Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors

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Carotenoids are key for plants to optimize carbon fixing using the energy of sunlight. They contribute to light harvesting but also channel energy away from chlorophylls to protect the photosynthetic apparatus from excess light. Phytochrome-mediated light signals are major cues regulating carotenoid biosynthesis in plants, but we still lack fundamental knowledge on the components of this signaling pathway. Here we show that phytochrome-interacting factor 1 (PIF1) and other transcription factors of the phytochrome-interacting factor (PIF) family down-regulate the accumulation of carotenoids by specifically repressing the gene encoding phytoene synthase (PSY), the main rate-determining enzyme of the pathway. Both in vitro and in vivo evidence demonstrate that PIF1 directly binds to the promoter of the *PSY* gene, and that this binding results in repression of *PSY* expression. Light-triggered degradation of PIFs after interaction with photoactivated phytochromes during deetiolation results in a rapid derepression of *PSY* gene expression and a burst in the production of carotenoids in coordination with chlorophyll biosynthesis and chloroplast development for an optimal transition to photosynthetic metabolism. Our results also suggest a role for PIF1 and other PIFs in transducing light signals to regulate *PSY* gene expression and carotenoid accumulation during daily cycles of light and dark in mature plants.

deetiolation | light | metabolism | seedling | transcription

Light provides photosynthetic organisms with a major source of energy to fix atmospheric carbon into organic matter that ultimately supports life on earth. But when the energy of the incoming light exceeds the photosynthetic capacity of plants, chlorophylls and some biosynthetic precursors, such as protochlorophyllide (Pchlide), interact with oxygen and produce highly reactive oxygen species that are potentially harmful to plants, causing irreversible damage to the photosynthetic machinery and cell death (1). Not surprisingly, plants continuously monitor external light conditions using complex photoreceptor and signaling systems that eventually regulate multiple aspects of their physiology and development. Immediately after germination of angiosperms, light availability already determines what developmental pathway will be followed: skotomorphogenesis in darkness or photomorphogenesis in the light (2, 3). Skotomorphogenic (etiolated) development results in seedlings that elongate in search of light. The plastids of such etiolated seedlings (etioplasts) accumulate chlorophyll precursors (Pchlide) as well as carotenoids, metabolites that facilitate greening when seedlings emerge from the soil (4, 5). After illumination, photomorphogenic development is derepressed and etioplasts differentiate into chloroplasts. This deetiolation process involves the production of high levels of chlorophylls and carotenoids. Chloroplast carotenoids can function as membrane stabilizers and accessory light-harvesting pigments, but their most important role is to channel excess energy away from chlorophylls to protect against photooxidative damage (6–8). To minimize the deleterious effects of light on the emerging photosynthetic apparatus, the production of carotenoids and chlorophylls during deetiolation occurs in a tightly regulated and interdependent fashion. Little is known about the specific factors involved in this coordinated control, however.

Although the main pathway for carotenoid biosynthesis in plants has been elucidated (9, 10), we still lack fundamental knowledge of the regulation of carotenogenesis in plant cells (11). In fact, to date no regulatory genes directly controlling carotenoid biosynthetic gene expression have been isolated. Nonetheless, it is known that a major driving force for carotenoid production in different plant species is the transcriptional regulation of genes encoding phytoene synthase (PSY), the first and main rate-determining enzyme of the pathway (5, 9, 11–14). Consistently, the burst in carotenoid biosynthesis that occurs during deetiolation of *Arabidopsis thaliana* seedlings is correlated with a very fast up-regulation of *PSY* transcripts (15, 16) and a concomitant increase in PSY protein levels and enzyme activity (17). This up-regulation of *PSY* gene expression is mediated by the phytochrome family of photoreceptors under red (R) and far-red (FR) light (12, 13, 16, 18).

