PHYTOCHROME-INTERACTING FACTOR 1 Is a Critical bHLH Regulator of Chlorophyll Biosynthesis

Enamul Huq,[1,2] Bassem Al-Sady,[2] Matthew Hudson,[2] Chanhong Kim,[3] Klaus Apel,[3] Peter H. Quail[2]*

Photosynthetic organisms must achieve a delicate balance between the light energy absorbed by chlorophyll and their capacity to channel that energy into productive photochemical reactions. Release of excess absorbed energy in the cell can cause lethal photooxidative damage. We identified a basic helix-loop-helix (bHLH) transcription factor, designated PHYTOCHROME-INTERACTING FACTOR 1 (PIF1), that negatively regulates chlorophyll biosynthesis. *pif1* mutant seedlings accumulate excess free protochlorophyllide when grown in the dark, with consequent lethal bleaching upon exposure to light. PIF1 interacts specifically with the photoactivated conformer of phytochromes A and B, suggesting a signaling pathway by which chlorophyll biosynthetic rates are tightly controlled during the critical initial emergence of seedlings from subterranean darkness into sunlight.

The colonization of land by terrestrial flowering plants has included the evolution of a capacity for the buried seed to germinate and grow upward in subterranean darkness toward the soil surface. This is accomplished with the use of a developmental strategy termed skotomorphogenesis, whereby germinated seedlings grow heterotrophically on seed reserves in the absence of chlorophyll accumulation and functional chloroplast development. Upon reaching the soil surface, the seedlings undergo a marked developmental transition, termed deetiolation, toward the normal photomorphogenic pattern of fully green plants. This transition is triggered by light and involves coordinate inhibition of hypocotyl cell elongation, unfolding of the apical hook, stimulation of cotyledon cell expansion, activation of functional chloroplast development, and chlorophyll accumulation.

The first exposure of emergent seedlings to sunlight is a point of particular vulnerability in the life cycle, requiring a sequence of tightly regulated responses. In preparation for the transition to photoautotrophic growth, subterranean seedlings accumulate a small pool of protochlorophyllide, the immediate precursor of chlorophyll, to permit rapid assembly of functional photosynthetic machinery upon initial light exposure. However, the size of this pool must be stoichiometrically linked to the level of the enzyme protochlorophyllide oxidoreductase, which catalyzes the light-induced conversion of protochlorophyllide to chlorophyll. Accumulation of free protochlorophyllide in excess over available protochlorophyllide oxidoreductase levels can result in lethal photooxidative damage, because light energy absorbed by these free molecules can be dissipated within the cell as reactive oxygen species or free radicals (*1*–*2*). Therefore, coordinate regulation of the chlorophyll biosynthetic pathway and the capacity for enzymatic conversion of protochlorophyllide to chlorophyll is particularly crucial during the initial deetiolation process.

The light signals that induce the deetiolation transition are perceived by members of the cryptochrome (cry) and phytochrome (phy) families of informational photoreceptors. We focused on defining the signaling and transcriptional networks involved in transducing the signals perceived by the five-member phy family (phyA through phyE) in *Arabidopsis*. These molecules monitor the incident red (R) and far-red (FR) light impinging on the plant by switching reversibly between two conformers—the inactive Pr and active Pfr forms—upon sequential R and FR photon absorption. The activated molecules subsequently transduce the perceived information to photoresponsive nuclear genes. Studies in recent years have defined and led to the cloning of a considerable number of genetic loci apparently involved in the signaling process (*3*–*5*), and a number of phytochrome-interacting factors (PIFs) that bind directly to the photoreceptor molecules have been identified (*3*, *6*). Current evidence suggests that the signal transduction mechanism involves light-induced translocation of the photoreceptor molecule from the cytoplasm into the nucleus, and subsequent physical interaction with PIF3, a member of the bHLH family of transcriptional regulators, with consequent modulation of target gene expression (*7*–*10*). Although initially reported to act positively in phy signaling (*9*, *11*, *12*), PIF3 has recently been found to have a complex role in seedling deetiolation, acting both positively and negatively in different facets of the process (*13*–*15*). In addition, recent data indicate that the translocated phy molecule induces degradation of PIF3 (*14*), thereby presumptively affecting transcriptional activity indirectly by reducing PIF3 abundance. Three other bHLH factors—PIF4 (*16*), HFR1 (*17*), and PIL1 (*18*)—have also been implicated in phy-regulated responses.

However, definitive identification of the mechanism involved in phy signaling to nuclear interactors is presently lacking, and the number of bHLH factors and their target genes that are potentially involved in phy-related activities remains to be defined. Based on the premise that closely related proteins may have similar functions, we investigated a new bHLH family member, designated PIF1 [*Arabidopsis* Genome Initiative (AGI) locus number At2g20180], with high sequence similarity to PIF3 (*11*), for possible involvement in the light-induced seedling deetiolation process.

