Identification of Primary Target Genes of Phytochrome Signaling. Early Transcriptional Control during Shade Avoidance Responses in Arabidopsis[1]

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The phytochrome (phy) photoreceptors modulate plant development after perception of light. Upon illumination of etiolated seedlings, phys initiate a transcriptional cascade by directly transducing light signals to the promoters of genes encoding regulators of morphogenesis. In light-grown plants, however, little is known about the transcriptional cascade modulated by phys in response to changes in light. The phy entry points in this cascade are completely unknown. We are particularly interested in the shade avoidance syndrome (SAS). Here we describe a subset of six genes whose expression is rapidly modulated by phys during both deetiolation and SAS in Arabidopsis (*Arabidopsis thaliana*). Using cycloheximide, we provide evidence that four of these *phy rapidly regulated* (*PAR*) genes are direct targets of phy signaling during SAS, revealing these genes as upstream components of the transcriptional cascade. Promoter-β-glucuronidase fusions confirmed that *PAR* genes are photoregulated at the transcriptional level. Analysis of gene expression in light signal transduction mutants showed that COP1 and DET1 (but not DET2 or HY5) play a role in modulating *PAR* expression in response to shade in light-grown seedlings. Moreover, genetic analyses showed that one of the genes identified as a direct target of phy signaling was *phy-interacting factor 3-like-1* (*PIL1*). PIL1 has previously been implicated in SAS in response to transient shade, but we show here that it also plays a key role in response to long-term shade. The action of PIL1 was particularly apparent in a *phyB* background, suggesting an important negative role for PIL1 under dense vegetation canopies.

Light regulates different aspects of plant growth and development, such as seed germination, stem elongation, and flowering time. Photoreceptors perceive light and transduce the signal to physiological responses. The red (R) and far-red (FR) light-absorbing phytochromes (phys) play a major role in controlling many of the aforementioned responses. Phys exist in two photointerconvertible forms. After synthesis of the R-absorbing form (Pr; λ_{max} 666 nm), photoconversion to the active FR-absorbing form (Pfr; λ_{max} 730 nm) is required for all responses. FR irradiation can subsequently reconvert Pfr to the Pr form. In Arabidopsis (*Arabidopsis thaliana*), phys are encoded by a small gene family of five members (*PHYA*–*PHYE*). PhyA is exclusively responsible for controlling seedling deetiolation under continuous FR (FRc) and phyB has the major role in this response under continuous R (Rc; Quail, 2002; Schäfer and Bowler, 2002; Chen et al., 2004). In light-grown plants, phyB, phyD, and phyE coregulate other responses, such as those known as the shade avoidance syndrome (SAS; Smith and Whitelam, 1997).

In dark-grown seedlings, phyA and phyB are cytosolic, inactive proteins that migrate to the nucleus upon light activation (Quail, 2002; Schäfer and Bowler, 2002). Both Pfr formation and nuclear translocation are necessary for phyB signaling activity (Huq et al., 2003). In the nucleus, phy-interacting factor-3 (PIF3), a basic helix-loop-helix (bHLH) protein, binds preferentially to the Pfr forms of phyA and phyB (Ni et al., 1998). PIF3 simultaneously binds to Pfr and a G-box motif located in the promoter region of several genes (Martínez-García et al., 2000) and exhibits phy-modulated transcriptional activity at target promoters (Ni et al., 1998; Martínez-García et al., 2000; Kim et al., 2003). This is moderated

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by rapid phy-induced degradation in the nucleus (Bauer et al., 2004; Monte et al., 2004). Further genomic analyses expanded this view and led to the proposal that, during deetiolation, light might implement the photomorphogenic program by regulating a complex transcriptional cascade, probably initiated by direct phy regulation of gene expression of a master set of transcriptional regulators via different PIFs (Tepperman et al., 2001). Indeed, different PIFs or PIF-likes (PILs), all belonging to the bHLH class of transcription factors (TFs), play important roles in phy signal transduction, very likely participating in the early steps of this transcriptional cascade (Huq and Quail, 2002; Kim et al., 2003; Bauer et al., 2004; Huq et al., 2004; Oh et al., 2004).

Most of the known phyA and phyB signaling components have been identified by genetic approaches based on analysis of seedling deetiolation. These screens have yielded two major classes of mutants: the *cop/det/fus* class of global regulators and a miscellaneous group, including components that appear to be specific for either phyA, phyB, or both phy signals. Null mutants of the COP/DET/FUS family of nuclear-localized factors display constitutive deetiolation in darkness. *COP1* encodes a repressor shown to be part of a large protein complex and to have E3 ubiquitin ligase activity toward some TFs (Saijo et al., 2003; Seo et al., 2003). In dark-grown seedlings, COP1 accumulates in the nucleus, where it interacts with TFs that trigger deetiolation, such as HY5, HYH, LAF1, and HFR1 (Holm et al., 2002; Seo et al., 2003; Duek et al., 2004), targeting them for proteasome-mediated degradation with the involvement of the COP9 signalosome and COP10, an E2 ubiquitin-conjugating enzyme variant (Suzuki et al., 2002). Soon after illumination, rapid changes in both gene expression (over the first hour of light treatment) and protein abundance (within 2 h) of these TFs initiate deetiolation. In the longer term (several hours), the slow light-mediated nuclear depletion of COP1 relieves the repression of the TFs, eventually resulting in seedling photomorphogenesis (Osterlund et al., 1999, 2000; Hardtke and Deng, 2000). DET1 and DDB1, a DET1-interacting factor (Schroeder et al., 2002), have been shown to form a complex with COP10, called the CDD complex, which interacts with the COP1 complex (Yanagawa et al., 2004). Therefore, it has been suggested that COP1 and DET1 act together to regulate ubiquitin proteasome-mediated degradation of photomorphogenesis-promoting TFs in darkness (Yanagawa et al., 2004). PhyA signaling is also directly regulated during deetiolation by light-induced degradation of the phyA photoreceptor itself, and by COP1 E3 activity in a process that implicates the proteasome-mediated degradation machinery (Seo et al., 2004).

An important gap exists in our understanding of phy action because the functioning of phys in established light-grown plants is very poorly understood. Under these conditions, phyB, rather than phyA, is most abundant; the photoequilibrium between the Pfr and Pr forms is already established; phys are already nuclear (Kircher et al., 2002); and the amount of nuclear COP1 is low (although it is still sufficient to modulate development; von Arnim et al., 1997). There are also many other differences between light-grown and etiolated seedlings, such as large changes in gene expression patterns (Tepperman et al., 2001; Ma et al., 2003). We have focused on the analysis of SAS, one of the best-characterized phy-dependent responses in light-grown plants. SAS refers to a set of responses (which affect hypocotyl and/or stem elongation, cotyledon expansion, petiole length, flowering time, etc.) triggered by a reduction in the R to FR ratio associated with the proximity of neighboring vegetation (Smith, 1982; Smith and Whitelam, 1997). Changes in the R to FR ratio are detected by plants as a change in the relative proportions of Pr and Pfr. Although phyB is the major phy controlling SAS, genetic and physiological analyses have shown that other phys act in conjunction with phyB in the control of some aspects of SAS-driven development, like flowering time (phyD and phyE), petiole elongation (phyD and phyE), and internode elongation between rosette leaves (phyE; Devlin et al., 1998, 1999). Downstream of the phys, information about the components involved in the SAS control is limited. Previous work showed that expression of three genes, *ATHB2/HAT4* (hereafter *ATHB2*), *ATHB4*, and *PIL1*, is quickly and reversibly regulated by simulated shade (Carabelli et al., 1993, 1996; Salter et al., 2003). Genetic approaches have demonstrated roles for *ATHB2* and *PIL1* in the SAS response (Steindler et al., 1999; Salter et al., 2003). ATHB2 has also been shown to affect morphology throughout the life history of Arabidopsis (Schena et al., 1993). A role for PIL1 has, thus far, only been demonstrated in the responses of hypocotyls of young seedlings to transient exposure to shade (Salter et al., 2003). Very recently, another gene, *HFR1*, has been shown to be rapidly upregulated by simulated shade and to negatively regulate SAS responses, likely contributing to a fitting response to canopy shade in nature (Sessa et al., 2005). Genomic analyses have also identified dozens of additional SAS-regulated genes (Devlin et al., 2003), suggesting that the SAS program is implemented by phy regulation of a complex transcriptional cascade, as is postulated for deetiolation. However, very little is known about how phy perception is translated into changes in gene expression, what the cellular factors or biochemical activities involved are, and whether the large-scale changes in gene expression after simulated shade are necessary for implementing the morphological and physiological modifications that result in the measured SAS responses. Ultimately, these plastic responses are initiated by the proximity of neighboring plants and evoke appropriate competitive or survival reactions by which the plant attempts to overgrow or to accelerate flowering and early seed production.