Phytochromes exist in two photoreversible forms: Pr, which absorbs R light, and Pfr, which absorbs FR light. When the inactive Pr form present in the dark absorbs R light, it is converted to the biologically active Pfr form and relocates to the nucleus. Once there, it interacts with signaling components that eventually translate the light signal into changes in gene expression and physiological responses (19). Some of these nuclear components are members of the phytochrome-interacting factor (PIF) subfamily of basic-helix–loop–helix (bHLH) transcription factors. PIFs are central mediators in a variety of light-mediated responses (20), and at least some of them (PIF1, PIF3, PIF4, and PIF5) are required to repress photomorphogenic development in the dark (21–24). On exposure of etiolated seedlings to R light, the direct interaction of the photoactivated Pfr form of phytochromes with PIFs results in the phosphorylation and proteasome-mediated degradation of PIFs, allowing photomorphogenic development to proceed (19–24). The photomorphogenic phenotype displayed in the dark by a quadruple mutant defective in PIF1, PIF3, PIF4, and PIF5 activities (*pifQ*) correlates with gene expression profiles similar to those of R light-grown WT seedlings (21, 23). Thus, genes involved in the biosynthesis of chlorophylls and chloroplast development are up-regulated in dark-grown seedlings with decreased levels of PIFs, consistent with the hypothesis that these factors play a negative role in the regulation of photosynthetic development (21, 23, 25). In particular, PIF1 has been shown to directly bind to the promoter of the *PORC* gene encoding Pchlide oxidoreductase (POR, an enzyme that enables

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the conversion of Pchlide into chlorophylls) and also to indirectly regulate other chlorophyll biosynthetic genes (26).

Derepression of photomorphogenesis in the dark also leads to an up-regulation of *PSY* gene expression and activity, resulting in a concomitant increase in carotenoid biosynthesis (5). Higher *PSY* transcript levels also have been observed in dark-grown *pifQ* seedlings before and after illumination with R light (23). Based on the available data on the molecular mechanisms regulating light-triggered deetiolation, we proposed that PIFs might participate in the control of carotenoid biosynthesis during this critical process of plant life, and that this effect could be regulated at the transcriptional level, particularly by repressing *PSY* gene expression (27). Here we present experimental data that validate this model and demonstrate that PIF1 binds directly to the *PSY* promoter *in planta*. We also show that PIF1 and other PIFs participate in the control of *PSY* gene expression and carotenoid biosynthesis in fully deetiolated, mature plants.

Results and Discussion

PIF1 and Other PIFs Are Negative Regulators of Carotenoid Accumulation. To test whether carotenoid biosynthesis could be controlled by PIF activity during the transition of dark-grown seedlings to photosynthetic development, we first monitored the level of these compounds in etiolated and deetiolating WT and mutant seedlings with decreased PIF activity. Seedlings were germinated and grown in the dark for 3 d and then illuminated with R light for 6 h. A comparison of single mutants defective in PIF1, PIF3, PIF4, or PIF5 activity with the WT found that only *pif1* seedlings had significantly ($P < 0.05$) increased carotenoid levels in the dark (Fig. 1*A*). Illumination led to an up-regulation of carotenoid biosynthesis that was strongest in the case of the *pif1* mutant (Fig. 1*B*). Deetiolating *pif3* seedlings also showed an increased accumulation of carotenoids, whereas smaller differences were observed in the *pif4* and *pif5* mutants relative to WT seedlings (Fig. 1*B*). Double *pif1-pif3* mutant seedlings accumulated higher levels of carotenoids compared with the single *pif1* and *pif3* mutants in the dark (Fig. 1*A*) and during deetiolation (Fig. 1*B*), suggesting that PIF3 acts together with PIF1 to negatively regulate carotenoid biosynthesis. Furthermore, seedlings of the quadruple *pifQ* mutant (22) showed the highest levels of carotenoids both before (Fig. 1*A*) and after illumination (Fig. 1*B*), consistent with PIF4 and PIF5 also playing a role in this process. Similar results were found regarding chlorophyll accumulation in deetiolating seedlings (Fig. 1*B*), in agreement with previous reports of the role of these PIFs in negatively regulating the production of chlorophylls and the assembly of photosynthetic complexes (21, 23, 25). These data together suggest that the production of chlorophylls and carotenoids is coordinately regulated by PIFs during deetiolation, with a major contribution of PIF1 and an overlapping role of PIF3 and other PIFs (i.e., PIF4 and PIF5).