PIF1 negatively regulates chlorophyll biosynthesis. To determine whether PIF1 is functionally involved in phy-regulated seedling development, we isolated homozygous *pif1* transferred DNA (T-DNA) insertion lines and generated *PIF1* overexpression lines. Sequence analyses showed that the T-DNA is inserted in the second exon, causing deletion of the bHLH domain in both the *pif1-1* and *pif1-2* alleles (Fig. 1A). Northern blot analyses showed a stable band of 0.9 kb for the *pif1-1* allele and no detectable band for the *pif1-2* allele (Fig. 1B), suggesting that at least the latter is a null allele.

Although the overexpression lines displayed some perturbation in normal seedling growth, neither T-DNA–insertion mutant showed any detectable alteration in photoresponsiveness under the extended irradiation conditions used from germination onward (fig. S1, A and B). These data suggest that PIF1 is not necessary for normal seedling deetiolation under these conditions but is capable of interfering with this process when overexpressed.

In contrast, we observed a marked bleaching phenotype in *pif1* mutant seedlings that had first been germinated and grown in the dark for several days before transfer to white light (Fig. 1C). Because this phenotype is qualitatively reminiscent of *flu* mutants, which bleach in light as a result of the accumulation of excess protochlorophyllide (*19*),

[1]Section of Molecular Cell and Developmental Biology and Institute of Molecular Biology, University of Texas at Austin, Austin, TX 78712, USA. [2]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA and U.S. Department of Agriculture/Agricultural Research Service, Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710, USA. [3]Institute of Plant Sciences, Swiss Federal Institute of Technology (ETH), Universitatstrasse 2, 8092 Zurich, Switzerland.

*To whom correspondence should be addressed. E-mail: quail@nature.berkeley.edu

we examined the *pif1* mutant for protochlorophyllide levels using low-temperature (77 K) fluorescence spectra analysis (*2*, *19*). The data show that the level of protochlorophyllide in dark-grown *pif1-1* is about two times as high as that in the wild-type seedlings (Fig. 1D). Importantly, the *pif1* mutants also show levels of free protochlorophyllide four to six times as high as wild-type seedlings in the short term after initial transfer to white light (up to 3 hours shown in Fig. 1, E and F). However, *pif1* seedlings showed little or no residual chlorophyll or chlorophyll precursors under prolonged incubation in light, reflecting the bleaching process (fig. S2, A and B). Thus, the bleaching phenotype of *pif1* is apparently due to the overaccumulation of free protochlorophyllide in the postgermination dark period and seems to indicate that photooxidative damage in light is caused by the photosensitizing activity of this tetrapyrrole intermediate.

In time-course experiments, we observed that the severity of the bleaching phenotype increased markedly with increasing time in darkness before transfer to light (Fig. 2A). Young seedlings germinated for 1 or 2 days in darkness before transfer to light exhibited no detectable bleaching, whereas seedlings held in darkness for 3 to 6 days displayed an increasingly severe phenotype. These data suggest that PIF1 may function as a negative regulator of the chlorophyll biosynthetic pathway, acting to prevent the accumulation of excess protochlorophyllide in prolonged darkness. To test this proposition more directly, we examined the rate of light-induced chlorophyll accumulation in young *pif1* mutant seedlings in which insufficient protochlorophyllide had previously accumulated to cause bleaching. The *pif1* seedlings accumulated chlorophyll significantly more rapidly than did wild-type seedlings under these conditions (Fig. 2B). This increase is not due to enhanced cotyledon expansion, given that the *pif1* mutants have cotyledon areas similar to those of the wild-type seedlings (fig. S3). Conversely, *phyA* and *phyB* null mutants accumulated chlorophyll more slowly than did the wild-type seedlings (Fig. 2C), consistent with a positive role for each photoreceptor in this process. Together, the data indicate that PIF1 does indeed negatively regulate the chlorophyll biosynthetic pathway and that phyA and phyB may act to repress PIF1 activity in the light (Fig. 2D).

The increasing lethality in *pif1* seedlings with increasing preillumination darkness suggests that PIF1 may have evolved to protect seedlings germinating at depth in subterranean darkness from the deleterious effects of uncontrolled protochlorophyllide accumulation before reaching the surface. This is more directly apparent when the seedling survival rate is quantified as a function of hypocotyl length at first exposure to light for wild-type compared with *pif1* seedlings (Fig. 3). The data show that the presence of PIF1 in wild-type seedlings confers a marked presumptive selective advantage for seeds germinating at 10 mm or more below the soil surface (Fig. 3). Consistent with this notion, PIF1 overexpressors exhibit a substantially enhanced capacity to resist lethal bleaching compared with that of the wild-type seedlings after considerably longer preillumination dark periods (>10 days) (*20*).

