bind to G-box elements (CACGTG) and regulate the expression of various genes associated with G-box–containing promoters (9–11). In the case of PIF1, a genome-wide analysis of its binding sites and an associated gene-expression analysis indicated that PIF1 binds to 748 sites and directly regulates the expression of at least 166 genes either positively (105 genes) or negatively (61 genes) during the seed imbibition period (12). The 166 genes include many hormone-signaling genes, such as *RGA*, *ABI3*, *ABI5*, *JAZ1*, and *ARF18*, as well as various cell wall-modifying enzyme genes. In addition to these direct target genes, PIF1 indirectly regulates some hormone metabolic genes. These analyses suggested that the phytochrome-PIF1 signaling module regulates seed germination by coordinating hormone signaling and cell wall properties in imbibed seeds. Molecular networks that link phytochrome-PIFs modules to other downstream light responses, such as hypocotyl negative gravitropism, are not clearly understood.

Plant gravitropic responses can be divided conceptually into four steps, consisting of gravity sensing, signal generation, signal transmission to the responding tissues, and asymmetric elongation (13). Among these steps, gravity sensing requires starch-filled amyloplasts in root columella cells for root gravitropism or in shoot endodermis for hypocotyl and shoot negative gravitropism (14, 15). Consistent with the critical role of endodermis in shoot gravity sensing, the *scarecrow* (*scr*) and the *shortroot* (*shr*) mutants, which do not develop an endodermis, do not display shoot negative gravitropism (16, 17). Starch levels in the amyloplasts also affect gravitropism of both the shoot and root, as indicated by the reduced gravitropic responses of a phosphoglucomutase mutant (*pgm*) that has a reduced level of amyloplast starch, and by the stronger gravitropic responses of starch-excessive mutant (*sex1*), with higher levels of amyloplast starch (18–22). A series of shoot gravitropic mutants named *shoot gravitropism* (*sgr1–sgr5*, *sgr7*) further demonstrate the importance of endodermis, endodermal vacuole biogenesis, and vacuolar membrane dynamics in shoot gravity sensing (13, 23–26). Mislocalization and altered movement of amyloplasts in these *sgr* mutants have been implicated in reduced gravity sensing. Once gravity is sensed by amyloplasts, the biophysical signal is presumed to be converted to a biochemical signal that is then transmitted to responding tissues for asymmetric elongation partly through auxin signaling (13, 27, 28).

The inhibition of hypocotyl negative gravitropism by light is one of the less-understood light responses. Hypocotyl negative gravitropism, which assists seedlings during their emergence from the soil, could hinder their growth toward light, particularly in the complex environment at the soil surface. Accordingly, plants may have adopted phytochrome signaling to inhibit hypocotyl negative gravitropism in light. In support of this inference, seedlings display stronger phototropic responses to directional blue light when negative gravitropism is inhibited (29). Under more natural conditions, the inhibition of hypocotyl negative gravitropism by light increases survival fitness when seeds are covered with mulch (30). It is not also clear how phytochromes inhibit hypocotyl negative gravitropism. The *pifQ* mutant displays completely disrupted hypocotyl negative gravitropism in the dark, indicating that phytochromes inhibit hypocotyl negative gravitropism by inhibiting PIFs (6). In this article, we investigated how phytochromes inhibit hypocotyl negative gravitropism through PIFs. We show that phytochromes promote the conversion of endodermal amyloplasts to

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plastids with etioplastic or chloroplastic features by inhibiting PIFs. We further demonstrate that the specific expression of *PIF1* in the endodermis of the dark-grown *pifQ* mutant is sufficient to inhibit the conversion of endodermal amyloplasts to etioplasts, thereby enabling hypocotyl negative gravitropism in this mutant.

Results

PIFs Inhibit the Conversion of Endodermal Amyloplasts to Etioplasts. Phytochromes inhibit hypocotyl negative gravitropism by inhibiting PIFs in red or far-red light (*SI Text* and Figs. S1 and S2). We investigated how phytochromes and PIFs regulate hypocotyl negative gravitropism. To simplify our analysis, we focused mainly on the inhibition of hypocotyl negative gravitropism by red light, as follows.

Inhibition of hypocotyl negative gravitropism could be caused by the disruption of gravity sensing, subsequent signaling, or the asymmetric elongation steps. We examined whether gravity sensing is disrupted by red light or by the *pifQ* mutation. In the analysis, we grew seedlings on vertical plates for 2 d either in the dark or in red light, and then incubated them for 2 more days under the same light conditions after changing the direction of gravity by 90°. Dark-grown wild-type hypocotyls curved against the direction of gravity upon alteration of the gravity vector, whereas red light-grown hypocotyls continued to grow in the same direction (Fig. 1). In contrast to hypocotyls, roots of both dark-grown and red light-grown seedlings curved toward the direction of gravity, indicating that red light inhibits hypocotyl negative gravitropism but does not inhibit root positive gravitropism. The *pifQ* mutant behaved like red light-grown wild-type seedlings. Irrespective of light conditions, hypocotyls of the *pifQ* mutant did not respond to the change in direction of gravity, whereas roots of the mutant curved toward the direction of gravity. The curving of roots suggested that both the light-grown wild-type and the *pifQ* mutant possess the ability to elongate asymmetrically. This finding was further supported by hypocotyl phototropism toward blue light both in the wild-type and the *pifQ* mutant (Fig. S1*C*). Taken together, our results indicated that hypocotyls of the *pifQ* mutant, like those of the light-grown wild-type, cannot sense gravity or cannot process subsequent gravity signaling. However, they do possess the ability to elongate asymmetrically.