Carotenoid Levels Correlate with PIF-Mediated Changes in *PSY* Gene Expression. We next investigated whether the changes in carotenoid accumulation observed in etiolated and deetiolating seedlings were paralleled by similar changes in *PSY* transcript accumulation seen on real-time quantitative RT-PCR (qPCR) assays. The results showed an increase in *PSY* transcript levels relative to the WT in etiolated *pif1* (1.5-fold), *pif1-pif3* (2-fold), and *pifQ* (3-fold) seedlings (Fig. 1*C*) that was closely correlated with the increase in carotenoid levels (Fig. 1*A*). Derepression of deetiolation after illumination with R light led to a rapid up-regulation of *PSY* transcript levels in all genotypes (Fig. 1*C*). Compared

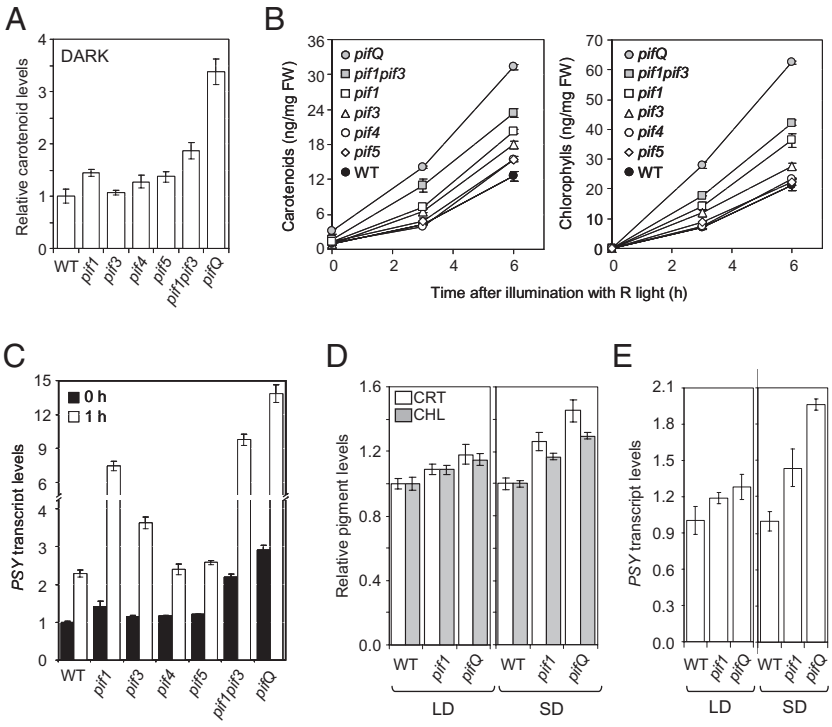

Fig. 1. PIFs negatively regulate the accumulation of carotenoids and the expression of the *PSY* gene. (*A*) Carotenoid levels in seedlings germinated and grown for 3 d in the dark. Values are shown relative to those in WT seedlings. (*B*) Carotenoid and chlorophyll accumulation after illumination of 3-d-old etiolated seedlings with R light for the indicated times. (*C*) qPCR analysis of *PSY* expression in seedlings germinated and grown for 3 d in the dark (black columns) and illuminated with R light for 1 h (white columns). *PSY* transcript accumulation normalized to *APT*1 levels is expressed relative to that in WT seedlings before illumination. (*D*) Carotenoid (CRT) and chlorophyll (CHL) accumulation in plants germinated and grown under long day (LD) or short day (SD) conditions for 3 wk. Values are represented relative to those in WT samples. (*E*) qPCR analysis of *PSY* transcript levels in the samples described in (*D*). Values are relative to the WT. In all cases, means ± SD ($n = 3$) are represented.

with the WT, however, a much stronger increase in *PSY* gene expression was seen in quadruple *pifQ*, double *pif1-pif3*, and single *pif1* seedlings. As a result, *PSY* transcripts in these seedlings were between 3- (*pif1*) and 6-fold (*pifQ*) higher than in the WT as soon as 1 h after illumination (Fig. 1*C*). Similar results were observed after 3 h of illumination (Fig. S1). Because the regulation of *PSY* gene expression is a critical factor controlling carotenoid biosynthesis during deetiolation (5, 16, 17), these results strongly hint that PIF1, and to a lesser extent other PIFs, control the production of carotenoids in etiolated and deetiolating seedlings by regulating *PSY* expression.