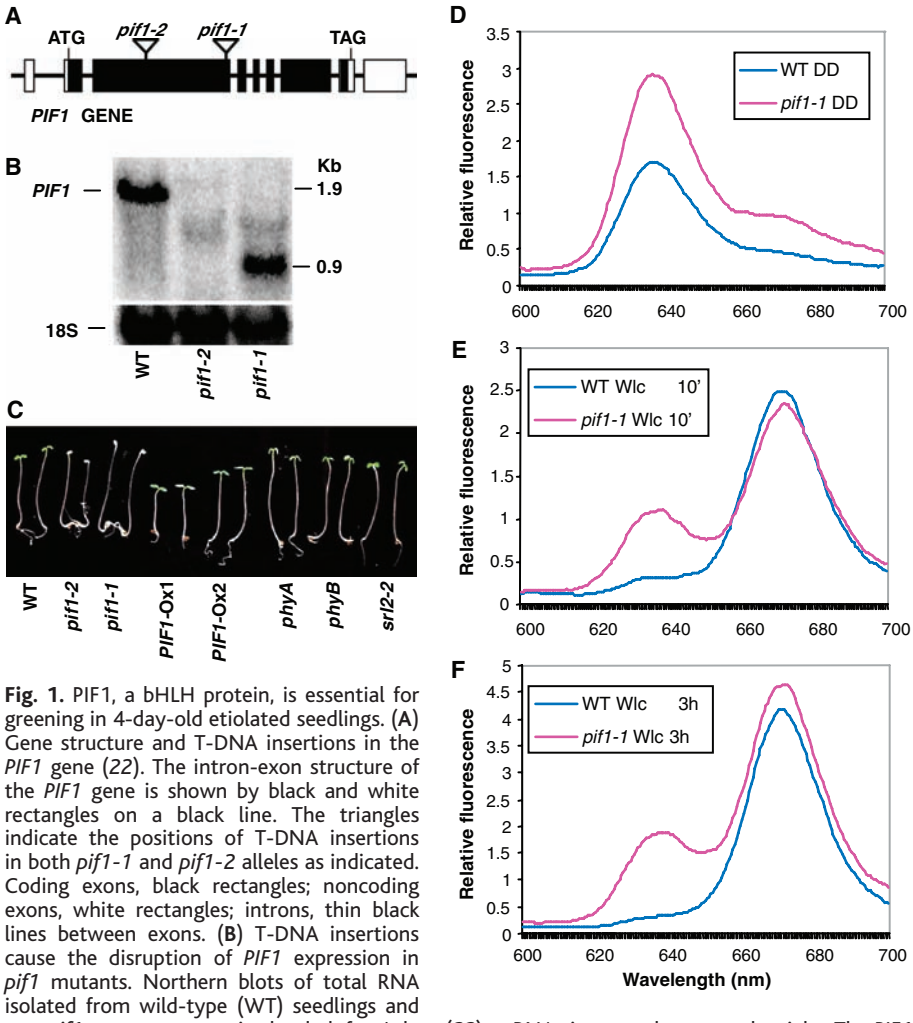

Fig. 1. PIF1, a bHLH protein, is essential for greening in 4-day-old etiolated seedlings. (**A**) Gene structure and T-DNA insertions in the *PIF1* gene (*22*). The intron-exon structure of the *PIF1* gene is shown by black and white rectangles on a black line. The triangles indicate the positions of T-DNA insertions in both *pif1-1* and *pif1-2* alleles as indicated. Coding exons, black rectangles; noncoding exons, white rectangles; introns, thin black lines between exons. (**B**) T-DNA insertions cause the disruption of *PIF1* expression in *pif1* mutants. Northern blots of total RNA isolated from wild-type (WT) seedlings and two *pif1* mutants grown in the dark for 4 days (*22*). mRNA sizes are shown on the right. The PIF1 open-reading-frame region was used as a probe. 18*S* ribosomal RNA was used to show the amount of RNA loaded in each lane. (**C**) Visible phenotypes of the *pif1* mutants, wild-type (Col), and *PIF1* overexpression (Ox) lines grown for 5 days in the dark and then transferred to white light (50 μmol m^{-2} s^{-1}) for 2 days (*22*). *phyA*, *phyB*, and *srl2-2* mutants were grown under the same conditions (controls). Seedling images are to scale. (**D** to **F**) *pif1* has a higher level of protochlorophyllide (635-nm peak) than the wild-type seedlings. Fluorescence spectra of acetone extracts from wild-type and *pif1-1* mutant seedlings that were grown for 4 days in the dark (D) or dark-grown seedlings transferred to white light for 10 min (E) or 3 hours (h) (F) with the use of an excitation wavelength of 440 nm. DD, dark; Wlc, white light.