Gravity sensing or subsequent signaling could be disrupted by altered expression of previously reported gravitropic genes, such as *SHOOT GRAVITROPISM* genes (*SGR1–SGR5*, *SGR7*), *ALTERED RESPONSE TO GRAVITY1* (*ARG1*) and its related genes (*ARL1*, *ARL2*), phosphoglucomutase (*PGM*), *STARCH EXCESS1*, *-4* (*SEX1, SEX4*), *GRAVITROPIC IN THE LIGHT1* (*GIL1*), and *PROTEIN KINASE SUBSTRATE1*, *-4* (*PKS1*, *PKS4*) (13, 30–33). Among these gravitropic genes, *GIL1*, *SEX1*, *SEX4*, and *PKS1*, *PKS4* are negatively acting gravitropic genes, whereas other genes are positively acting genes for shoot or hypocotyl negative gravitropism. We examined two previously reported *pifQ* microarray datasets to determine if the expression of these gravitropic genes is altered by the *pifQ* mutation in both microarray datasets (5, 6). The positively acting gravitropic genes are expressed similarly in dark-grown wild-type and in the *pifQ* mutant when a 1.5-fold criterion [>1.5-fold, false-discovery rate (FDR) < 0.05) is applied (Fig. S3). The negatively acting gravitropic genes are also expressed similarly. Our results suggest that agravitropism of the *pifQ* mutant is not caused by severe repression or activation of these previously identified shoot gravitropic genes, with potential exceptions of *PKS1* and *-4* that show higher expression in the *pifQ* in one set of microarray data.

Gravity sensing could be compromised by a defect in the development of endodermal amyloplasts. Amyloplasts filled with starch granules are required to sense gravity both in endodermis and in columella cells of root caps. Because PIFs have been shown to regulate chloroplast development (6, 34), we investigated whether PIFs regulate hypocotyl negative gravitropism by modulating the development of amyloplasts in the endodermis. We first examined endodermal amyloplasts by staining them with I_2-KI. In dark-grown wild-type seedlings, amyloplasts in the endodermis of the hypocotyl elongation zone and in the columella cells of root cap were stained dark by I_2-KI (Fig. 2*A*). In the dark-grown *pifQ* mutant, however, no amyloplasts could be detected by I_2-KI staining in the endodermis of the hypocotyl elongation zone, but amyloplasts were still detected in the columella cells of the root cap (Fig. 2*B*). The *nph4-3* mutant, which is caused by a mutation in the *AUXIN-RESPONSE FACTOR 7* (*ARF7*) gene, also displayed hypocotyl agravitropism in the dark (35). Unlike the *pifQ* mutant, the *nph4-3* mutant was stained strongly by I_2-KI (Fig. 2*C*), in-

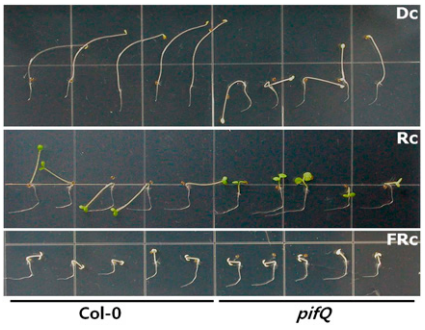

Fig. 1. Hypocotyls of *pifQ* mutant seedlings do not respond to changes in the direction of gravity. The direction of gravity was altered by turning plates 90° after the wild-type (Col-0) and the *pif* quadruple mutant (*pifQ*) were grown for 2 d either in the dark or in continuous red/far-red light on vertical agar plates. The plates were incubated for another 2 d under the same light conditions. Dc, Rc, and FRc indicate continuous dark, continuous red light (20 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and continuous far-red light (2.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions.

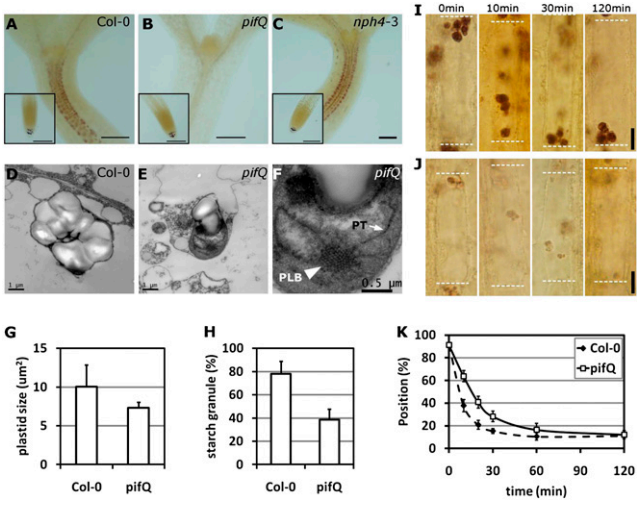

Fig. 2. Amyloplasts are partially converted to etioplasts in the endodermis of the dark-grown *pif* quadruple mutant (*pifQ*). (*A–C*) I_2-KI staining patterns of the wild-type (Col-0) (*A*), the *pifQ* mutant (*B*), and the *nph4-3* mutant (*C*). (Scale bars, 100 μm.). (*D* and *E*) TEM images of endodermal plastids of the wild-type (*D*) and the *pifQ* (*E*). (*F*) A magnified image of a *pifQ* plastid shows the prolamellar body (PLB) and prothylakoids (PT). (*G*) Endodermal plastid sizes of wild-type and *pifQ* mutant. Data are mean with 95% confidence intervals indicated; $n = 10$. (*H*) Plastid areas occupied by starch granules in wild-type and *pifQ* mutant. Data are mean with 95% confidence intervals indicated; $n = 10$. (*I* and *J*) Sedimentation of wild-type amyloplasts (*I*) and mutant plastids (*J*) in response to the changing gravity vector. Time indicates minutes after changing the gravity vector by 180°. White broken lines indicate the top and bottom of each endodermal cell. (Scale bars, 10 μm.) (*K*) Quantification of plastid sedimentation in response to the changing gravity vector. Data are mean with 95% confidence intervals indicated; $n = 100$.