Because PIFs are also known to mediate light (phytochrome) signaling in fully deetiolated plants (20), we evaluated whether PIFs also influenced the accumulation of carotenoids and the regulation of *PSY* gene expression in plants grown for 3 wk under long-day (LD) or short-day (SD) conditions. Higher levels of carotenoids (Fig. 1*D*) and *PSY* transcripts (Fig. 1*E*) were observed in SD-grown *pifQ* and, to a lesser extent, *pif1* plants compared with the WT. Although these loss-of-function mutations also resulted in increased chlorophyll levels, the effect was weaker than that observed for carotenoids (Fig. 1*D*). These phenotypes were attenuated in LD-grown plants (Fig. 1 *D* and *E*). PIF1, PIF3, PIF4, and PIF5 are known to degrade during the day and to reaccumulate during the night in recurring light-dark cycles (20). Thus, it is possible that the longer night period in SD-grown plants allows greater accumulation of PIFs compared with LD-grown plants. This would eventually result in increased differences in terms of PIF-regulated phenotypes between WT and mutant plants under SD. Future experiments should clarify this issue. Together, these results suggest that PIF1 and other PIFs (i.e., PIF3, PIF4, and PIF5) contribute to regulate the expression of *PSY* and the accumulation of carotenoids in response to daily oscillations of light signals in fully deetiolated, mature plants.

PIF1 Efficiently Binds to a G-Box in the *PSY* Promoter. Based on their sequence in the basic domain, most members of the PIF family are predicted to bind to a G-box sequence (CACGTG), a subtype of the canonical E-box (CANNTG) binding sites for bHLH transcription factors (28). The *PSY* promoter contains several G-box–like sequences close to the transcription initiation site (18), but only two canonical G-boxes (numbered I and II) in an upstream region (Fig. 2*A*). Because PIF1 has been shown to preferentially bind to G-boxes (26, 29, 30), we tested the binding of the PIF1 protein to the G-box I and II motifs in the *PSY* promoter using the electrophoretic mobility shift assay (EMSA), as described previously (31). In brief, complementary oligonucleotides spanning the corresponding G-boxes and flanking regions were designed to be used as templates (Table S1). Similar versions in which the G-box had been removed by mutations were included as controls (Fig. 2*A*). We found that recombinant PIF1 bound efficiently and specifically to the labeled fragment encompassing G-box I (Fig. 2*B*), but not to that containing G-box II even at high PIF1 concentrations, confirming that the region flanking the G-box can modulate binding of this transcription factor to DNA (29, 30). The *PSY* promoter sequence with a mutated G-box I element did not compete with the WT G-box I fragment for PIF1 binding (Fig. 2*B*). These results demonstrate that PIF1 can directly bind to the *PSY* promoter in vitro in a sequence-specific manner.

PIF1 Binds Directly to the *PSY* Promoter to Repress Gene Expression *in Planta*. To investigate whether PIF1 binds directly to the *PSY* promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays as described previously (26). Transgenic lines constitutively expressing either a PIF1 fusion protein with a tandem affinity purification (TAP) tag harboring nine copies of the myc repeat (TAP-PIF1) in a *pif1* background or a TAP-tagged GFP protein (TAP-GFP) in a WT background were used for triplicate