By exploring available genomic and molecular information in Arabidopsis, we have identified in this work a subset of genes whose expression is rapidly

regulated by phys after SAS induction by simulated shade. Pharmacological evidence strongly suggests that some of these *phy rapidly regulated* (*PAR*) genes are primary targets of phy action during SAS. Promoter-β-glucuronidase (*GUS*) fusions confirmed that *PAR* genes are photoregulated at the transcriptional level, with COP1 and DET1 (but not HY5) playing a role in modulating their expression during SAS. Finally, we show that one of the *PAR* genes, *PIL1*, controls SAS responses in addition to the previously reported effect on hypocotyl elongation upon transient exposure to shade.

RESULTS

Early Phy-Regulated Genes during Both Deetiolation and SAS as Candidates for Primary Targets of Phy Action

We aimed to identify primary target genes of phy signaling within the transcriptional cascade operating after induction of SAS in light-grown Arabidopsis plants. We reasoned that at least some of these genes should be rapidly regulated by phys in other physiological contexts, such as seedling deetiolation. Indeed, we observed that some of the Arabidopsis genes known to be rapidly up-regulated by simulated shade in light-grown plants (*ATHB2*, *ATHB4*, and *PIL1*) were also rapidly down-regulated after seedling deetiolation. Although light regulates the expression of these genes during deetiolation and SAS in opposite directions (repression and activation, respectively), in both cases their expression is down-regulated by phy action. To identify other *PAR* genes showing this pattern of expression during both processes, we first looked for genes that were rapidly down-regulated during seedling deetiolation under FRc (Tepperman et al., 2001). Besides *ATHB2*, *ATHB4*, and *PIL1*, we identified genes encoding an unknown factor (At2g42870; hereafter PAR1), a putative pectinesterase (At4g25260; RIP), a β-expansin (At2g20750; β-EXP), and three TFs originally classified as late repressed, but nonetheless showing a clear down-regulation only 1 h after illumination: SCL21 (At2g04890), HAT2 (At5g47370), and HAT7 (At5g15150; Tepperman et al., 2001). Subsequent microarray experiments showed that some of these genes were also rapidly up-regulated by simulated shade (Devlin et al., 2003).

To confirm the microarray data, expression of the selected *PAR* genes was evaluated by RNA-blot analysis in seedlings grown under continuous white light (W) before and 1 h after illumination with W enriched with FR (W + FR, simulated shade). As expected (Carabelli et al., 1993, 1996; Salter et al., 2003), expression of *ATHB2*, *ATHB4*, and *PIL1* was up-regulated by simulated shade in the three different ecotypes used (data not shown). W + FR also induced expression of *HAT2*, *PAR1*, and *RIP* (Fig. 1). The up-regulated expression of these genes was sustained in seedlings left for up to 3 h under simulated shade (data not shown). Changes in the R to FR ratio, however, did not affect

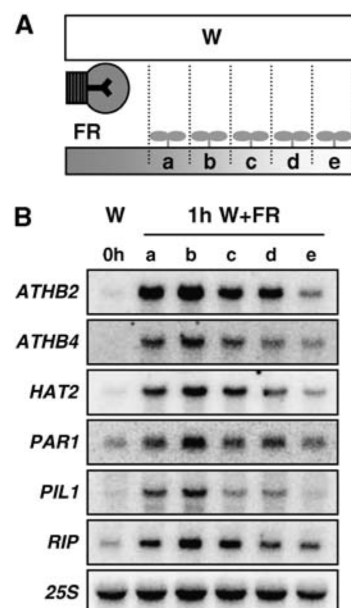

Figure 1. Expression of *PAR* genes in response to simulated shade. A, Experimental configuration used to study the effect of different R to FR ratios on *PAR* expression. No-0 seedlings grown for 7 d (d7) under continuous white light (W; white box), were treated for 1 h with W enriched in FR applied laterally. The resulting R to FR ratios were 0.07 (a), 0.09 (b), 0.13 (c), 0.20 (d), and 0.31 (e). B, RNA analysis of expression of *PAR* genes in seedlings grown as indicated in A.

HAT7 and *SCL21* expression, whereas β-EXP was undetectable in the light-grown seedlings used in this study (data not shown). The six *PAR* genes demonstrated to be rapidly up-regulated by simulated shade (*ATHB2*, *ATHB4*, *HAT2*, *PAR1*, *PIL1*, and *RIP*) were selected for further study.

Changes in the R to FR Ratio and Phy Levels Impair *PAR* Gene Expression

To further substantiate the dependence on light quality of the observed changes in *PAR* gene expression, seedlings were exposed to different R to FR ratios for 1 h (Fig. 1A). The level of simulated shade-induced up-regulation ranged from a maximal response for the two lowest R to FR ratios (0.07–0.09) to a lesser effect for the highest R to FR ratio (0.31) in all genes (Fig. 1B). The results indicate that the observed rapid up-regulation of *ATHB2*, *ATHB4*, *HAT2*, *PAR1*, *PIL1*, and *RIP* is truly dependent on simulated shade and proportional to the degree of shading, consistent with physiological SAS responses (Smith, 1982).

The role of phys in controlling the expression of the identified *PAR* genes in Arabidopsis was confirmed using transgenic lines overexpressing oat (*Avena sativa*) phyA (AOX; Boylan and Quail, 1991) or Arabidopsis phyB (ABO; Wagner et al., 1991). Seedlings grown in W were either maintained in W or transferred to W + FR for 24 h. Wild-type seedlings under simulated shade showed elongated hypocotyls relative to

those maintained in W, which is evidence of an active SAS response (Fig. 2A). As expected, such a response was significantly attenuated in phy-overexpressing seedlings, with ABO seedlings displaying stronger inhibition of the response than AOX seedlings (Fig. 2A). *PAR* gene expression was also affected in both AOX and ABO lines because reduced *PAR* transcript levels were detected before and after simulated shade treatment compared to wild-type seedlings (Fig. 2B). Again, ABO seedlings displayed the strongest effect. Together, the results indicate that high phy levels maintain a strong repression of *PAR* expression in light-grown seedlings.