dicating that the lack of amyloplast staining by I_2-KI is not a common feature of all agravitropic mutants and that I_2-KI can be used to distinguish different defects in gravity sensing and response.

To further examine the status of endodermal amyloplasts in the dark-grown *pifQ* mutant, we performed transmission electron microscopy (TEM) of endodermal amyloplasts. Wild-type endodermal amyloplasts were filled with large starch granules (Fig. 2*D*). In contrast, endodermal plastids of the *pifQ* mutant contained only small starch granules (Fig. 2*E*). In addition, they also contained a prolamellar body and prothylakoids (Fig. 2*F*), which are characteristics of etioplasts. Quantification further showed that plastids sizes are not significantly different between wild-type and the *pifQ*, but starch granules occupy less area in the *pifQ* (Fig. 2 *G* and *H*). The results indicate that endodermal amyloplasts are converted to plastids with etioplastic features in the dark-grown *pifQ* mutant, and that the hypocotyls of *pifQ* mutants are agravitropic because of the conversion of gravity-sensing amyloplasts to other forms of plastids in the endodermis.

We determined if the endodermal plastids of wild-type and *pifQ* settle equally well in response to changing gravity vector. For this experiment, we first settled plastids of *pifQ* by reorienting and incubating dark-grown *pifQ* seedlings vertically. After the plastids settled, we rotated the seedlings 180° and then determined the plastid movement in response to this changed gravity vector. At 10 min after changing the gravity vector, amyloplasts of wild-type moved more than half a cell length (62%), but plastids of *pifQ* only moved an average of 35% of the cell length (Fig. 2 *I–K*). Two hours after rotation, plastids of both wild-type and *pifQ* had moved to the new cell bottoms (89 and 88%, respectively). The results indicate that plastids of *pifQ* mutant settle more slowly to changing gravity vector.

The conversion of endodermal amyloplasts to other forms of plastids with etioplastic or chloroplastic features and the concomitant reduction of starch granules were also observed in light-grown wild-type seedlings. When wild-type seedlings were grown in monochromatic light, red light-grown endodermal plastids contained small starch granules and developed thylakoids (Fig. 3*A*). Similarly, far-red light-grown endodermal plastids also contained small starch granules, a prolamellar body, and prothylakoids. Overall, negatively gravitropic dark-grown wild-type seedlings possessed endodermal amyloplasts that were characterized by large starch granules and the lack of thylakoids or prolamellar bodies, whereas seedlings that exhibited agravitropism possessed endodermal plastids that were characterized by relatively small starch granules and the presence of thylakoids or a prolamellar body. During seedling development, I_2-KI stains both wild-type and *pifQ* equally well, irrespective light condition until 36 h after germination induction. At 48 h after the germination induction, however, dark-grown wild-type are stained darker than light-grown wild-type and dark-grown *pifQ* seedlings, and at 60 h, only dark-grown wild-type seedlings are stained darkly (Fig. S4). The results imply that PIFs inhibit the conversion of amyloplasts to other plastids in the dark.

The reduction in the size of endodermal starch granules by light was dependent on phytochrome signaling. When the I_2-KI solution was used to stain endodermal starch granules in seedlings, I_2-KI strongly stained endodermal starch granules in dark-grown seedlings, but not in red or far-red light-grown seedlings (Fig. 3*B*). These results were consistent with the TEM images of endodermal starch granules of seedlings grown under different light conditions (Fig. 3*A*). In the *phyA* mutant, I_2-KI stained endodermal starch granules not only in dark-grown seedlings but also in far-red light-grown seedlings. In contrast, in the *phyB* mutant, I_2-KI stained both the dark-grown and red light-grown seedlings (Fig. 3*B* and Fig. S5). These findings indicated that phytochromes mediate red or far-red light reduction of endodermal starch granule size.

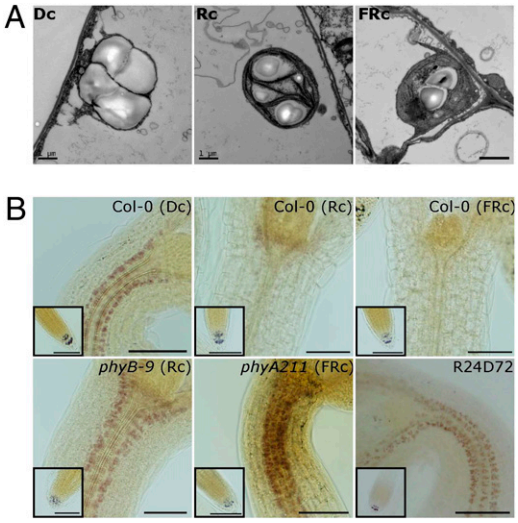

Fig. 3. Phytochromes promote the conversion of amyloplasts to other forms of plastids in the endodermis. (*A*) TEM images of endodermal plastids of the wild-type grown in the dark (Dc), in continuous red light (Rc), and in continuous far-red light (FRc). (Scale bars, 1 μm.) (*B*) I_2-KI staining of the wild-type (Col-0), the *phyA* mutant (*phyA*-211), and the *phyB* mutant (*phyB*-9) grown in the dark (Dc), in continuous red light (Rc), or in continuous far-red light (FRc). R24D72 indicates I_2-KI staining of wild-type seedlings grown in red light for 24 h and transferred to the dark for 72 h. (Scale bars, 100 μm.)