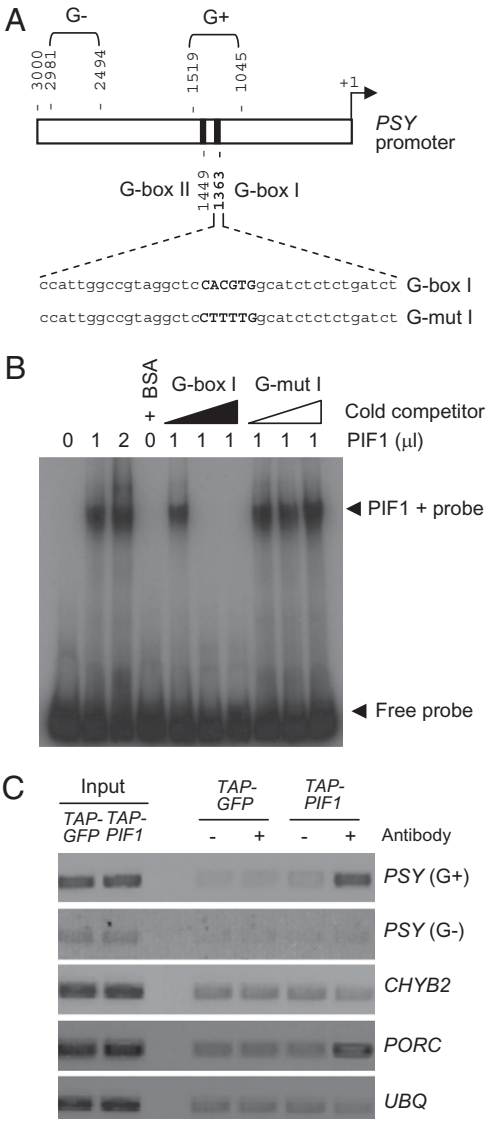

Fig. 2. PIF1 binds to the *PSY* promoter. (*A*) A scheme of the *PSY* promoter upstream the transcription initiation site (+1) (18). The position of the G-box motifs, the EMSA probes with WT (G-box I) and mutant (G-mut I) sequences, and the regions amplified in ChIP experiments (G+ and G–) are shown. (*B*) EMSA assay with labeled G-box I probes incubated with the indicated amounts of recombinant PIF1 protein or BSA as a negative control. A competition assay for PIF1 binding was performed using 5×, 50×, and 125× cold G-box I or G-mut I probes. (*C*) ChIP assay using lines constitutively expressing recombinant TAP-GFP or TAP-PIF1 proteins. Transgenic seedlings grown for 6 d under W light were treated with a saturating FR pulse and incubated in the dark for 24 h before sampling to inactivate the phytochromes. An anti-myc antibody was used for the immunoprecipitation of DNA fragments bound to TAP-PIF1 or TAP-GFP. Samples processed in the same way but without including the antibody were used as negative controls. Immunoprecipitated DNA was amplified by PCR using primers specific for the promoters of the indicated genes. In the case of *PSY*, the amplified regions included the sequence harboring the G-boxes (G+) as well as sequences lacking G-boxes (G–). Only a region containing the G-box motif was amplified in the case of *CHYB2*. Primers for *PORC* and *UBQ* were used as positive and negative (loading) controls, respectively, for PIF1 target genes. The gels show the PCR-amplified products from the ChIP assay. "Input" corresponds to the sample before immunoprecipitation; "+" and "–" indicate the presence or absence of antibody in the assay.

ChIP assays. After immunoprecipitation of protein–DNA complexes using an antibody against the myc epitope, enriched DNA sequences were amplified by PCR using primers that annealed

on the *PSY* promoter (Fig. 2*C*). The promoter region of *PORC* (a gene known to be a direct PIF1 target) also was amplified as a positive control, and the promoter of the ubiquitin *UBQ* gene was amplified as a negative and loading control (26). As shown in Fig. 2*C*, G-box–containing sequences from the promoters of both *PSY* and *PORC* genes were efficiently amplified from the immunoprecipitated fraction of the TAP-PIF1 samples, but not from either the TAP-GFP samples or controls without antibody. When PCR reactions were performed with primers for other regions of the *PSY* promoter lacking G-boxes, no specific amplification was obtained (Fig. 2*C*).

The binding of PIF1 to the region encompassing the G-box motifs in the *PSY* promoter could function as a mechanism to rapidly transduce light signals to control carotenoid biosynthesis by regulating *PSY* gene expression. To test this possibility, we conducted experiments using *pif1* mutant lines overexpressing a PIF1 chimeric protein fused to the glucocorticoid receptor sequence (PIF1-GR) that required treatment with exogenous dexamethasone (DEX) for nuclear transport and biological activity. These transgenic lines and WT controls were germinated on filter paper and grown for 3 d under continuous white (W) light and then transferred to plates with or without DEX for 3 h. DEX treatment had no effect on the accumulation of *PSY* transcripts in WT plants (Fig. 3*A*). In contrast, *PSY* transcript levels were lower in DEX-treated PIF1-GR seedlings compared with mock-treated controls (Fig. 3*B*). This result confirms that *PSY* expression can be rapidly down-regulated in response to sudden changes in the levels of active PIF1. Moreover, treatment of transgenic lines with the protein synthesis inhibitor cycloheximide (CHX) had no effect on the DEX-mediated decrease of *PSY* transcript levels (Fig. 3*B*). We confirmed CHX activity by monitoring the expression of *SAUR68* (Fig. 3*A*), a CHX-induced gene (32). As an additional control, we analyzed the expression of genes previously found to be regulated by direct binding of PIF1 to their promoters, including *PORC* (26) and *GAI* (29). These genes were confirmed to be induced by DEX treatment even in the presence of CHX in transgenic PIF1-GR lines, but not in WT control seedlings (Fig. 3). The observation that PIF1 binding eventually results in activation of some genes (*PORC*, *GAI*) but repression of others (*PSY*) is consistent with the fact that particular bHLH transcription factors can have both positive and negative effects on gene expression (28).