PIF1 interacts with photoactivated phyA and phyB. To explore the mechanism(s) by which PIF1 might regulate the chlorophyll biosynthetic pathway, we investigated the molecular interactions between PIF1 and the two principal phytochromes regulating seedling deetiolation. Figure 4, A and B, shows that PIF1 interacts strongly and specifically with the biologically active Pfr form of both phyA and phyB, as determined by an in vitro coimmunoprecipitation assay. PIF1 has reduced affinity for signaling-compromised missense mutant forms of phyA and phyB, suggesting that this interaction is biologically important (Fig. 4, A and B). Because the other two phy-interacting bHLH proteins thus far reported, PIF3 and PIF4, both displayed strongly preferential binding to phyB compared with phyA (*16*, *21*), we directly compared the apparent binding affinities of these two PIFs with those of PIF1 toward the two phy's. The data show that PIF1 and PIF3 have similar affinities for phyB, which are somewhat higher than PIF4 (Fig. 4C). In

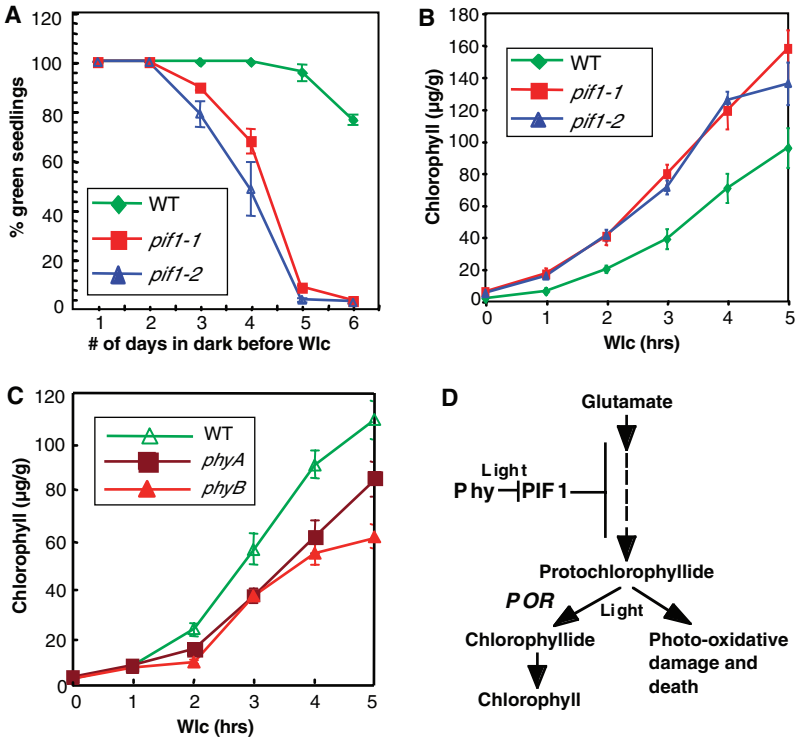

Fig. 2. Perturbation of chlorophyll biosynthesis is responsible for the bleaching phenotype of *pif1* mutants. (**A**) *pif1* seedlings fail to green if grown in the dark for 4 to 6 days before transfer to white light. (**B**) PIF1 modulates control of chlorophyll biosynthesis in response to light. Young *pif1* mutant seedlings accumulate chlorophyll at higher rates compared with accumulation rates of wild–type seedlings in response to light. Two-day-old dark-grown wild-type or *pif1* mutant seedlings were transferred to continuous white light (Wlc) for different periods of time and total chlorophyll was extracted in N′N′-dimethylformamide, according to Inskeep and Bloom (*23*). Standard error of means for three different experiments are shown. (**C**) Photoreceptor mutants accumulate chlorophyll at a reduced rate compared with that of wild-type seedlings. Three-day-old etiolated wild-type (Col-O), *phyA*, and *phyB* mutant seedlings (in Col-O background) were transferred to Wlc for different time periods and total chlorophyll was measured as described in (B). Error bars in (A) to (C) show mean ± SEM. (**D**) Model outlining potential role of PIF1 in controlling chlorophyll biosynthesis. PIF1 either directly or indirectly represses the biosynthesis of protochlorophyllide in the dark, or increases its stability. Phy's directly interact with PIF1 upon light activation, potentially repressing PIF1 transcriptional activity in a light-dependent manner and thereby increasing chlorophyll biosynthesis in response to light. POR, photochlorophyllide oxidoreductase.