The Rapid Phy-Regulated Expression of Some *PAR* Genes Does Not Require de Novo Protein Synthesis

To address whether any of the identified *PAR* genes might be a primary phy target, we used the protein synthesis inhibitor cycloheximide (CHX). The rationale behind this experiment was that the light response of primary phy target genes would be unaffected by CHX

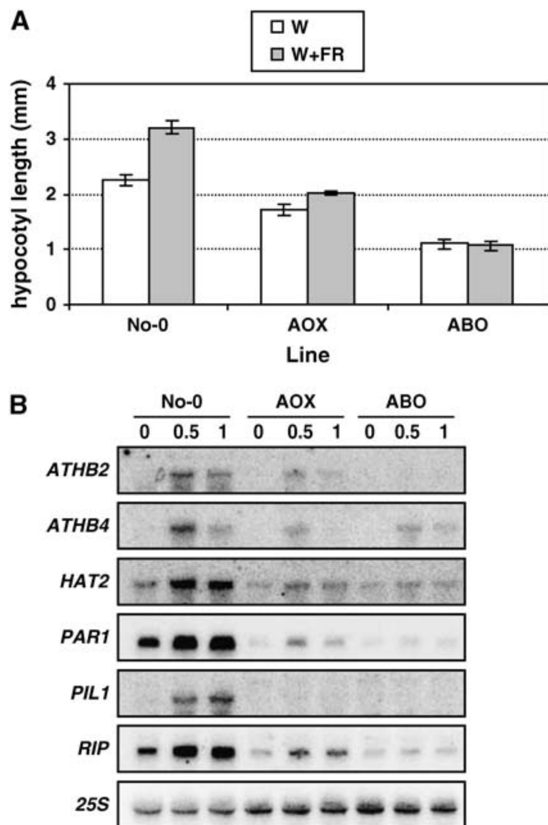

Figure 2. Effect of increased levels of phyA (AOX) and phyB (ABO) on *PAR* expression and hypocotyl length induced by simulated shade. A, Changes in hypocotyl length in response to simulated shade were analyzed in wild-type (No-0), AOX, and ABO seedlings. Seedlings grown for 7 d under W were either maintained in W (white bars) or transferred to W + FR (gray bars) for 24 h, after which mean (±SE) hypocotyl lengths were measured. B, RNA analysis of *PAR* gene expression in Arabidopsis wild-type, AOX, and ABO seedlings harvested at 0, 0.5, and 1 h after W + FR treatment.

because protein synthesis would not be required. To validate our experimental conditions, we used the previously characterized LhGR-N(4c) line in which the *GUS* reporter gene is a direct target gene of the TF LhGR (Craft et al., 2005). Nuclear translocation (hence, transcriptional activity) of LhGR is dependent on treatment with dexamethasone (DEX), a synthetic glucocorticoid. In the absence of CHX, seedlings exhibited strong DEX-dependent GUS staining, as expected (Fig. 3A). However, when CHX was coapplied with DEX, it completely blocked GUS activity after 24 h (Fig. 3A), indicating that CHX treatment efficiently inhibited de novo synthesis of the GUS protein. The inhibition of GUS synthesis by CHX was observed as early as 2 h after coapplication of CHX and DEX (data not shown). However, the application of DEX 1 h before CHX treatment resulted in much more reproducible results (Fig. 3B) and confirmed that treatment of seedlings with CHX for 2 h efficiently blocked protein synthesis. In subsequent experiments, we treated seedlings with CHX for 2 h before initiating simulated shade treatments for target-gene analysis in planta.

Seedlings grown under W were transferred to W + FR for 1 h and then returned to W for an additional hour. In the absence of CHX (−CHX), the levels of *PAR* mRNAs increased after simulated shade and decreased upon transferring the seedlings back to W (Fig. 3C), confirming that *PAR* gene expression is, indeed, rapidly and reversibly regulated by changes in light quality. In the presence of CHX (+CHX), the expression levels of a number of *PAR* genes were altered even before the simulated shade treatment (Fig. 3C). The strongest effect was a clear increase in the expression of *HAT2*, a gene previously shown to be induced by CHX treatment (Sawa et al., 2002). Transcript levels of *HAT2* and *RIP* were unaltered by simulated shade in CHX-treated seedlings. By contrast, up-regulation of *ATHB2* and *ATHB4* transcript levels by simulated shade was dramatically increased in CHX-treated seedlings, whereas a weaker up-regulation was observed for *PAR1* and *PIL1* compared to mock-treated seedlings. Most significantly, the reversible and photoregulated response of these latter four genes was qualitatively independent of the CHX treatment. We concluded that the shade-mediated up-regulation of a subset of *PAR* genes (*ATHB2*, *ATHB4*, *PAR1*, and *PIL1*) does not require de novo protein synthesis, consistent with these being direct targets of phy action. *RIP* can be considered as a secondary target of phy action. The high sensitivity of *HAT2* expression to CHX does not allow us to ascertain whether this is a phy primary target.

SAS-Associated Changes in *PAR* Gene Expression Are Impaired in *cop1* Mutants

COP1, a master integrator of light signaling during seedling deetiolation, has also been shown to participate in shade-induced hypocotyl elongation (McNellis et al., 1994) and to regulate the abundance of HFR1 (Duek et al., 2004), a TF encoded by a gene recently identified

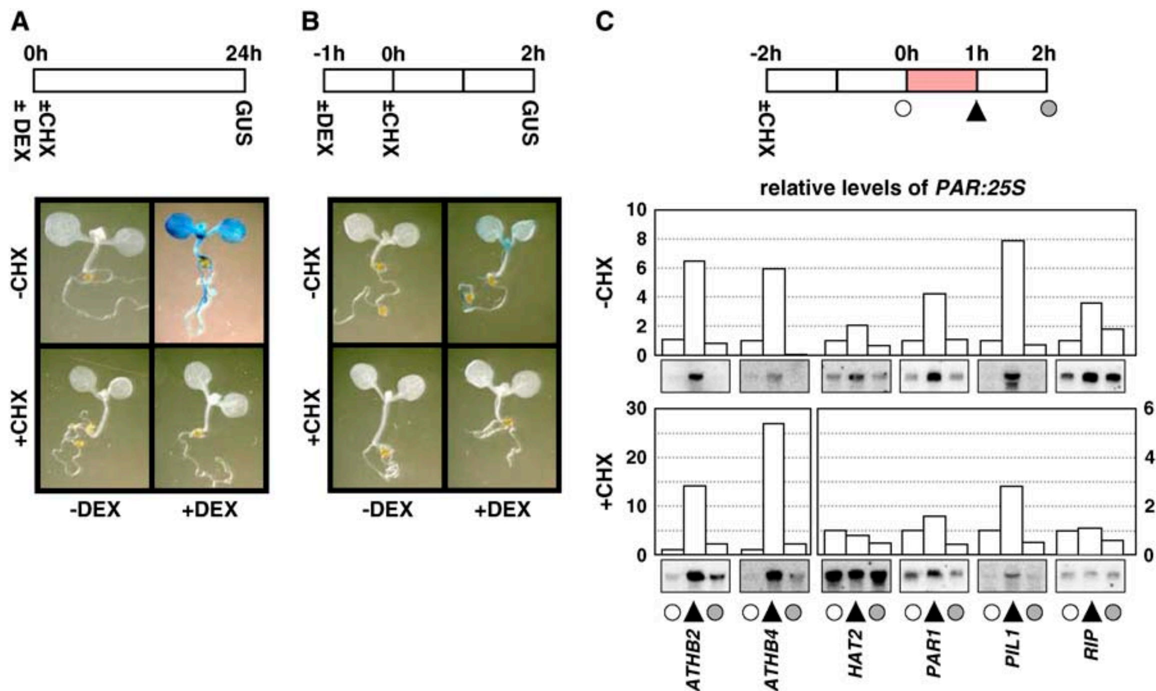

Figure 3. Identification of phy primary target genes within the *PAR* genes. A, GUS activity in seedlings of the DEX-inducible LhGR-N(4c) line 24 h after simultaneous ±CHX and ±DEX treatment, as shown in the top diagram. B, GUS activity in seedlings of the DEX-inducible LhGR-N(4c) line 2 h after ±CHX treatment and 3 h after ±DEX treatment, as schematized in the top diagram. C, Analysis of the effect of CHX on light-regulated *PAR* gene expression. Two hours before altering light quality, 7-d-old No-0 seedlings were treated without CHX (−CHX) or with CHX (+CHX). W-grown seedlings were irradiated for 1 h with W + FR and then transferred to W for 1 h, as schematized in the top diagram. Plant material was harvested immediately before (0 h; white circle), 1 h (triangle), and 2 h (gray circle) after beginning light treatments. RNA-blot analyses of *PAR* expression in these samples, as well as the normalized relative levels of expression for one representative experiment, are shown.