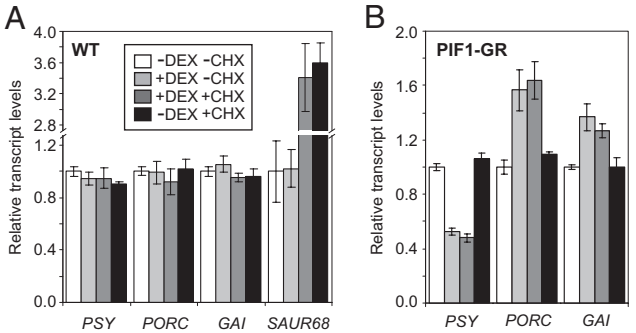

Fig. 3. PIF1 rapidly down-regulates *PSY* expression in vivo. WT (*A*) and transgenic *pif1* + PIF1-GR (*B*) seedlings were germinated and grown on filter paper under W light for 3 d and transferred to new plates either supplemented (+) or not (–) with dexamethasone (DEX) and/or cyclohexymide (CHX). Samples were collected 3 h after transfer and used for RNA extraction and qPCR analysis of *PSY*, *PORC*, and *GAI* transcript levels. CHX activity was verified by analyzing the levels of the CHX-induced *SAUR68* gene. Transcript levels are normalized to those of *APT1* and shown relative to mock-treated samples. Data in columns are mean ± SD (n = 3).

Our data provide evidence that *PSY* is a primary direct target of PIF1 *in planta*, and that PIF1 binds to the *PSY* promoter to negatively regulate its activity in *Arabidopsis*. The involvement of phytochromes in the control of carotenoid accumulation and *PSY* gene expression in different plant species, including tomato, maize, and rice (12, 13, 33), along with the presence of G-boxes in the promoters of light-responsive maize and rice *PSY* genes (13, 34), suggest that the participation of PIFs in the light-mediated regulation of *PSY* gene expression might be a general mechanism in plants.

PIF1 Does Not Regulate Other Genes Involved in Carotenoid Biosynthesis. A recent microarray analysis comparing transcript profiles of dark-grown WT and *pifQ* seedlings before and after illumination with R light included *PSY* in a group designated class 7 (23). These class 7 genes, which demonstrated robust derepression in the mutant when grown in the dark, rapid R light–triggered induction, and sustained expression after 48 h under R light, were considered the best candidates for direct targets of PIF-regulated deetiolation (23). In addition to *PSY*, most genes involved in the biosynthesis of carotenoids and their isoprenoid precursors are light-induced during deetiolation (35) (Fig. S2). Among these genes, *DXS*/*CLA1* (At4g15560), *CHYB2* (At5g52570), and *ZEP*/*ABA1* (At5g67030) belong to class 7 as well (23). Although the carotenoid biosynthetic gene *LCYE* (At5g57030) is not included in this class, it also exhibited up-regulated transcript levels in dark-grown *pifQ* seedlings and rapid derepression on light exposure (Fig. S2). Of these four potential PIF target genes (*DXS*/*CLA1*, *CHYB2*, *ZEP*/*ABA1*, and *LCYE*), only *CHYB2* contained a G-box motif in its promoter (as deduced from the analysis of 3 kb of the 5′-upstream region). However, ChIP assays performed to investigate a possible role of PIF1 in the regulation of this gene showed no enrichment in the TAP-PIF1 sample compared with the controls (Fig. 2*C*). Together, these results suggest that PIF1 specifically targets the *PSY* gene for the control of carotenoid biosynthesis during deetiolation. Consistently, the up-regulation of *PSY* expression has been demonstrated to be sufficient by itself to increase carotenoid production in various plant systems, including deetiolating seedlings (5, 9, 36).