When etiolated seedlings were transferred to red light, seedlings showed slightly weaker staining at 6 h after the transfer, had noticeably fainter staining at 9 h, and exhibited no staining at 12 h after the transfer (Fig. S6). The results indicate that amyloplasts are converted to other plastids rather slowly and further suggest that seedlings should lose their ability to sense gravity rather slowly when transferred to red light. To investigate how long it takes for red light-transferred seedlings to lose the ability to sense gravity, we transferred etiolated seedlings to red light for various times and then transferred them back to dark, and changing the gravity vector by 90°. The ability to sense gravity decreased gradually with increased exposure to red light; if seedlings were incubated more than 9 h, they did not respond to the changing gravity (Fig. S6). The slow loss of gravity sensing is consistent with the slow conversion of amyloplasts under red light. The ability of red light-transferred seedlings to sense gravity soon after transfer was also previously observed (33).

The irreversible inhibition of hypocotyl gravitropism by light (Fig. S2) was associated with irreversible reduction of endodermal starch granules by light. When seedlings were grown in red light for 24 h and transferred to the dark for 3 d, endodermal starch granules were strongly stained by I_2-KI, indicating that the first 24 h of red light were not effective in reducing endodermal starch granules (Fig. 3*B*). However, when seedlings were grown in red light for 36 h and transferred to the dark for 60 h, they were stained only very weakly by the I_2-KI solution, indicating that treatment with red light for 36 h causes an irreversible reduction in the size of starch granules and irreversible inhibition of hypocotyl negative gravitropism (Fig. S5). Taken together, our results suggest that the conversion of starch-filled endodermal amyloplasts to other forms of plastids by phytochromes is one of the main light responses associated with the inhibition of hypocotyl negative gravitropism.

Endodermis-Specific *PIF1* Is Sufficient to Inhibit the Conversion of Amyloplasts to Etioplast and Hypocotyl Negative Gravitropism. Because light and the *pifQ* mutation have been shown to disrupt the development of endodermal amyloplasts, we investigated whether hypocotyl negative gravitropism in the *pifQ* mutant could

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be rescued by endodermis-specific *PIF*s. *PIF1* was expressed under the endodermis-specific *SCR* promoter in the *pifQ* mutant background (Fig. 4*A*). The *SCR* promoter has been shown to be active in the endodermis of roots, hypocotyls, and cotyledons (36). Two independent homozygous lines were established and used for further analysis (*SCRpro::PIF1*/*pifQ* #1, #2).

Expression of *PIF1* by the *SCR* promoter restored hypocotyl negative gravitropism of the *pifQ* mutant in the dark. Two *SCRpro::PIF1*/*pifQ* lines displayed wild-type–like hypocotyl negative gravitropism in the dark, and also displayed wild-type–like hypocotyl agravitropism in red and far-red light (Fig. 4*B*). The restoration of hypocotyl negative gravitropism by endodermis-specific expression of *PIF1* was accompanied by the restoration of endodermal amyloplasts (Fig. 4*C*). Taken together, the results indicate that endodermis-specific *PIF1* is sufficient to inhibit the conversion of endodermal amyloplast to etioplasts and subsequent hypocotyl negative gravitropism in the dark.

We also investigated whether endodermis-specific *PIF1* can rescue other phenotypes associated with the *pifQ* mutant. Among light responses, *SCRpro::PIF1* partially rescued hook formation and cotyledon-opening phenotypes of the *pifQ* mutant in the dark (Fig. 4*D*), indicating that endodermal *PIF*s contribute to hook formation and cotyledon closure in the dark. Unlike hook and cotyledon phenotypes, the *SCRpro::PIF1* lines still had short hypocotyls in the dark (Fig. 4*E*). These lines also exhibited high germination frequency in the dark (Fig. 4*F*), as well as a higher degree of bleaching during the dark-to-light transition (Fig. 4*G*). These results indicated that endodermis-specific *PIF1* can rescue some *pifQ* mutant phenotypes either fully (e.g., hypocotyl gravitropism) or partially (e.g., hook formation and cotyledon opening), but cannot rescue others (e.g., hypocotyl elongation, bleaching during the dark-to-light transition, and germination in the dark). It should be noted, however, that, although the *SCR* promoter was shown to be specific to the endodermis, one cannot completely rule out the possibility of weak expression of *PIF1* in other tissues.