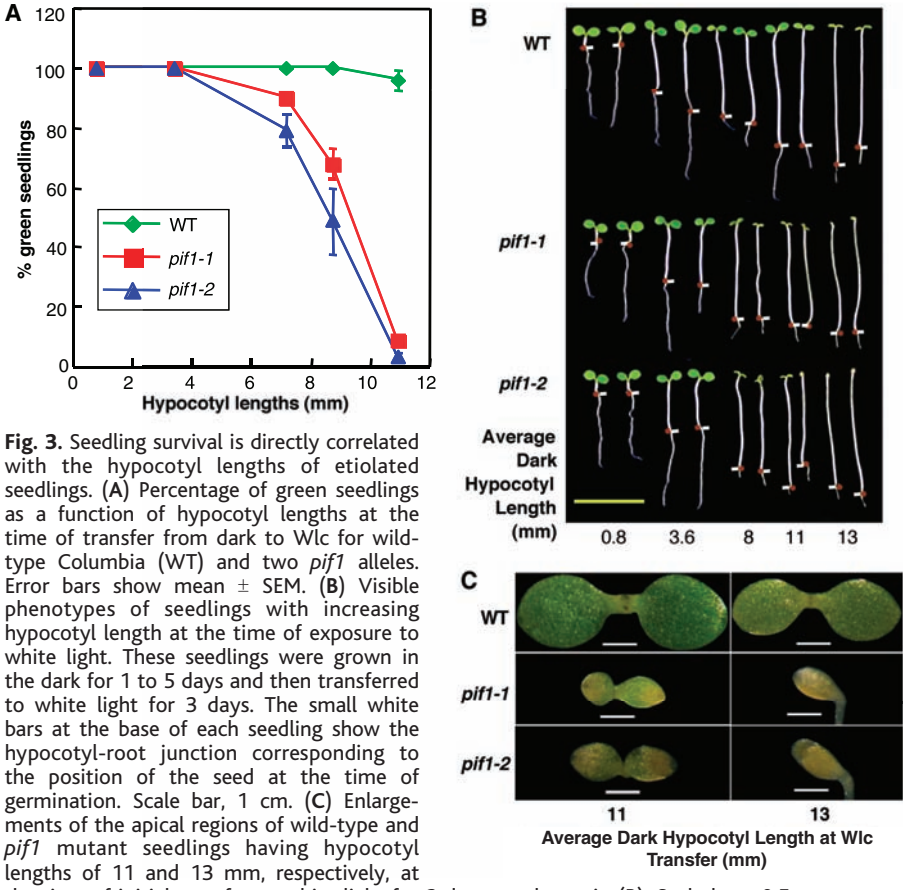

Fig. 3. Seedling survival is directly correlated with the hypocotyl lengths of etiolated seedlings. (**A**) Percentage of green seedlings as a function of hypocotyl lengths at the time of transfer from dark to Wlc for wild-type Columbia (WT) and two *pif1* alleles. Error bars show mean ± SEM. (**B**) Visible phenotypes of seedlings with increasing hypocotyl length at the time of exposure to white light. These seedlings were grown in the dark for 1 to 5 days and then transferred to white light for 3 days. The small white bars at the base of each seedling show the hypocotyl-root junction corresponding to the position of the seed at the time of germination. Scale bar, 1 cm. (**C**) Enlargements of the apical regions of wild-type and *pif1* mutant seedlings having hypocotyl lengths of 11 and 13 mm, respectively, at the time of initial transfer to white light for 3 days, as shown in (B). Scale bars, 0.5 mm.

contrast, PIF1 has affinity for phyA more than 10 times as high as that of PIF3 or PIF4 (Fig. 4C). These results establish PIF1 as a distinct member of the phy-interacting bHLHs thus far described, with a potential role in both phyA and phyB signaling. The data suggest that different bHLH proteins may interact differentially with different phy family members, thereby providing the potential for selective channeling of photosensory signaling information to different subsets of target genes.

As PIF1 is a member of the bHLH family, we investigated its subcellular localization and whether it can directly bind to DNA. Not unexpectedly, the data indicate that PIF1 localizes to the nucleus (fig. S4). Previously, we have shown with gel-shift assays that both PIF3 and PIF4 bind to the G-box DNA sequence motif (CACGTG) found in many light-regulated promoters and that phyB can bind DNA-bound PIF3 in the Pfr form to generate a specific super-shifted complex in these assays (*9*, *16*). Our present data show that PIF1 can also bind to this G-box motif in sequence-specific fashion (fig. S5A). However, in contrast to PIF3, DNA-bound PIF1 displayed no detectable interaction with either phyB or phyA by gel-shift assay (fig. S5B). Thus, PIF1 apparently cannot bind to both the G-box DNA and activated phy molecules simultaneously under these experimental conditions, which is similar to previously reported behavior for PIF4 (*16*). These data suggest that the mechanism of phy-mediated control of gene expression through PIF1 and PIF3 might be different. Alternatively, if the recently reported light-induced degradation of PIF3 (*14*) is general among other phy-interacting bHLHs, it is possible that this basic process can occur regardless of whether the factor is DNA-bound or free and that it is different in this respect between the different bHLHs.