to be rapidly up-regulated by simulated shade (Sessa et al., 2005). To investigate whether COP1 might also have a role in the regulation of the *PAR* genes identified here as direct targets of phy signaling during SAS, we used the nonlethal loss-of-function alleles *cop1-4* and *cop1-6* (Deng et al., 1992). Mutant and wild-type seedlings grown in W were transferred to W + FR and samples were collected at 0-, 0.5-, and 1-h time points. RNA-blot analysis showed that *PAR1* and *PIL1* displayed reduced photomodulation in both *cop1* mutants compared to that observed in wild-type seedlings (Fig. 4A). The same was true for *ATHB2* and *ATHB4*, but the effect was weaker (Fig. 4A). These results reveal that COP1 has a role in regulating expression of the identified phy primary target genes in response to simulated shade.

To evaluate whether phy-regulated expression of these *PAR* genes is also affected by COP1 during deetiolation, wild-type and *cop1-6* seedlings were grown for 4 d in the dark and then illuminated with FRc. Samples were harvested before (0 h) and 1 h after FRc treatment and used for RNA-blot analysis (Fig. 4B). As expected, all four genes were rapidly and strongly down-regulated after light treatment in the wild type. In etiolated *cop1-6* seedlings, *PAR1* mRNA levels were higher than those in wild-type seedlings both before and after treatment, whereas minor differences were observed for *ATHB2* and *PIL1*. Transcript levels of *ATHB4* in mutant seedlings were hardly detectable in *cop1* mutants under any conditions (Fig. 4B). Most importantly, *cop1-6* seedlings showed clear phy-mediated repression of *ATHB2*, *PAR1*, and *PIL1*, as was observed in wild-type seedlings (Fig. 4B). The low levels of *ATHB4* mRNAs made it difficult to draw any conclusion as to the role of COP1 in the phy-mediated changes of this gene during FRc-induced deetiolation. These results indicate that, unlike the situation observed for SAS, COP1 does not play a major role in the early repression of at least three of the analyzed *PAR* genes during FRc-mediated deetiolation.

Photomodulation of Phy Primary Target Genes Is Attenuated in *det1*, But Not in *det2* or *hy5* Mutants

COP1 directly interacts with HY5, another photomorphogenic regulator with a role in seedling deetiolation (Oyama et al., 1997; Ang et al., 1998). Because HY5 is a TF, we aimed to investigate whether it might also participate in controlling the expression of the identified direct target genes of phy signaling during SAS. Mutant *hy5-1* (a null allele) and wild-type seedlings were grown in W and then transferred to W + FR.

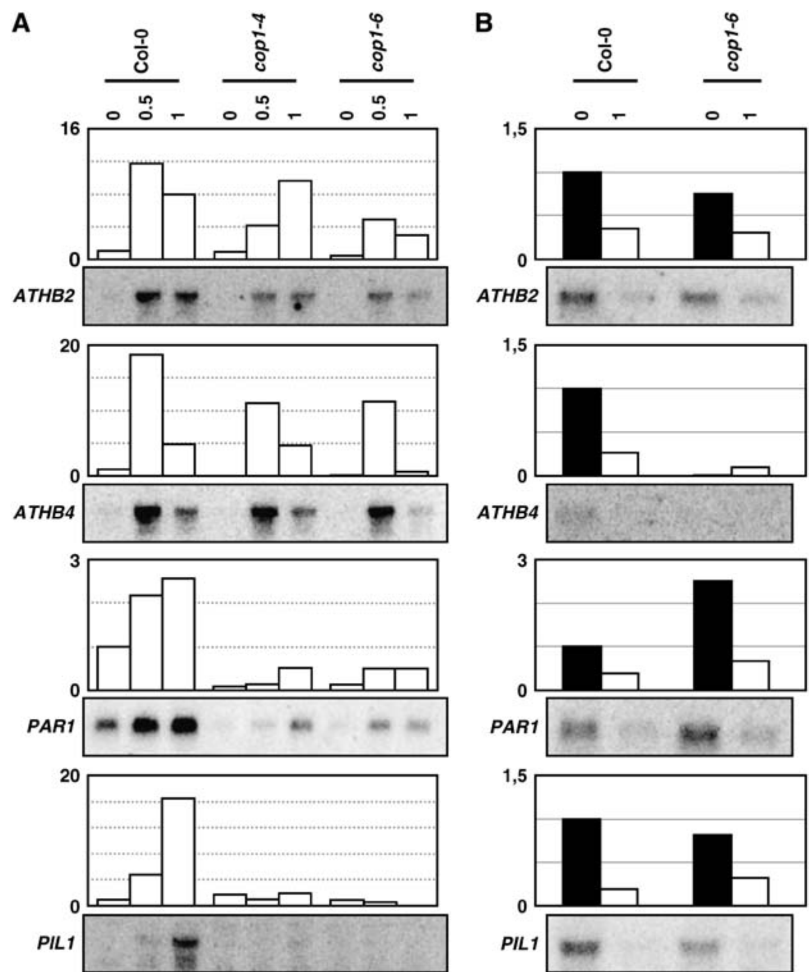

Figure 4. Role of COP1 on *PAR* expression induced by simulated shade or after deetiolation under FRc. Only *PAR* genes identified here to be direct targets of phy action are analyzed. A, RNA-blot analysis of *PAR* expression in Arabidopsis wild-type (Col-0), *cop1-4*, and *cop1-6* seedlings harvested at 0, 0.5, and 1 h after W + FR treatment. B, RNA-blot analysis of *PAR* expression in Arabidopsis wild-type and *cop1-6* seedlings harvested at 0 and 1 h after deetiolation.

After collecting samples at 0-, 0.5-, and 1-h time points, RNA-blot analyses were performed. The *hy5-1* mutation did not dramatically affect expression of the analyzed *PAR* genes under W or their photomodulated expression after simulated shade (Fig. 5A). These results suggest that, unlike COP1, HY5 is not required for transducing shade-triggered signals to early molecular SAS responses. Regarding physiological SAS responses, transfer of mutant *hy5-1* seedlings to W + FR for 24 h still resulted in a significant response (Fig. 5B), suggesting that HY5 is not needed for hypocotyl elongation in response to simulated shade.