Discussion

In previous work, we reported that *pif1* and *pif3* single mutants and their double mutants have reduced levels of hypocotyl negative gravitropism in the dark, whereas the *pifQ* mutant is completely agravitropic in the dark. This finding led us to conclude that phytochromes inhibit hypocotyl negative gravitropism by inhibiting these four PIFs. Other studies have shown that phytochromes inhibit PIFs by destabilizing PIF proteins. It is unclear, however, how PIFs promote hypocotyl negative gravitropism. In this report, we show that the *pifQ* mutant possesses endodermal plastids with small starch granules and etioplastic features, such as a prolamellar body and prothylakoids, instead of starch-filled amyloplasts, indicating that PIFs are necessary for the development of endodermal amyloplasts for the sensing of gravity in the dark. We also show that phytochromes promote the partial conversion of endodermal amyloplasts to other forms of plastids with small starch granules and etioplastic or chloroplastic features in far-red or red light conditions, indicating that endodermal amyloplasts are also targets of phytochrome action during seedling development. By analyzing transgenic plants expressing *PIF1* under the *SCR* promoter, we further demon-

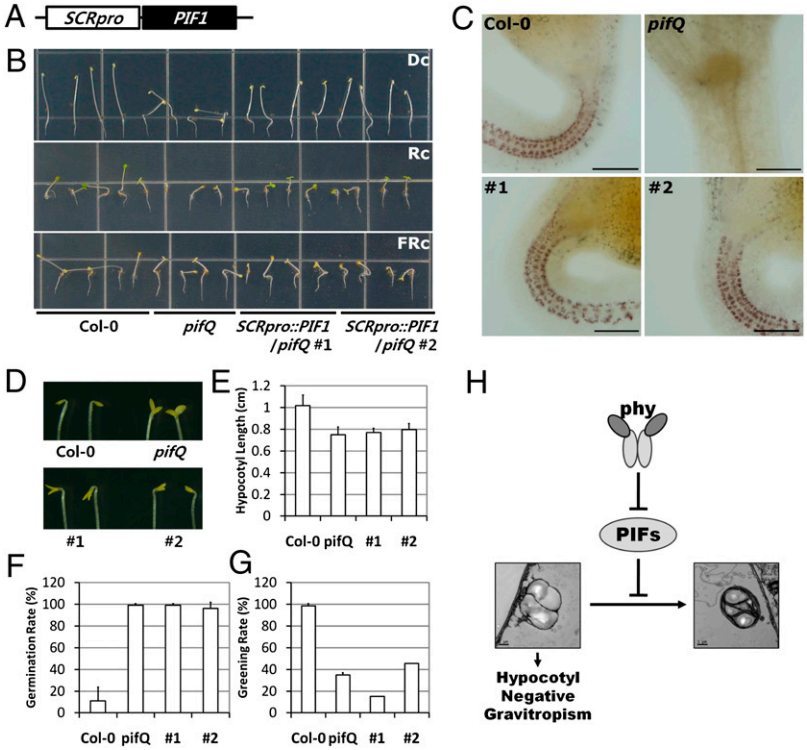

Fig. 4. Endodermis-specific expression of PIF1 restores hypocotyl negative gravitropism in the *pifQ* mutant. (*A*) Schematic diagram showing the *SCRpro::PIF1* construct. (*B*) Restoration of hypocotyl negative gravitropism by endodermal PIF1. The wild-type (Col-0), the *pifQ* mutant, and two transgenic lines (*SCRpro::PIF1*/*pifQ* #1, #2) were grown in the dark (Dc), in red light (Rc), and in far-red light (FRc) on vertical agar plates. (Scale bars, 100 μm.) (*C*) I_2-KI staining of the dark-grown wild-type, the *pifQ* mutant, and two transgenic lines (#1, #2). (*D*) Partial restoration of hook formation and cotyledon opening in the dark-grown *SCRpro::PIF1*/*pifQ* transgenic lines (#1, #2). (*E*–*G*) No restoration of hypocotyl length in the dark (*E*), germination frequency in the dark (*F*), and greening during the dark-to-light transition (*G*) in two *SCRpro::PIF1*/*pifQ* transgenic lines (#1, #2). (*H*) A diagram summarizing the role of PIFs on hypocotyl negative gravitropism. PIFs suppress the conversion of starch-filled endodermal amyloplasts to plastids with small starch granules and more developed thylakoids in the dark. In the light, phytochromes inhibit PIFs by targeting them for protein degradation, which in turn promotes the conversion of gravity sensing amyloplasts to other plastid forms.

strated that endodermal *PIF1* is sufficient to restore endodermal amyloplasts and hypocotyl negative gravitropism in the *pifQ* mutant. Our results link hypocotyl negative gravitropism to endodermal plastid development and suggest that the molecular mechanisms regulating these two seemingly distinct processes are closely related (Fig. 4*H*).

The conversion of endodermal amyloplasts to other forms of plastids by light or by mutations in *PIF* genes might be partially associated with changes in the expression of various genes, such as starch metabolic enzyme genes, chlorophyll biosynthetic genes, and photosynthetic genes. As shown in Figs. 2 and 3, dark-grown endodermal amyloplasts are characterized by large starch granules and the lack of a noticeable prolamellar body and prothylakoids. In contrast, endodermal plastids in the dark-grown *pifQ* mutant and the far-red light-grown wild-type are characterized by small starch granules and conspicuous prolamellar bodies and prothylakoids, whereas endodermal plastids in the red light-grown wild-type are characterized by small starch granules and well-developed thylakoids. Consistent with these morphological changes in plastids, previous microarray data indicate that both red light-grown wild-type and the dark-grown *pifQ* mutant seedlings express higher levels of chlorophyll biosynthetic genes and photosynthetic genes compared with dark-grown wild-type seedlings (5, 6). Expression patterns of starch metabolic genes are also consistent with the reduced levels of starch in the *pifQ* mutant (Fig. S7). Among starch metabolic genes, some starch degrading enzymes genes, including the *debranching enzyme* gene (*LDA1*) and *glucan phosphorylase* (*PHS1*) are expressed higher (criterion: >1.5-fold, FDR < 0.05) in the dark-grown *pifQ* mutant seedlings in two independent microarray data (5, 6), indicating that smaller starch granules in the *pifQ* seedlings are associated with higher expression of starch-degrading enzyme genes. Whether the up-regulation of these starch metabolic genes is sufficient to reduce the size of starch granules in endodermal amyloplsts needs further testing.