PIF1 transcriptional activation activity is negatively modulated by phyA and phyB. To determine whether PIF1 can function in phy-modulated gene regulation, we investigated whether it can activate transcription in a transient assay in a light-responsive manner. For this purpose, PIF1 was expressed as a fusion protein with the GAL4 DNA binding domain under the control of the strong cauliflower mosaic virus 35S (CaMV35S) promoter (Fig. 5A). The reporter construct consisted of a minimal promoter with the GAL4 DNA binding site driving a Luciferase reporter gene. A Renilla Luciferase gene under the control of a CaMV35S promoter was also used as an internal control. These constructs were expressed transiently in 3-day-old dark-grown *Arabidopsis* seedlings after particle bombardment, and Luciferase activity was assayed after 16 hours of incubation under pulses of red light (Rp), continuous red light (Rc) (*20*), continuous far-red light (FRc), or dark. PIF1 stimulated up to seven times as much Luciferase expression as that of the controls in dark-incubated seedlings, indicating that this factor does indeed have the capacity to function as a transcriptional activator in vivo (Fig. 5, B to D). Intriguingly, both Rp and FRc light treatments significantly suppressed the transcriptional activator activity of PIF1 in a phyB- and phyA-dependent manner, respectively (Fig. 5, B to D), indicating that each of these two photoreceptors, endogenously present in the target cells, was capable of negatively regulating the transcriptional activity of PIF1 in response to the relevant light signals. The yeast GAL4 transcriptional activator DNA binding domain (DBD)–GAL4 activation domain fusion (DBDAD), although active in the *Arabidopsis* cells, showed no such responsiveness to the light signals (Fig. 5B). Because DNA-bound PIF1 did not interact with either phy molecule (fig. S5B), these data suggest that the function of the direct physical interaction between unbound PIF1 and phyA and phyB might be to modulate PIF1 transcriptional activity through interference, sequestration, or degradation to modulate chlorophyll biosynthesis in response to light.

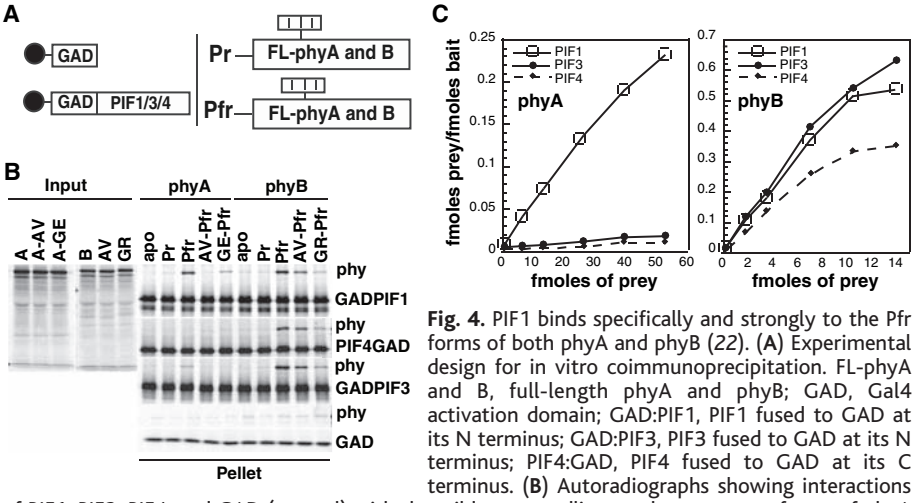

Fig. 4. PIF1 binds specifically and strongly to the Pfr forms of both phyA and phyB (*22*). (**A**) Experimental design for in vitro coimmunoprecipitation. FL-phyA and B, full-length phyA and phyB; GAD, Gal4 activation domain; GAD:PIF1, PIF1 fused to GAD at its N terminus; GAD:PIF3, PIF3 fused to GAD at its N terminus; PIF4:GAD, PIF4 fused to GAD at its C terminus. (**B**) Autoradiographs showing interactions of PIF1, PIF3, PIF4, and GAD (control) with the wild-type seedlings and two mutant forms of phyA and phyB, respectively. Left panel shows the input, and right panel shows the pellet fraction from the in vitro coimmunoprecipitation assay. A and B under input represent phyA and phyB, respectively; apo, phy without chromophore; AV/GR and AV/GE are missense mutants of phyA and phyB, respectively. GAD, GAD:PIF1, GAD:PIF3, and PIF4:GAD are described in (A). (**C**) Concentration curves showing the increased binding with increasing amount of phyA or phyB in the Pfr form (prey) with either GAD:PIF1, GAD:PIF3, or PIF4:GAD (bait). The same amount of bait (~12 fmol) was used for each construct in each tube. The absolute amount of each bait and prey used was calculated from a standard curve with the use of known amounts of ^{35}S-methionine. fmoles of prey/fmoles of bait is plotted against increasing amount of prey used.