Unlike the loss of HY5 function, the loss of COP1 function results in a strong pleitropic phenotype that might somehow be responsible for the observed effects on *PAR* expression after simulated shade. To evaluate this possibility, photomodulated *PAR* expression was analyzed in two further mutants of the same constitutively photomorphogenic class as *cop1*: *det1-1* (Pepper et al., 1994) and *det2-1* (Li et al., 1996). When grown in

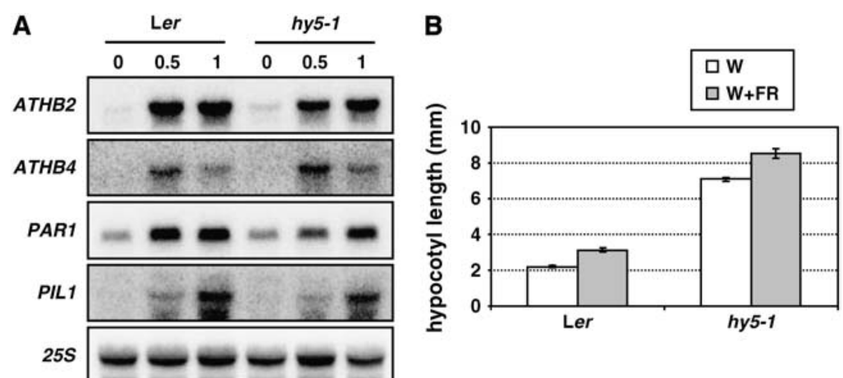

Figure 5. Role of HY5 on hypocotyl length and *PAR* expression induced by simulated shade. A, RNA-blot analysis of the expression of *PAR* genes in Arabidopsis wild-type (Landsberg *erecta*) and hy5-1 seedlings. Only *PAR* genes identified here to be direct targets of phy action are analyzed. B, Changes in hypocotyl length in response to simulated shade in wild-type and hy5-1 seedlings.

the dark, both *cop* and *det* mutants exhibit an obvious photomorphogenic phenotype (inhibition of hypocotyl growth, expansion of cotyledons, development of primary leaves, and accumulation of anthocyanins). However, the molecular lesions involved affect very different biochemical and physiological processes. The nuclear DET1 protein has been suggested to participate with COP1 in the degradation of positive regulators of photomorphogenesis via the proteasome system (Yanagawa et al., 2004), whereas DET2 is an enzyme involved in brassinosteroid biosynthesis (Li et al., 1996). A similar experiment to that described for *cop1* (Fig. 4A) and *hy5* (Fig. 5A) mutants was carried out using *det1-1* and *det2-1* seedlings (Fig. 6). The reduced photomodulation observed for *PAR1* and *PIL1* and, to a lesser extent, for *ATHB2* and *ATHB4* in *cop1* seedlings compared to the wild type (Fig. 4A), was also observed in *det1-1* seedlings (Fig. 6A). By contrast, *det2-1* seedlings displayed wild-type (*PAR1* and *PIL1*) or slightly increased (*ATHB2* and *ATHB4*) photomodulation of *PAR* expression (Fig. 6B), confirming that the attenuated photoregulated *PAR* expression in *cop1* and *det1* is not a secondary effect of the constitutively photomorphogenic phenotype, but a direct effect of the molecular lesions in the latter mutants. Together, these results show that not all of the factors genetically identified to have a role in seedling deetiolation participate in the regulation of SAS responses. Furthermore, those that do participate, such as COP1 and DET1, appear to target a different set of primary genes of phy action.

The Promoter Regions of *ATHB2* and *PAR1* Confer Simulated Shade Responsiveness to a Reporter Gene

To address whether the observed changes in transcript levels were the result of altered promoter activity (transcriptional regulation), transgenic plants expressing a *GUS* reporter gene driven by the 1-kb promoter region of *ATHB2* and *PAR1* were generated. These genes were selected because they represented both types of responses to simulated shade observed in our pharmacological (Fig. 3C) and genetic (Figs. 4A and 6A) experiments. The resulting transgenic plants were referred to as Pro_{ATHB2}:*GUS* and Pro_{PAR1}:*GUS* lines. As a control, we also analyzed Pro_{35S}:*GUS* plants. Several independent transgenic lines were obtained for each construct. In the T_2 generation, GUS histochemical assays were performed and lines displaying GUS activity in seedlings were selected for further analysis. *GUS* transcript levels were quantified before and 1 h after simulated shade treatment in four to six selected lines. Although the analyzed lines displayed variable levels of basal *GUS* expression (i.e. before transferring the seedlings to simulated shade; data not shown), all the lines but the Pro_{35S}:*GUS* controls showed a clear photoregulated expression of the *GUS* reporter (Fig. 7). Pro_{ATHB2}:*GUS* lines displayed the highest degree of shade-induced up-regulation of *GUS* mRNA levels (Fig. 7). In all cases, the expression of the endogenous *PAR* gene analyzed was normally photoregulated (data not shown). These data showed that the selected *PAR* promoter regions are sufficient to confer simulated shade responsiveness to an unrelated reporter gene.

Long-Term SAS Responses Are Impaired in a *pil1* Mutant

From the four *PAR* genes identified in this work as direct targets of phy signaling, only *ATHB2* and *PIL1* have been shown to be instrumental in implementing SAS responses (Steindler et al., 1999; Salter et al., 2003). In the case of PIL1, however, the phenotype observed in a loss-of-function *pil1* mutant is more subtle than might be expected for a primary gene within the transcriptional cascade modulating SAS responses. Despite extensive phenotypic characterization, PIL1 has only been shown to affect hypocotyl elongation in response to transient simulated shade (Salter et al., 2003; Yamashino et al., 2003). However, our analysis of hypocotyl elongation after prolonged (5 d) simulated shade treatment revealed that the novel *pil1-4* mutant, a T-DNA insertion allele that we characterized from the public Salk collection (Fig. 8A), displayed a subtle, but significantly stronger, response compared to the wild type (Fig. 8B), suggesting that PIL1 may play a role in moderating this shade avoidance response.

PIL1 expression still responds strongly to simulated shade in a *phyB* mutant background in which the expression of this *PAR* gene is promoted (Devlin et al.,

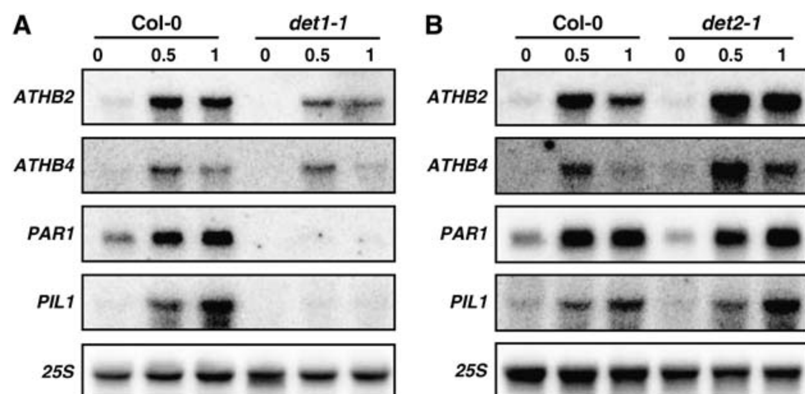

Figure 6. Role of DET1 and DET2 on *PAR* expression during simulated shade. A, RNA-blot analysis of the expression of *PAR* genes in Arabidopsis wild-type (Col-0) and *det1-1* seedlings. B, RNA-blot analysis of the expression of *PAR* genes in Arabidopsis wild-type (Col-0) and *det2-1* seedlings. Only *PAR* genes identified here to be direct targets of phy action are analyzed.

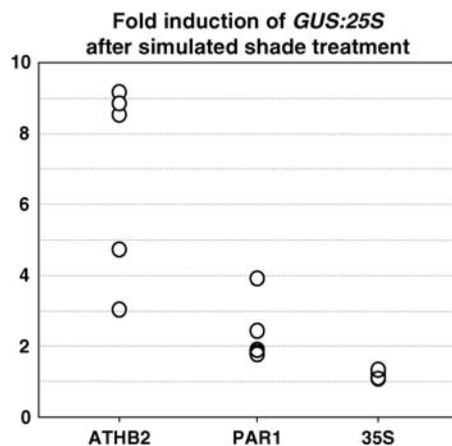

Figure 7. Photoresponse activity of the 5′ promoter regions of *ATHB2*, *PAR1*, and *35S*. Analyses of *GUS* expression were performed in 7-d (d7) seedlings using four (Pro_{35S}) or five (Pro_{ATHB2} and Pro_{PAR1}) independent transgenic lines for each construct. *GUS*:*25S* expression levels are shown as fold induction 1 h after simulated shade treatment.