Our data do not exclude the possibility that PIFs regulate hypocotyl negative gravitropism also partly through auxin. Auxin is responsible for the tropic elongation downstream of gravity sensing or photosensing, as shown by altered gravitropism or phototropism of various auxin-related mutants, including *nph4*, *pgp19*, *shy2-1D*, *pin2*, *pin3*, and *aux1* (28, 37–44). Phytochromes have been shown to regulate auxin biosynthetic genes, transport genes, and signaling genes either at the transcriptional level or at the protein level (37, 45–47), suggesting that phytochromes may regulate some of these genes through PIFs. Consistent with this idea, an analysis of two previously reported microarray datasets (5, 6) indicate that PIFs regulate an auxin transport gene (*PGP4*) and two auxin signaling genes (*ARF18*, *IAA29*) (criterion: >1.5-fold, FDR < 0.05) in two independent microarray data (Table S1). In imbibed seeds, PIF1 was shown to directly bind to promoters of *ARF18* and *PGP4* (12), indicating that PIFs may regulate *ARF18* and *PGP4* by directly binding to their promoters also in seedlings. Currently, it is not clear if these auxin-related genes regulate the hypocotyl negative gravitropism. It should be also noted that the criterion we applied is an arbitrary criterion; thus, genes that do not pass the criterion might still play important roles in the hypocotyl negative gravitropism. In regard to this theory, it is noteworthy that the expression of *PGP19* that was shown to regulate tropic elongation downstream of phytochromes (37) is only mildly altered in the microarray data. Thus, further analyses are required to determine if PIFs regulate hypocotyl negative gravitropism also through changes in auxin levels or signaling.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana* plants were grown in a growth room with a 16-h light/8-h dark cycle at 22 to 24 °C for general growth and seed harvesting. To express *PIF1* under endodermis-specific *SCR* promoter, a *SCR* promoter (~1.7 kb) and *PIF1* cDNA were amplified with specific primer sets (SCRpro LP: 5′-GAGAA AGCTT CTATT CAAAT ATGGA CTTGG AGAA-3′, SCRpro RP: 5′-GAGAT CTAGA GGAGA TTGAA GGGTT GTTG-3′, PIF1 LP: 5′-GAGAC CTAGG ATGCA TCATT TTGTC CC T-3′ PIF1 RP: 5′-GAGAT GATCA AACCT GTTGT GTGGT TTCCG TGA-3′) and cloned into pCAMBIA1300 vector to generate a *SCRpro::PIF1* vector. The *SCRpro::PIF1* vector was then transformed into the *pifQ* mutant and two independent homozygous lines were subsequently selected for the analysis. All *Arabidopsis* plants used in the experiments (Col-0, *phyA*-211, *phyB*-9, *pifQ*, *SCRpro::PIF1/pifQ* #1, #2) are Col-0 ecotype.

Hypocotyl Negative Gravitropism, Photobleaching, and Germination Assays. For hypocotyl negative gravitropism assay and photobleaching assay, surface-sterilized seeds were plated on MS agar (1/2 MS, 0.8% phytoagar, and 0.05% Mes, pH 5.7), imbibed for 3 d at 4 °C in the dark, and irradiated with white light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 3 h for the induction of germination. For the hypocotyl negative gravitropism assay, plates were incubated vertically for 3 d in the dark, continuous red light (20 $\mu mol \cdot m^{-2} \cdot s^{-1}$), or continuous far-red light (2.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22 °C. Growth orientations of hypocotyls were determined by the degrees from vertical axis. For the dark-to-red or red-to-dark transfer experiments, seedlings were incubated either in the dark or red light for various hours and transfered to either red light or dark, respectively. Total incubation time after the induction of germination was 96 h. To investigate if seedlings can respond to the changing direction of gravity, seedlings were incubated vertically for 2 d either in the dark or red light, turned by 90° counterclockwise, and incubated 2 more days in the same light conditions. Photobleaching assay and germination assay were performed as described previously (6, 48).

Visualization of Endodermal Amyloplasts. To visualize endodermal amyloplasts by iodine staining, seedlings were fixed in FAA (5% Formaldehyde, 45% Ethanol, 5% Acetic acid) solution for 24 h at 4 °C. After fixation, seedlings were rinsed in 50% (vol/vol) ethanol once and stained in I_2-KI solution [2% (wt/vol) iodine, 5% (wt/vol) potassium iodine and 20% (wt/vol) chloral hydrate] for 1 min. Samples were de-stained in 1:1:1 trichloroacetic acid: phenol: lactic acid = 1:1:1) for 5 min and carefully mounted on slides with a drop of destaining solution for the light microscopic observation.