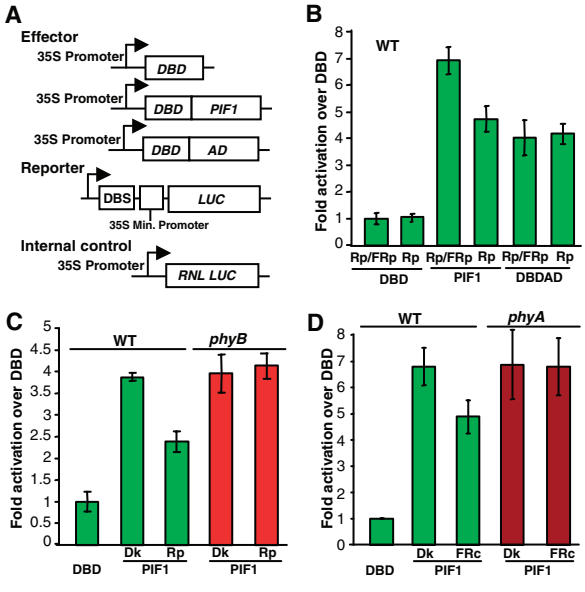

Fig. 5. PIF1 transcriptional activation activity is regulated by phyA and phyB in a light-dependent manner (*22*). (**A**) Constructs used for the experiment. AD, GAL4 activation domain; DBS, GAL4 DNA binding site; LUC, firefly Luciferase; RNL LUC, Renilla Luciferase. Three-day-old etiolated *Arabidopsis* seedlings of Col wild type (WT) (**B**), wild type and *phyB* (**C**), or wild-type and *phyA* mutants (**D**) were bombarded with effector constructs constitutively expressing a DBD-PIF1 fusion (DBDPIF1), DBDAD, or DBD alone. Seedlings were treated for 15 min with FR light and then exposed to 5-min pulses of R light alone (Rp) every 2 hours (9×40 μmol m^{-2} s^{-1}) [(B) and (C)] or R pulses followed immediately by a FR pulse (Rp/FRp) (9×55 μmol m^{-2} s^{-1}) (B), or continuous FR light (FRc) (20 μmol m^{-2} s^{-1}) (D) or darkness (Dk) for 14 hours [(C) and (D)]. Each histogram column represents the mean of eight biological replicates and the variation is expressed as standard error. Transcriptional activity was measured with the use of a dual-Luciferase assay system (Promega). The photon-count ratio of the reporter firefly (LUC+ in pGLL reporter plasmid) and *Renilla* (RNL internal control plasmid) luciferases measured in the presence of the effector DBD alone (pMN6 plasmid) is set as 1× fold activation. Fold transcriptional activity is determined by the photon-count ratios of these two reporter luciferases driven by the DBDPIF1 or DBDAD effectors, divided by this ratio for DBD alone.

Fine-tuning chlorophyll biosynthesis: Mechanisms and potential evolutionary implications. Collectively, the data suggest that PIF1 may function as a negative regulator of the chlorophyll biosynthetic pathway in the dark, and that this activity is negatively regulated by light (Fig. 2D). According to this model, light-induced photoconversion of phy molecules to the Pfr form would trigger interaction with PIF1, in some way reducing the transcriptional activation activity of PIF1. This would, in turn, result in partial release of the negative regulation of PIF1, which would allow plants to produce higher rates of chlorophyll synthesis in the light (Fig. 2B). On this basis, PIF1 would appear to function as a critical modulator by which plants optimize chlorophyll biosynthesis in response to environmental light conditions and protect against accumulation of potentially toxic levels of intermediates. Given the similarity of the *pif1* phenotype to that of the *flu* mutants (*19*), we examined whether PIF1 might regulate *FLU* gene expression. However, no difference in *FLU* transcript levels between wild-type and *pif1-1* seedlings was detected on Northern blots (*20*), suggesting that other components in the pathway are potential targets.

The proposed protective biological function of PIF1 may have emerged early in angiosperm evolution to provide a selective advantage in certain environments where seed burial was critical to survival. The capacity to germinate and emerge from subterranean darkness may have been particularly important in the successful radiation of the seed producers into drier, more hostile environments. Seed burial can provide long-term survival through protection from predators and hostile surface conditions until environmental conditions are favorable for germination and can facilitate establishment of a robust underground root system before seedling emergence, thereby increasing survival potential.

phy signaling through a bHLH transcriptional network. The reverse-genetic strategy of targeting *Arabidopsis* bHLH-family members, which are phylogenetically related to PIF3, for potential involvement in phy-regulated development is providing emerging evidence of a small network of these factors differentially involved in regulating and integrating different facets of the seedling deetiolation process. PIF3 (*11*), PIF4 (*16*), HFR1 (*17*), PIL1 (*18*), and PIF1 are all involved in this process, but each appears to have a differential role. Moreover, together with the recent evidence of phy-induced PIF3 degradation (*14*), our data here for PIF1 suggest that modulation of constitutively active transcription factor activity might be one of the mechanisms by which the phy family of photoreceptors induces photomorphogenesis in response to light.