Because short versions of the *Arabidopsis PSY* promoter lacking the G-boxes appear to remain light-responsive (18), it is likely that other transcription factors besides PIFs participate in the light-mediated regulation of *PSY* expression during seedling deetiolation. These as-yet unidentified transcription factors might be responsible for the up-regulation of *PSY* expression observed after illumination of mutant *pifQ* seedlings. They also might be involved in regulating other light-induced carotenoid biosynthetic genes that lack G-boxes, as well as those with G-boxes for which binding of PIF1 was not observed in our ChIP assays, such as *CHYB2*. The identity of the transcription factors that directly regulate carotenoid biosynthetic genes has remained elusive until now. The identification of a common *cis* element (ATCTA) in the promoters of *PSY* and other *Arabidopsis* genes involved in photosynthesis and photoprotection (18) suggested a simple mechanism for the coordinated control of these two critical processes based on the existence of common *cis* and *trans* factors. However, the only *trans* factor found to bind to this motif does not appear to be instrumental for the control of *PSY* expression or carotenoid synthesis (37). In contrast, the results reported here validate the existence of such a mechanism for a safe transition of etiolated (heterotrophic) seedlings to photomorphogenic (photoautotrophic) development based on direct or indirect regulation of the expression of key genes of both the carotenoid and the chlorophyll pathways by PIF1 and other PIFs in response to light signals.

Multilevel Role for PIF1 and Other PIFs in Regulating Photosynthetic Metabolism. A model for the PIF-mediated control of photosynthetic metabolism during seedling deetiolation emerges from the

available data (Fig. 4). When *Arabidopsis* seedlings germinate in the dark, high PIF levels prevent photomorphogenic development (21–23). Under these conditions, low carotenoid levels are produced due to the repression of the *PSY* gene by direct binding of PIF1 and possibly other PIFs as well. The accumulation of PIFs in dark-grown seedlings also appears to indirectly down-regulate other genes involved in carotenoid biosynthesis (Fig. S2), and to repress genes required for chlorophyll biosynthesis and chloroplast development (21, 23, 25, 26). At the dark-to-light transition stage, the levels of PIFs are dramatically reduced by their degradation on interaction with photoactivated phytochromes, causing a common derepression of all these genes. This triggers a coordinated biosynthesis of carotenoids and chlorophylls in parallel with the production of components of the photosynthetic machinery and the differentiation of etioplasts into chloroplasts. The assembly of photosynthetic complexes and the buildup of thylakoid membranes in developing chloroplasts increase the capacity to sequester the newly synthesized carotenoid molecules, which also improves PSY enzyme activity (17). As a result, carotenoid production and accumulation increase rapidly, protecting the emerging photosynthetic apparatus from photooxidative damage when underground seedlings emerge from the soil into sunlight. PIF1 and other PIFs also repress *PSY* gene expression and carotenoid biosynthesis in fully deetiolated plants (Fig. 1). In mature plants grown under daily dark and light cycles (SD), the effect of the loss-of-function *pif1* and *pifQ* mutations on the accumulation of chlorophylls is weaker than that on the accumulation of carotenoids. This observation supports the conclusion that PIFs contribute to coordinate the production of chlorophylls and carotenoids throughout the plant's life.

Along with their essential role in photosynthesis, carotenoids are also of significant economic interest as natural pigments and food additives. Their presence in the human diet provides health benefits as nontoxic precursors of vitamin A and antioxidants (9, 36). In this context, manipulating the levels of PIF transcription factors by transgenic or marker-assisted breeding approaches might help improve carotenoid accumulation in plants for the production of varieties with enhanced agronomical, industrial, or nutritional value.