2003; Salter et al., 2003). We examined the effect of loss of PIL1 function in a *phyB-9* mutant background in the hope that this might more clearly show the role for PIL1 in prolonged SAS responses. As known, the *phyB* mutation resulted in a long hypocotyl phenotype in both single and double *phyB-9 pil1-4* seedlings. In response to W + FR, the long *phyB* hypocotyls showed less elongation than under W, in agreement with previous findings (Devlin et al., 2003). This was concluded to be the result of a moderating (i.e. negative) factor only apparent in the absence of phyB (Devlin et al., 2003). By contrast, this reduction in hypocotyl elongation in response to simulated shade was not apparent in *phyB-9 pil1-4* seedlings (Fig. 8B), confirming a role for PIL1 in this moderation of hypocotyl elongation in response to shade. Together, these data indicate that the *pil1* mutation impairs long-term SAS responses, such as hypocotyl elongation, in addition to the previously observed effect on the response to transient shade conditions (Salter et al., 2003).

DISCUSSION

SAS generally refers to a broad set of physiological and developmental changes in light-grown plants in response to shade perceived by the phys (Smith, 1982; Smith and Whitelam, 1997). Simulated shade also results in up-regulation of *PAR* genes in Arabidopsis (Fig. 1), which can be considered as an authentic SAS response. The inverse correlation between Pfr (R to FR ratio) and *PAR* transcript levels (Fig. 1) supports the idea that the observed up-regulation of *PAR* expression by simulated shade is actually a release of repression by the active Pfr form of the phys. Consistently, high phy levels in AOX and ABO lines result in low *PAR* transcript levels both before and after simulated shade treatment (Fig. 2B). In this work, we additionally show that some of the selected *PAR* genes are direct targets of phy action and unveil a role for COP1 and DET1 in regulating their expression during SAS. Furthermore, we report that one of the identified primary target genes, *PIL1*, affects long-term SAS responses.

As proposed for seedling deetiolation, it was expected that phys transduce light signals to implement SAS responses by rapidly modulating a transcriptional cascade. The genes directly targeted by phy signaling, however, are unknown. Here we report that four *PAR* genes are authentic direct targets of phy signaling in light-grown seedlings based on four main lines of evidence: (1) the negative correlation between Pfr levels and their expression (Figs. 1 and 2); (2) the rapid kinetics (min) of their light-dependent regulation; (3) the fast responsiveness of their promoters to simulated shade, indicative of a transcriptional control (Fig. 7); and (4) the CHX-independent pattern of their photoregulated expression (Fig. 3). A common strategy to identify primary target genes of a TF is to control its transcriptional activity by regulating the DEX-dependent nuclear translocation of TF-glucocorticoid receptor fusions combined with CHX treatments to block de novo protein synthesis. When CHX is applied together with DEX, only transcript levels of the TF primary targets are affected: Expression of the immediate targets is therefore CHX independent, whereas expression of downstream targets is CHX dependent. Although this approach has most often been used for TFs (Sablowski and Meyerowitz, 1998; Wagner et al., 1999; Ohgishi et al., 2001; Sawa et al., 2002), it has also given successful results for proteins without known DNA-binding domains that need to be nuclear for signaling activity, like

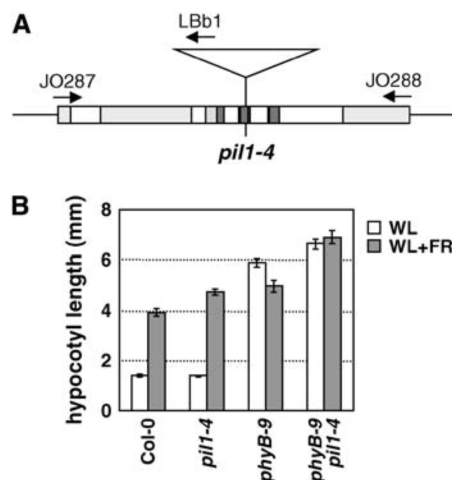

Figure 8. Role of PIL1 on SAS seedling responses. A, Schematic representation of *PIL1* (At2g46970) genomic sequence and T-DNA insertion in *pil1-4*. Introns (white), exons (light gray), and the bHLH domain (dark gray) are indicated with boxes. B, Changes in hypocotyl length in response to simulated shade in wild-type (Col-0), *pil1-4*, *phyB-9*, and *pil1-4 phyB-9* double-mutant seedlings. Seedlings grown for 2 d under W were either maintained in W (white bars) or transferred to W + FR (gray bars; R to FR ratio 0.05) for 5 d, after which mean (±SE) lengths were measured.

CONSTANS (Samach et al., 2000). The experimental configuration presented in this article to identify primary target genes of phys in vivo incorporates a novel aspect: Transcriptional activity is not controlled by nuclear translocation, but by simulated shade, which modulates phy photoequilibrium and, subsequently, its binding ability to different PIFs (the shade signal would eventually also regulate nuclear translocation of the phys, of course, but this would not be the primary factor regulating activity of the light-stable phys over the short time scale involved here [Kircher et al., 2002]). These PIFs are TFs whose transcriptional activity has been hypothesized to be regulated by Pfr action (Quail, 2002). Therefore, in the presence of CHX, we are monitoring the immediate (translation independent) effects of the shade-triggered disappearance of Pfr-PIF complexes on *PAR* gene expression. By using this experimental design, we observed that the rapid photoregulated response of *ATHB2*, *ATHB4*, *PAR1*, and *PIL1* was CHX independent (Fig. 3), strongly suggesting that these genes are primary targets of phy action in light-grown seedlings. Our results do not discriminate whether the active Pfr form binds to the promoters of these genes via specific PIFs, prevents other PIFs from accessing these promoters, or requires additional biochemical steps to transduce light signals to changes in *PAR* expression.

Although we have not directly investigated whether *ATHB2*, *ATHB4*, *PAR1*, and *PIL1* are also primary targets of phy action during seedling deetiolation, their similar pattern of phy-mediated regulation during both SAS and deetiolation supports this possibility in both physiological contexts. This is in agreement with the notion of functional gene cassettes, which was developed in animal systems after observing that groups of genes with functions in a given developmental process were also used to serve similar functions in other stages of development (Jan and Jan, 1993). The possible existence of a functional *PAR* gene cassette working in both seedling deetiolation and SAS, however, does not necessarily imply that all the molecular mechanisms required for its photoregulation are fully conserved. For instance, nuclear COP1 participates in the photoregulation of *ATHB2*, *ATHB4*, *PAR1*, and *PIL1* by simulated shade, whereas it does not play a major role in the early repression of *ATHB2*, *PAR1*, and *PIL1* during deetiolation (Fig. 4).