To visualize endodermal amyloplasts by EM, seedlings were fixed with 3% glutaraldehyde in PBS for 2 h at room temperature. After fixation, seedlings were washed five times with 0.1 M cacodylate buffer (pH 7.2) containing 0.1% $CaCl_2$ at 4 °C and then postfixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) containing 0.1% $CaCl_2$ for 3 h at 4 °C. After rinsing with cold distilled water, samples were dehydrated slowly with an ethanol series and propylene oxide at 4 °C. The samples were embedded in Spurr's epoxy resin. After the polymerization of the resin at 70 °C for 36 h, serial sections were made with a diamond knife and mounted on formvar-coated slot grids. Sections were stained with 4% uranyl acetate for 10 min and lead citrate for 7 min. Samples were observed by a Tecnai G2 Spirit Twin transmission electron microscope (FEI Company) and JEM ARM 1300S high-voltage electron microscope (JEOL).

To determine the sedimentation speed, both wild-type and *pifQ* mutants were grown in the dark on vertical agar plates. Two-d-old dark-grown *pifQ* seedlings were then reoriented vertically and incubated 12 more hours in the dark to settle down plastids to the bottom. Plates were turned upside down to change the gravity vector by 180°. At various times after the changing gravity vector, seedlings were transferred to FAA solution and stained using I_2-KI solution. The relative positions of plastids were determined by assigning the original bottom of a cell to 0 and the top of the cell to 1.

Microarray Analysis. Microarray data from Shin et al. were analyzed as reported previously (6). For reanalysis of microarray data from Leivar et al. (5), RMA normalized data were downloaded from the National Center for Biotechnology Information GEO database http://www.ncbi.nlm.nih.gov/geo/query/browse.cgi (accession no. GSE17159) and analyzed in R/Bioconductor [R Development Core Team, 2010 (49)] using the packages GEOquerry and limma (50–52). We contrasted expression values for wild-type and *pif* quadruple mutant seedlings grown for 2 d in dark conditions. Genes with a FDR < 0.05 and a fold-change > 1.5 in both microarray data were considered significant.