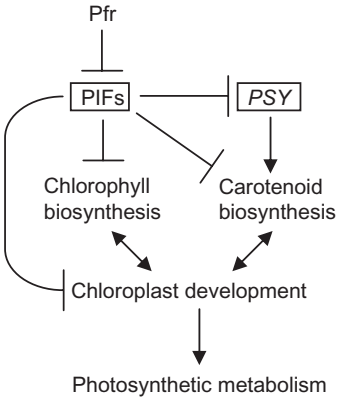

Fig. 4. A model for the role of PIFs in regulating photosynthetic metabolism during seedling development. In dark-grown seedlings, high PIF1 levels repress *PSY* gene expression by direct binding to its promoter. PIFs also repress other genes involved in carotenoid biosynthesis (likely by indirect pathways), as well as genes required for the biosynthesis of chlorophylls and the differentiation of etioplasts into chloroplasts (such as those encoding components of the photosynthetic apparatus). All of these genes are rapidly and coordinately derepressed when PIFs levels drop on illumination, when photoactivated phytochromes (Pfr form) migrate to the nucleus and interact with PIFs to promote their degradation. This leads to a rapid production of carotenoids and chlorophylls together with components of the photosynthetic machinery in an interdependent fashion, eventually resulting in the development of functional chloroplasts and the transition to photosynthetic metabolism.

Materials and Methods

Plant Material and Growth Conditions. All of the *A. thaliana* lines used in this work are in the Columbia background. Seeds from *pif1-2, pif3-3*, *pif4-2*, *pif5-2*, *pif1-2 pif3-3,* and *pifQ* were kindly provided by P. Quail (University of California Berkeley). The *35S::TAP-PIF1* and *35S::TAP-GFP* transgenic lines were produced as described previously (24). The PIF1-GR construct was created by cloning PIF1 (At2g20180) cDNA into the *Xba*I-*Bam*HI sites of pBI-ΔGR (38). The 35S::PIF1-GR cassette was removed from pBI-ΔGR by restriction digestion using *Hin*dIII-*Eco*RI and then cloned into the pPZP100 binary vector (39). The resulting construct was introduced into *Agrobacterium tumefaciens* GV3101 (MP90) and used for transformation of *pif1-2* plants by floral dip. Transgenic seeds were selected on 100 μg mL^{-1} of gentamycin. Homozygous lines containing a single T-DNA insertion were selected by segregation of the resistance marker.

Fresh seeds were surface sterilized and sown on sterile filter paper on top of sterile MS media in Petri dishes. Plates were kept for 4 d at 4 °C in darkness for stratification. For deetiolation experiments, a 3-h W light treatment (35 μmol m^2 s^{-1}) at 21 °C was given to induce and synchronize germination. The plates were then wrapped in aluminum foil and kept in darkness at 21 °C for 3 d before illumination with R light (30 μmol m^2s^{-1}). For the experiments with DEX and CHX, plates were incubated at 21 °C under W light (35 μmol m^2 s^{-1}) for 3 d, before the filter papers with the seedlings were transferred to fresh MS plates containing DEX and/or CHX as described previously (32). For the analysis of mature plants and seed generation, plants were germinated and grown on soil at 22 °C under long-day (8 h of dark and 16 h of W light) or short-day (16 h of dark and 8 h of W light) conditions.

Measurement of Carotenoid and Chlorophyll Levels. Total carotenoid and chlorophylls were extracted and quantified spectrophotometrically, as described previously (40, 41). Concentration in a given sample was estimated relative to fresh weight.

RNA Isolation and Analysis of Transcript Levels by qPCR. Total RNA was isolated from seedlings using an RNA purification kit (Sigma-Aldrich) and reverse-transcribed using SuperScript II (Invitrogen). The qPCR protocol was as described previously (5), but using Fast Start Universal SYBR Green Master Mix (Roche) on a Light Cycler 480 apparatus (Roche). The *APT1* (At1g27450) gene was used for normalization. The primer sequences for the qPCR reactions are listed in Table S1.

DNA Gel Shift Assays. EMSAs were conducted as described previously (31). For the experiment, PIF1 recombinant protein was produced in bacteria and incubated with a *PSY* promoter fragment containing the G-box motif labeled with ^{32}P-dCTP. Cold competitor probe was generated from the same promoter region using dimerized oligos containing a mutated G-box sequence (see Fig. 2, and Table S1 for more details).

Chromatin Immunoprecipitation Assays. ChIP assays were performed as described previously (26), except that dark-adapted seedlings were used for the assay. Seeds were sterilized and stratified as described above, and then grown for 6 d under W light (50 μmol m^2 s^{-1}). After this, an FR light-saturating pulse was given to return the phytochromes to the inactive form, and seedlings were immediately transferred to darkness for 24 h before tissue collection. The sequence of the primers used in these experiments to amplify promoter sequences of individual genes is shown in Table S1.

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