Genetic screens of Arabidopsis seedling deetiolation have originated the concept of early and late, phy-specific or common, light-signaling intermediates. The constitutive deetiolated *cop/det/fus* class of mutants, shown to be mostly epistatic to all the photoreceptor mutants, has been proposed to participate in the later stages of light signaling (for review, see Quail, 2002; Schäfer and Bowler, 2002; Chen et al., 2004). More recent data indicate, however, that COP1 regulates at least three different and consecutive processes during early phy signaling: (1) accumulation of phy-interacting factors like PIF3 in etiolated seedlings (Bauer et al., 2004); (2) simulated shade-dependent changes in the expression of genes directly regulated by phy action in light-grown plants (Fig. 4A); and (3) degradation of their encoded gene products, such as HFR1 (Duek et al., 2004; Sessa et al., 2005). The multilevel participation of COP1 in early phy signaling implies that some phy-mediated responses may be COP1 independent. Indeed, control of seed germination by phys is unaffected by *cop1* mutations (Deng et al., 1992) and *phyB* is epistatic to *cop1* for the reverse cotyledon angle response during seedling deetiolation under both R and FR (Boccalandro et al., 2004). Also, our data consistently show that COP1 is differentially required for early *PAR* gene expression during SAS and deetiolation (Fig. 4). Our conclusions, however, do not exclude the possibility that COP1 might also act at downstream steps of phy signaling.

DET1, which acts together with COP1 in regulating proteolysis of TFs involved in light signaling (Yanagawa et al., 2004), also participates in the photoregulation of *ATHB2*, *ATHB4*, *PAR1*, and *PIL1* by simulated shade (Figs. 4A and 6A). A corollary of these observations is that *cop1* and *det1* mutants sense the differences between various light conditions (dark versus light and light versus shade), although they are clearly impaired in transducing this information to regulate growth and development. On the other hand, other factors, such as DET2 or HY5, have little or no effect on the photoregulation of the selected *PAR* genes (Figs. 5A and 6B). HY5 is not required for hypocotyl elongation in response to simulated shade (Fig. 5B). By contrast, defective physiological SAS responses have been observed in brassinosteroid-deficient mutants *det2-1* (data not shown) and *eve1/dwf1* (Luccione et al., 2002). This suggests that brassinosteroids affect SAS responses by mechanisms other than the regulation of *PAR* gene expression (likely acting downstream of the identified genes).

One of the proposed mechanisms of phy signaling is the activation of transcriptional cascades by both PIF3-dependent and -independent pathways (Tepperman et al., 2001). PIF3 belongs to the large and complex bHLH family of DNA-binding proteins, many of which have been shown to bind to the G-box motif GAGCTC in vitro (Martínez-García et al., 2000; Huq and Quail, 2002; Huq et al., 2003). The functional relevance of the G-box motif for the activity of PIF3 and related bHLHs in planta, however, has not been demonstrated yet. In addition to gene activation pathways, microarray data suggest the existence of at least another pathway initiated by early repression of the transcriptional cascade (Tepperman et al., 2001), as exemplified by the *PAR* genes. It is likely that the PIFs involved in this repression pathway were different from those acting as transcriptional activators, as initially postulated for PIF3 (Ni et al., 1998). Alternatively, the same PIFs may function as either transcriptional activators or repressors, depending on the specific promoters (Kim et al., 2003). The 1-kb region upstream of the ATG codon of *ATHB2* and *PAR1* is sufficient to confer rapid photoregulation to a reporter gene (Fig. 7). Neither these nor the corresponding upstream regions of *ATHB4* and *PIL1* contain G-box elements, supporting the proposal that

PIF3-like factors are not involved in the early repression of these *PAR* genes by phys.

A relevant contribution of our work is the demonstration that direct target genes of phy action, such as *PIL1*, can negatively regulate shade-induced hypocotyl elongation in response to sustained (5 d) reductions in the R to FR ratio (Fig. 8B) in addition to the previously reported transient (2 h) response (Salter et al., 2003). The negative role of *PIL1* is also consistent with previous data from wild-type plants in which transient low R to FR treatment at subjective dawn results in a maximal increase in *PIL1* transcript levels and inhibition of elongation. Conversely, the same signal given at dusk results in a lower increase in *PIL1* transcripts and in maximal elongation promotion (Salter et al., 2003). It is also interesting that *pil1* mutant seedlings show hyposensitivity to both Rc and FRc at lower fluence rates (Salter et al., 2003). This observation might be ecologically relevant because reductions in both the R to FR ratio and light quantity occur in nature under vegetation canopies (Smith, 1982).

Simulated shade also rapidly induces *HFR1* expression, another SAS negative regulator (Sessa et al., 2005) that encodes for a bHLH protein (Fairchild et al., 2000). All PIFs and PILs tested so far can bind in vitro to the core G-box motif, except HFR1, which appears to be a non-DNA-binding variant (Fairchild et al., 2000; Huq and Quail, 2002; Huq et al., 2004; Khanna et al., 2004). Furthermore, there is evidence that closely related Arabidopsis bHLH members can form heterodimers such as HFR1-PIF3 and PIF3-PIF4 (Fairchild et al., 2000; Toledo-Ortiz et al., 2003). By these various mechanisms, shade-induced *PIL1* and *HFR1* transcript changes can, in theory, rapidly feed back into the phy-regulated network of bHLH proteins and alter shade-induced changes in gene expression.

In summary, our work has identified candidate factors potentially representing entry points for the phy signal into the shade-modulated transcriptional cascade and has uncovered functions for one of them, PIL1.

MATERIALS AND METHODS

Plant Material and Growth Conditions

AOX (Boylan and Quail, 1991) and ABO (Wagner et al., 1991) lines are in the Arabidopsis (*Arabidopsis thaliana*) Nossen (No-0) ecotype; *cop1-4*, *cop1-6*, *det1-1*, *det2-1*, *pil1-4* (SALK_043937; Alonso et al. 2003), and *phyB-9* mutants are in the Columbia (Col-0) background; and *hy*5-1 is in the Landsberg *erecta* background. The SALK_043937 line was named *pil1-4* to distinguish it from three previously described mutants, *pil1-1* and *pil1-2* (Yamashino et al., 2003; Kazusa collection), and garlic line 438c01 (Salter et al., 2003). The *pil1-4* mutant allele contains a T-DNA insertion at position 1,102 in the middle of the bHLH domain (position 1 corresponds to the first nucleotide of the starting ATG codon; Fig. 8A). This mutant expressed truncated transcripts of *PIL1* (data not shown), as has been described for other alleles (Yamashino et al., 2003). A *pil1-4 phyB-9* double mutant was generated by crossing the two single mutants, allowing the F_1 progeny to self fertilize, and then selecting the F_2 plants. Seedling mutants for *phyB*, selected as those displaying long hypocotyls under W, were subsequently genotyped for *pil1-4* homozygous by using specific oligos (JO287, 5′-ATGGAAGCAAAACCCTTAGCATC-3′; JO288, 5′-TTAGTTTGGCGAGCGATAATAAC-3′; and LBb1, 5′-GCGTGGACCGCTTGCTGCAACT-3′) and standard PCR analysis. Different oligo combinations were used to discriminate between the wild type (JO287 + JO288) and the mutant (JO287 + LBb1) alleles of the *PIL1* gene.

For analyses of gene expression, seeds were surface sterilized and sown on top of a filter paper circle deposited on growth medium (GM; Valvekens et al., 1988) without Suc (GM−). For the simulated shade treatments, after stratification at 4°C for 2 to 5 d in the dark, seeds were germinated under W (40 μmol m^{-2} s^{-1}; R to FR ratio of 3.2–4.5) at 22°C. On day 7 after germination, seedlings were given a light treatment (W supplemented with FR, W + FR; R to FR ratio of 0.03–0.12, unless otherwise stated), harvested, frozen in liquid nitrogen, and stored at −80°C until processing. For the deetiolation treatments, after stratification as before, seeds were induced to germinate by a brief (0.5–3 h) W treatment and then transferred to the dark at 22°C. On day 4, etiolated seedlings were given a 1-h FR treatment (8 μmol m^{-2} s^{-1}), harvested under a green safelight, frozen in liquid nitrogen, and stored at −80°C until processing.