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1. Bae G, Choi G (2008) Decoding of light signals by plant phytochromes and their interacting proteins. *Annu Rev Plant Biol* 59:281–311.
2. Franklin KA, Quail PH (2009) Phytochrome functions in *Arabidopsis* development. *J Exp Bot* 61:11–24.
3. Huq E (2006) Degradation of negative regulators: A common theme in hormone and light signaling networks? *Trends Plant Sci* 11:4–7.
4. Leivar P, et al. (2008) Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. *Curr Biol* 18:1815–1823.
5. Leivar P, et al. (2009) Definition of early transcriptional circuitry involved in light-induced reversal of PIF-imposed repression of photomorphogenesis in young *Arabidopsis* seedlings. *Plant Cell* 21:3535–3553.
6. Shin J, et al. (2009) Phytochromes promote seedling light responses by inhibiting four negatively-acting phytochrome-interacting factors. *Proc Natl Acad Sci USA* 106: 7660–7665.
7. Lorrain S, Trevisan M, Pradervand S, Fankhauser C (2009) Phytochrome interacting factors 4 and 5 redundantly limit seedling de-etiolation in continuous far-red light. *Plant J* 60:449–461.
8. Lorrain S, Allen T, Duek PD, Whitelam GC, Fankhauser C (2008) Phytochrome-mediated inhibition of shade avoidance involves degradation of growth-promoting bHLH transcription factors. *Plant J* 53:312–323.
9. Martínez-García JF, Huq E, Quail PH (2000) Direct targeting of light signals to a promoter element-bound transcription factor. *Science* 288:859–863.
10. Huq E, et al. (2004) Phytochrome-interacting factor 1 is a critical bHLH regulator of chlorophyll biosynthesis. *Science* 305:1937–1941.
11. Shin J, Park E, Choi G (2007) PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in *Arabidopsis*. *Plant J* 49:981–994.
12. Oh E, et al. (2009) Genome-wide analysis of genes targeted by PHYTOCHROME INTERACTING FACTOR 3-LIKE5 during seed germination in *Arabidopsis*. *Plant Cell* 21: 403–419.
13. Morita MT, Tasaka M (2004) Gravity sensing and signaling. *Curr Opin Plant Biol* 7: 712–718.
14. Sack FD (1997) Plastids and gravitropic sensing. *Planta* 203(Suppl 1):S63–S68.
15. Stanga JP, Boonsirichai K, Sedbrook JC, Otegui MS, Masson PH (2009) A role for the TOC complex in *Arabidopsis* root gravitropism. *Plant Physiol* 149:1896–1905.
16. Fukaki H, et al. (1998) Genetic evidence that the endodermis is essential for shoot gravitropism in *Arabidopsis thaliana*. *Plant J* 14:425–430.
17. Benfey PN, et al. (1993) Root development in *Arabidopsis*: Four mutants with dramatically altered root morphogenesis. *Development* 119:57–70.
18. Kiss JZ, Hertel R, Sack FD (1989) Amyloplasts are necessary for full gravitropic sensitivity in roots of *Arabidopsis thaliana*. *Planta* 177:198–206.
19. Weise SE, Kiss JZ (1999) Gravitropism of inflorescence stems in starch-deficient mutants of *Arabidopsis*. *Int J Plant Sci* 160:521–527.
20. Tanimoto M, Tremblay R, Colasanti J (2008) Altered gravitropic response, amyloplast sedimentation and circumnutation in the *Arabidopsis* shoot gravitropism 5 mutant are associated with reduced starch levels. *Plant Mol Biol* 67:57–69.
21. Kiss JZ, Guisinger MM, Miller AJ, Stackhouse KS (1997) Reduced gravitropism in hypocotyls of starch-deficient mutants of *Arabidopsis*. *Plant Cell Physiol* 38:518–525.
22. Vitha S, Yang M, Sack FD, Kiss JZ (2007) Gravitropism in the *starch excess* mutant of *Arabidopsis thaliana*. *Am J Bot* 94:590–598.
23. Kato T, et al. (2002) SGR2, a phospholipase-like protein, and ZIG/SGR4, a SNARE, are involved in the shoot gravitropism of *Arabidopsis*. *Plant Cell* 14:33–46.
24. Morita MT, et al. (2006) A C2H2-type zinc finger protein, SGR5, is involved in early events of gravitropism in *Arabidopsis* inflorescence stems. *Plant J* 47:619–628.
25. Saito C, Morita MT, Kato T, Tasaka M (2005) Amyloplasts and vacuolar membrane dynamics in the living graviperceptive cell of the *Arabidopsis* inflorescence stem. *Plant Cell* 17:548–558.
26. Yano D, et al. (2003) A SNARE complex containing SGR3/AtVAM3 and ZIG/VTI11 in gravity-sensing cells is important for *Arabidopsis* shoot gravitropism. *Proc Natl Acad Sci USA* 100:8589–8594.
27. Takahashi H, Miyazawa Y, Fujii N (2009) Hormonal interactions during root tropic growth: Hydrotropism versus gravitropism. *Plant Mol Biol* 69:489–502.
28. Esmon CA, Pedmale UV, Liscum E (2005) Plant tropisms: Providing the power of movement to a sessile organism. *Int J Dev Biol* 49:665–674.
29. Lariguet P, Fankhauser C (2004) Hypocotyl growth orientation in blue light is determined by phytochrome A inhibition of gravitropism and phototropin promotion of phototropism. *Plant J* 40:826–834.
30. Allen T, Ingles PJ, Praekelt U, Smith H, Whitelam GC (2006) Phytochrome-mediated agravitropism in *Arabidopsis* hypocotyls requires GIL1 and confers a fitness advantage. *Plant J* 46:641–648.
31. Boonsirichai K, Sedbrook JC, Chen R, Gilroy S, Masson PH (2003) ALTERED RESPONSE TO GRAVITY is a peripheral membrane protein that modulates gravity-induced cytoplasmic alkalinization and lateral auxin transport in plant statocytes. *Plant Cell* 15:2612–2625.
32. Guan C, Rosen ES, Boonsirichai K, Poff KL, Masson PH (2003) The ARG1-LIKE2 gene of *Arabidopsis* functions in a gravity signal transduction pathway that is genetically distinct from the PGM pathway. *Plant Physiol* 133:100–112.
33. Schepens I, Boccalandro HE, Kami C, Casal JJ, Fankhauser C (2008) PHYTOCHROME KINASE SUBSTRATE4 modulates phytochrome-mediated control of hypocotyl growth orientation. *Plant Physiol* 147:661–671.
34. Stephenson PG, Fankhauser C, Terry MJ (2009) PIF3 is a repressor of chloroplast development. *Proc Natl Acad Sci USA* 106:7654–7659.
35. Harper RM, et al. (2000) The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial *Arabidopsis* tissue. *Plant Cell* 12:757–770.
36. Wysocka-Diller JW, Helariutta Y, Fukaki H, Malamy JE, Benfey PN (2000) Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. *Development* 127:595–603.
37. Nagashima A, et al. (2008) Phytochromes and cryptochromes regulate the differential growth of *Arabidopsis* hypocotyls in both a PGP19-dependent and a PGP19-independent manner. *Plant J* 53:516–529.
38. Moon J, et al. (2007) A new CULLIN 1 mutant has altered responses to hormones and light in *Arabidopsis*. *Plant Physiol* 143:684–696.
39. Kim BC, Soh MS, Hong SH, Furuya M, Nam HG (1998) Photomorphogenic development of the *Arabidopsis* shy2-1D mutation and its interaction with phytochromes in darkness. *Plant J* 15:61–68.
40. Chen R, et al. (1998) The *Arabidopsis thaliana AGRAVITROPIC 1* gene encodes a component of the polar-auxin-transport efflux carrier. *Proc Natl Acad Sci USA* 95: 15112–15117.
41. Luschnig C, Gaxiola RA, Grisafi P, Fink GR (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes Dev* 12: 2175–2187.
42. Müller A, et al. (1998) AtPIN2 defines a locus of *Arabidopsis* for root gravitropism control. *EMBO J* 17:6903–6911.
43. Friml J, Wiśniewska J, Benková E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* 415:806–809.
44. Marchant A, et al. (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO J* 18:2066–2073.
45. Tao Y, et al. (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell* 133:164–176.
46. Carabelli M, et al. (2007) Canopy shade causes a rapid and transient arrest in leaf development through auxin-induced cytokinin oxidase activity. *Genes Dev* 21: 1863–1868.
47. Colón-Carmona A, Chen DL, Yeh KC, Abel S (2000) Aux/IAA proteins are phosphorylated by phytochrome in vitro. *Plant Physiol* 124:1728–1738.
48. Oh E, et al. (2004) PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key negative regulator of seed germination in *Arabidopsis thaliana*. *Plant Cell* 16: 3045–3058.
49. Gentleman RC, et al. (2004) Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol* 5:R80.
50. Sean D, Meltzer PS (2007) GEOquery: A bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinformatics* 23:1846–1847.
51. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3: Article3.
52. Smyth GK (2005) Limma: Linear models for microarray data. *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, eds Gentlemen R, Carey V, Dudoit S, Irizarry R, Huber W (Springer, New York), pp 397–420.