References and Notes

1. S. Reinbothe, C. Reinbothe, K. Apel, N. Lebedev, *Cell* **86**, 703 (1996).
2. R. G. L. op den Camp *et al.*, *Plant Cell* **15**, 2320 (2003).
3. P. H. Quail, *Nature Rev. Mol. Cell. Biol.* **3**, 85 (2002).
4. H. Wang, X.-W. Deng, *The Arabidopsis Book*, C. R. Somerville, E. M. Meyerowitz, Eds. (American Society of Plant Biologists, Rockville, MD, 2004), 10.1199/tab.0074.1, http://aspb.org/publications/arabidopsis.
5. M. M. Neff, C. Fankhauser, J. Chory, *Genes Dev.* **14**, 257 (2000).
6. P. H. Quail, *Sem. Cell Dev. Biol.* **11**, 457 (2000).
7. F. Nagy, E. Schaefer, *Annu. Rev. Plant Biol.* **53**, 329 (2002).
8. S. Kircher *et al.*, *Plant Cell* **14**, 1541 (2002).
9. J. Martinez-Garcia, E. Huq, P. H. Quail, *Science* **288**, 859 (2000).
10. M. Ni, J. M. Tepperman, P. H. Quail, *Nature* **400**, 781 (1999).
11. M. Ni, J. M. Tepperman, P. H. Quail, *Cell* **95**, 657 (1998).
12. K. J. Halliday, M. Hudson, M. Ni, M.-M. Qin, P. H. Quail, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5832 (1999).
13. J. Kim *et al.*, *Plant Cell* **15**, 2399 (2003).
14. D. Bauer *et al.*, *Plant Cell* **16**, 1433 (2004).
15. E. Monte, P. Quail, unpublished data.
16. E. Huq, P. H. Quail, *EMBO J.* **21**, 2441 (2002).
17. C. D. Fairchild, M. A. Schumaker, P. H. Quail, *Genes Dev.* **14**, 2377 (2000).
18. M. G. Salter, F. A. Keara, G. C. Whitelam, *Nature* **426**, 680 (2003).
19. R. Meskauskiene *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12826 (2001).
20. E. Huq, P. H. Quail, data not shown.
21. Y. Zhu, J. M. Tepperman, C. D. Fairchild, P. H. Quail, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13419 (2000).
22. Materials and methods are available as supporting material on *Science* Online.
23. W. P. Inskeep, P. R. Bloom, *Plant Physiol.* **77**, 483 (1985).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/305/5692/1937/DC1
Materials and Methods
Figs. S1 to S5
References

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REPORTS

Observation of Superflow in Solid Helium

E. Kim and M. H. W. Chan*

We report on the observation of nonclassical rotational inertia in solid helium-4 confined to an annular channel in a sample cell under torsional motion, demonstrating superfluid behavior. The effect shows up as a drop in the resonant oscillation period as the sample cell is cooled below 230 millikelvin. Measurement of 17 solid samples allows us to map out the boundary of this superfluid-like solid or supersolid phase from the melting line up to 66 bars. This experiment indicates that superfluid behavior is found in all three phases of matter.

At temperatures below 2.176 K, liquid ^{4}He enters into a superfluid state and flows without any friction (*1*, *2*). The onset of superfluidity is associated with Bose-Einstein condensation (*3*, *4*), where the ^{4}He atoms, which are bosons, condense into a single momentum state and acquire quantum mechanical coherence over macroscopic length scales. Bose-Einstein condensation of alkali atoms in the vapor phase was achieved (*5*) in 1995, and there is strong evidence for superfluidity in these systems (*6*–*8*). Perhaps counter to intuition, superfluid-like behavior is thought possible even in solid helium (*9*–*14*). A recent torsional oscillator measurement found evidence of superfluid flow in solid helium confined in porous Vycor glass (*15*) with pore diameter of 7 nm. There is, however, concern that the observed effect may be due to a liquid-like layer of helium atoms adsorbed on the surface of the pores (*16*). Here we report observation of superflow in bulk solid helium. Our experiment shows that the superfluid-like behavior is a general and intrinsic property of solid helium and not the result of confinement in any particular medium.

The resonant period of the high mechanical quality factor torsional oscillator (*17*) (Fig. 1) is given by $2\pi[(I/G)^{1/2}]$, where I is the moment of inertia of the torsion cell, which contains ^{4}He, and G is the torsion spring constant of the Be-Cu torsion rod. The torsion cell has an annular channel in which ^{4}He can be introduced and pressurized to the solid phase. Ultrahigh-purity ^{4}He, with a stated ^{3}He impurity of 0.3 parts per million, is used in our experiment. When ^{4}He enters the superfluid or supersolid phase and acquires nonclassical rotational inertia (NCRI), a fraction of the helium decouples from the oscillation, thereby reducing the rotational inertia I and the resonant period. We have measured a total of 17 solid ^{4}He samples with pressure ranging from 26 bars, close to the melting boundary, to 66 bars; all showed supersolid decoupling below 230 mK.

The resonant period as a function of temperature is shown for a solid sample pressurized to 51 bars (Fig. 2A). Measurements

Department of Physics, Pennsylvania State University, University Park, PA 16802, USA.

*To whom correspondence should be addressed. E-mail: chan@phys.psu.edu