For analyses of the hypocotyl elongation response in Figures 2 and 5, plant material was prepared and sown as indicated elsewhere (Devlin et al., 2003). For analyses of hypocotyl elongation to long-term simulated shade treatment (Fig. 8), 50 seeds were individually sown directly onto the GM−; on day 2, seedlings were either maintained under W or transferred to W + FR for 5 additional days. For measuring hypocotyl lengths, seedlings were laid out flat on agar plates. Hypocotyl lengths were measured by using National Institute of Health (NIH) IMAGE software to analyze the digital images of these seedlings. Data represent the mean (±SE) of at least 15 seedlings for each treatment. Experiments were repeated at least twice and a representative one is shown.

Light Sources and Treatments

W was provided by two cool-white fluorescent tubes (36 W, Sylvania standard; R to FR ratio of 3.2–4.5). Supplementary FR light was provided by QB1310CS-670-735, LED hybrid lamps (Quantum Devices). Plants were harvested immediately before (0 h) and after 0.5 and 1 h of W + FR treatment. The fluence rates were measured using a quantum radiometer photometer (188b; LI-COR), fitted with a quantum sensor (Li-190 SB) for R and a near-infrared sensor (Li-220 SB) for FR.

CHX and DEX Treatments

CHX (Sigma-Aldrich) was dissolved at 50 mM in 50% (v/v) ethanol and DEX (Sigma-Aldrich) was dissolved at 5 mM in 100% (v/v) ethanol and kept at −20°C until use. Fifty micromolar CHX and/or 5 μM DEX in water were prepared prior to the treatments. Day 7 seedlings growing on filter paper circles were transferred to new plates containing 4 mL of different combinations of ±CHX and ±DEX. In Figure 3A, ±CHX and ±DEX were applied at the same time and seedlings were assayed for GUS activity 24 h later. In Figure 3B, ±DEX was applied 1 h before ±CHX and seedlings were assayed for GUS activity 2 h after ±CHX application. In Figure 3C, ±CHX was applied 2 h before light treatments. Seedlings were kept in these conditions during the light treatments until harvesting.

Construction of Pro_{ATHB2}:*GUS* and Pro_{PAR1}:*GUS* Promoter Fusion Lines

The binary vector pCAMBIA1304 (GenBank accession no. AF234300; Pro_{35S}:*green fluorescent protein-GUS*) was used to subclone all promoter fusions. This plasmid confers hygromycin resistance to transgenic plants. We selected 1,000 bp located 5′ of the translation start. To get the corresponding promoter sequences, specific oligos were designed after the available sequence databases: JO281 (5′-GGAAGCTTTCAACCGTTTTTGTTTAGTTCTTC-3′); JO280 (5′-GTCGGATCCACCATCTTCTGTTGAACTTTCTCAAG-3′); JO301 (5′-GGAAGCTTACCAGGCACCACCCGAATGGC-3′); and JO302 (5′-CGGATCCACCATTGAAAGAAAGAGAGAGATG-3′). From standard Col-0 DNA preparation as template, different combination of oligos were used for PCR of *ATHB2* (JO281 + JO280) and *PAR1* (JO301 + JO302) promoters. These oligos generated fragments containing 1,000 bp of the corresponding promoter flanked by a *Hin*dIII site in the 5′ end and a *Bam*HI site after the translation start. The resulting PCR fragments were directly subcloned into a pGemT-Easy (Promega) plasmid, generating pJF278 and pJF297, and sequenced. The *Hin*dIII-*Bam*HI fragments from these plasmids were subcloned into pCAMBIA1304 digested with *Hin*dIII-*Bgl*II (this digestion removes the original 35S promoter that drives the expression of the GUS reporter) to generate pJF279 and pJF299, respectively. pJF279 corresponds to the 1,000-bp *ATHB2* promoter driving expression of *GUS*

(Pro_{ATHB2}:*GUS*) and pJF299 corresponds to the 1,000-bp *PAR1* promoter driving expression of *GUS* (Pro_{PAR1}:*GUS*).

The binary vectors pCAMBIA1303 (GenBank accession no. AF234299; Pro_{35S}: *GUS-green fluorescent protein*), pJF279, and pJF299 were introduced in *Agrobacterium tumefaciens* strain $C_{58}C_1$ (pGV2260) by electroporation, and transformed colonies were selected in kanamycin (50 μg mL^{-1}). Arabidopsis (Col-0) was transformed by floral dipping (Clough and Bent, 1998) and transgenic plant selection (T_1 generation) was done in GM plates containing hygromycin (30 μg mL^{-1}). The presence of the transgene in the selected T_1 plants was verified by PCR analysis using specific transgene primers on plant genomic DNA isolated from young leaves (Edwards et al., 1991). Promoter activity was verified by GUS histochemical assays of the T_2 hygromycin-resistant seedlings.

GUS Assays

Histochemical GUS assays were performed essentially as described (Craft et al., 2005). Seedlings were cleared with 70% (v/v) ethanol washes to improve contrast. Finally, whole-mount preparations were made in 50% (v/v) glycerol to visualize GUS activity using a Leica MZFLIII stereoscopic microscope and a Leica DC200 digital camera (Leica Microsistemas).

RNA Isolation and Northern Analysis

Total RNA was isolated from the frozen tissue essentially as described (Rodríguez-Concepción and Gruissem, 1999). Ten micrograms of total RNA were separated on 1.2% (w/v) agarose denaturing formaldehyde gels and transferred onto Hybond N nylon membranes. Hybridization was carried out as described (Martínez-García et al., 2002). The probes for the RNA blot were made by amplifying Col-0 genomic DNA with specific primers: JO282 (5′-CAGAAGATGATGTTCGAGAAAGAC-3′) and JO283 (5′-AAAGACT-TAGGACCTAGGACGAAG-3′) for *ATHB2*; JO284 (5′-AGGACAATGGGG-GAAAGAGATGAT-3′) and JO285 (5′-CCTTCCCTAGCGACCTGATTTTTG-3′) for *ATHB4*; JO289 (5′-TCAATGGAAGAAACTCTAGCCAC-3′) and JO290 (5′-TCAACCTCCGAACTTCATGTCTTC-3′) for *PAR1*; RO3 (5′-AACATGAT-GATGGGCAAAGAAG-3′) and RO4 (5′-AAATCACGATCGTGGACG-CAAGGC-3′) for *HAT2*; JO287 (5′-ATGGAAGCAAAACCCTTAGCATC-3′) and JO288 (5′-TTAGTTTGGCGAGCGATAATAAC-3′) for *PIL1*; and JO293 (5′-ATGGCTAGAAATTTCGAGCTT-3′) and JO294 (5′-TCAATGCTTGGAAG-CAAAGTC-3′) for *RIP*. PCR products were subcloned into pGemT-Easy (Promega) or PTZ57T/R (Fermentas) to give pJF281, pJF282, pJF285, pIR4, pJF284, and pJF290, respectively. Inserts were sequenced for identity confirmation. A partial fragment of 1 kb corresponding to the GUS coding region was PCR amplified using specific oligos (GUS-upper, 5′-CAACGAACT-GAACTGGCAGA-3′; GUS-lower, 5′-GGCACAGCACATCAAAGAGA-3′) and pCAMBIA1304 as a template. DNA inserts, isolated by restriction digestion or by PCR using specific primers, were radioactively labeled with [$^{\alpha 32}$P]dCTP by using a random primed DNA-labeling kit (Roche Molecular Biochemicals), and a purified trough Sephadex G-50 column (Amersham). Images were visualized by using a molecular imager FX (Bio-Rad), and band intensities were quantified by using QUANTITY ONE (Bio-Rad) software. Expression levels were calculated relative to the lowest value of each set of samples after normalization with the 25S rRNA signal.

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